APPLICATION NOTE

Inhibition of FGFR signaling pathways studied in cancer cell lines using HTRF.

Author

Maud Pratlong

Revvity, Inc.
Codolet, France

Co-Author

Alexis Aquilina

Revvity, Inc.
Codolet, France

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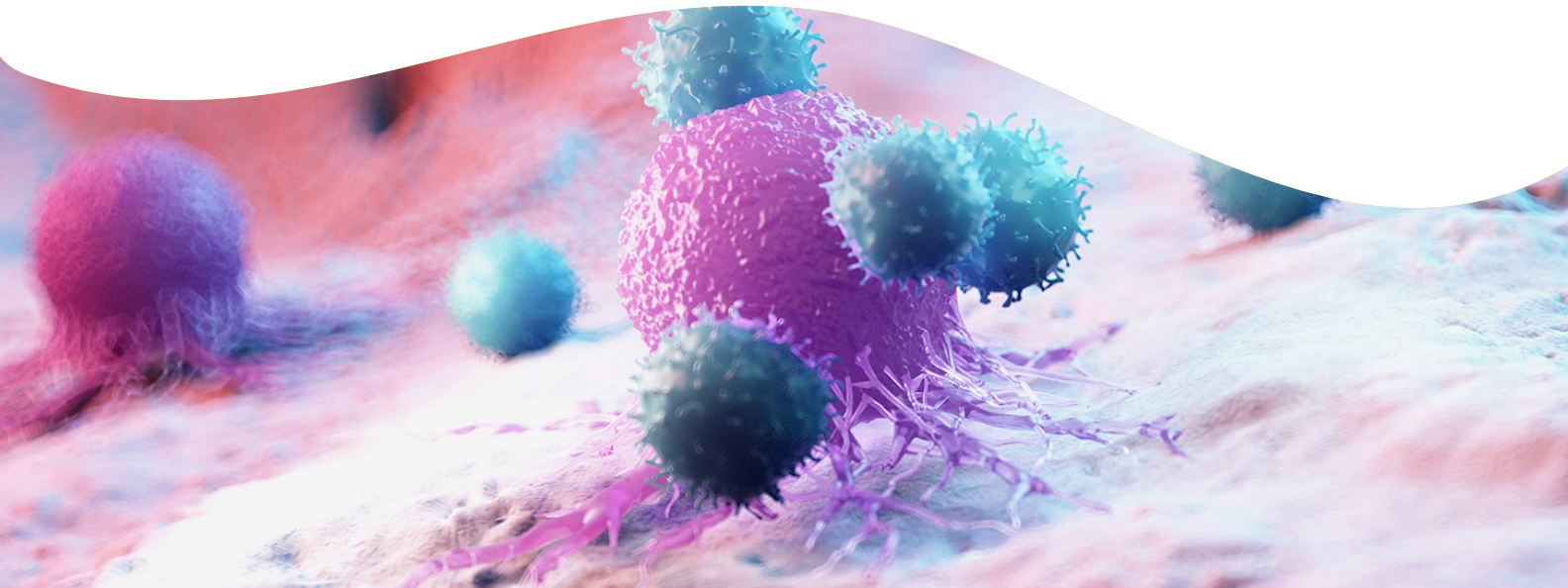
Fibroblast Growth Factor Receptors (FGFRs) 1 to 4 belong to the Receptor Tyrosine Kinase family that transmits signals through the cell membrane by activating different pathways, including RAS-MAPK, PI3K-AKT, PLCγ, and STATs. The propagation of signal occurs via the phosphorylation of FGFRs and associated adaptor proteins that control multiple physiological functions such as proliferation, survival, migration, and adhesion or differentiation. FGFR genetic alterations (overexpression, mutations, translocation, abnormal FGF ligand binding, etc.) have been reported in a wide variety of cancers, including breast, lung, ovarian, gastric, and prostate tumors. Deregulation via a constitutive activation of these pathways is frequently observed and studied in cancers, which explains why FGFRs are preferential targets for Tyrosine Kinase Inhibitors in anti-cancer strategies. The evaluation of these compounds is challenging and requires in depth exploration of signaling events associated with FGFR inhibition.

Revvity offers HTRF® (Homogeneous Time-Resolved Fluorescence) cell-based assay kits dedicated to cellular signaling activation through the phosphorylation of proteins. These assays enable the rapid and accurate measurement of the activation status of cell-surface receptors, as well as of their downstream key kinases and transcription factors. HTRF kits measuring both FGFRs and downstream effectors have been used to elucidate pathways in several cancer cell lines. This work confirmed that the regulation of FGFR activation is characteristic of each human cancer cell line, and can be evaluated with multiple HTRF assays targeting downstream proteins.



Here, we show that a pan-FGFR inhibitor, AZD4547, used in different clinical phases depending on the cancer type, is able to inhibit or even suppress the abnormal phosphorylation of FGFRs and their key effectors in 3 different types of cancer cell lines: KG-1 (blood cancer), SNU-16 (gastric cancer), and KMS-11 (multiple myeloma).

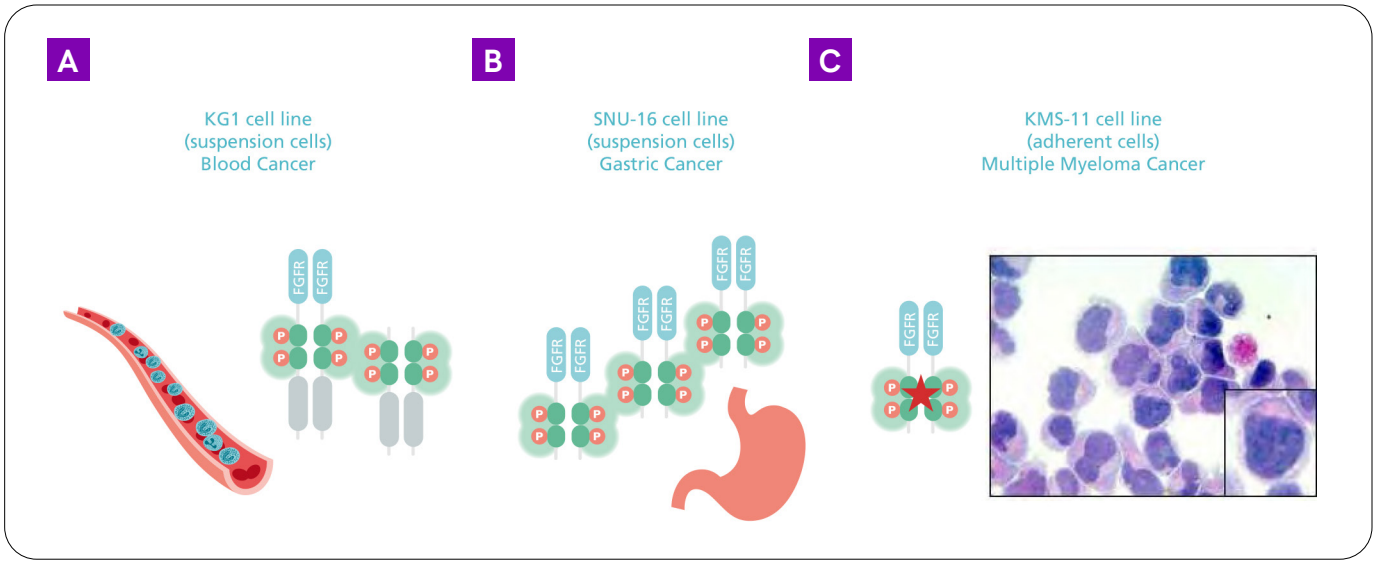
Introduction: FGFRS in cancer cell lines

Three major classes of FGFR genetic alterations in human cancers are known today: gene amplification, mutations, and gene fusion. The alterations and receptors involved differ depending on the type of cancer.

FGFR1 amplification preferentially occurs in squamous cell lung and metastatic breast cancers, whereas FGFR2 overexpression is most common in gastric cancer. For mutations, there can be a gain or loss of function in FGFRs. The mutations and the types of mutated receptor are characteristic for some cancers, such as FGFR2 mutations D530N, I642V, and A648T in melanoma which involve loss of function, whereas FGFR3 gain of function mutations R248C and S249C have been observed in bladder cancer.

Two types of FGFR fusions in human cancers are described as type 1 fusions caused by chromosomal translocations, particularly in myeloproliferative syndrome, and type 2 fusions caused by chromosomal rearrangements in solid tumors. In all cases, aberrant FGFR signaling may promote the proliferation and survival of tumor cells.

A representative panel of cancer cell lines was chosen to illustrate the variety of genetic alterations and cancers. KG-1 is a myeloid leukemia cell line which is characterized by a fusion of the FGFR1OP2 gene that disrupts the FGFR1 gene and involves an aberrant tyrosine phosphorylation of FGFR1, and especially a constitutive phosphorylation of STAT5 [1]. SNU-16 is a FGFR2-dependent gastric cancer cell line harboring an FGFR2 amplification [2]. KMS-11 cell line is a useful model of multiple myeloma and which presents a FGFR3 gene mutation (Y373C). This mutation involves a high level of phosphorylation of the receptor FGFR3 indicating a constitutive activation. It has been well established that the stimulation of cells increases the level of receptor phosphorylation which activates the MAP kinase signaling pathway, but does not seem to involve STAT cascade [3]. These three cell lines provide *in vitro* models for the study of FGFR inhibitors against FGFR1 fusion, FGFR2 amplification, and FGFR3 mutation.



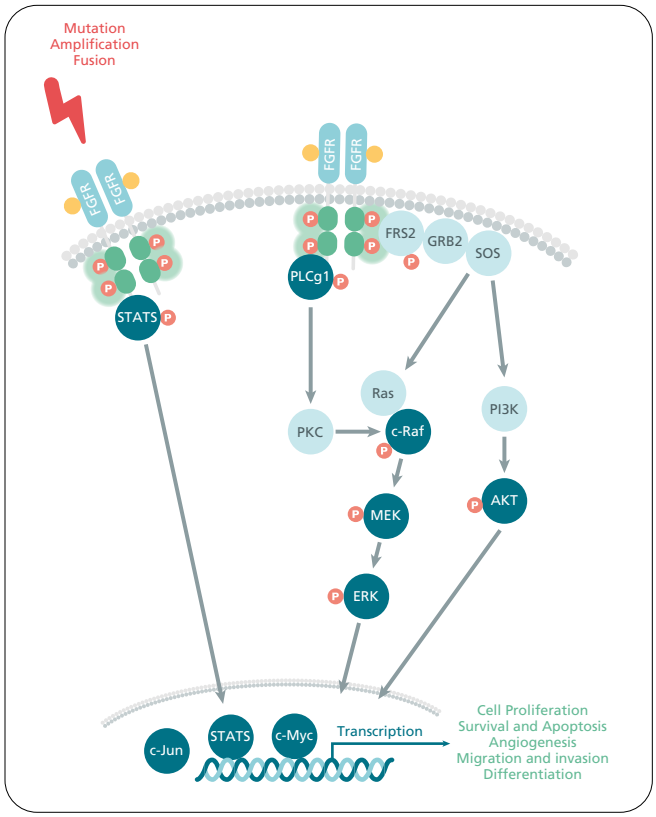
Representation of 3 cancer cell lines and their FGFR1-2 and 3 receptors *in vitro* models, respectively used in this application note: (A) KG1 cell line - Myeloid leukemia cell line (B) SNU-16 cell line - Gastric cancer cell line (C) KMS-11 cell line - Multiple Myeloma Cancer

FGFR signaling pathway

Fibroblast Growth Factor Receptors (FGFR 1 to 4) are trans-membrane receptors with an extracellular part activated by different types of ligands. These transduce signals to a variety of intracellular signaling pathways through phosphorylation of the cytoplasmic tyrosine kinase domain of receptors and adaptor associated proteins. FRS2α is a key adaptor protein constitutively associated with FGFR. This protein, via the recruitment of GAB1 and GRB2, induces the activation of the RAS-MAPK pathway, particularly ERK1/2 and PI3K. PI3K then phosphorylates AKT. The phosphorylation of FGFR also activates the STAT cascade by phosphorylating STAT1, 3, and 5. Activation of PLCγ1 hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2), generating inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C (PKC), whereas IP3 alters the cellular calcium levels. Finally, the transduction of signals through FGFRs can activate several transcription factors, like c-myc, c-jun, and c-fos [4].

This entire signal transduction mechanism is involved in the regulation of cellular functions and their deregulation is found in various types of cancers. Furthermore, genetic alterations of FGFRs in a wide variety of cancers are associated with the overexpression or hyperactivity of FGFRs, making the receptors and their signaling pathways key targets for anti-cancer therapies.

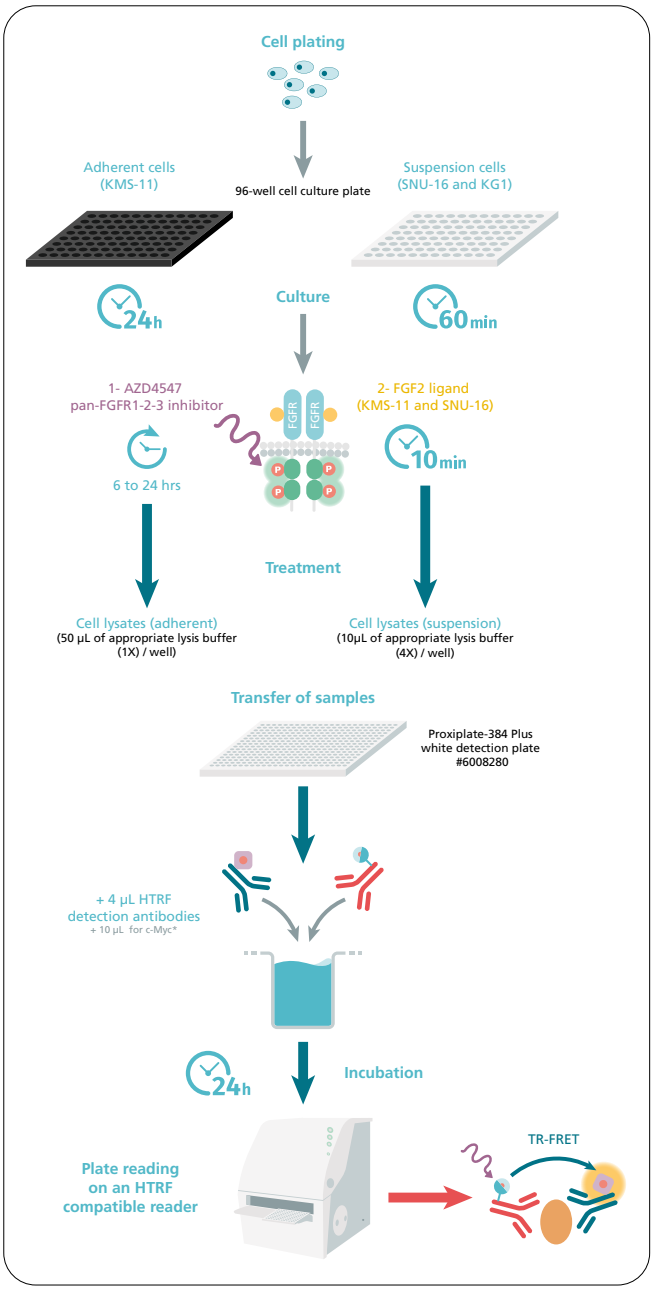
In this work, we selected AZD4547, a potent ATP-competitive small molecule inhibitor of FGFR1-2 and 3 currently studied in clinical trials (Phase II/III depending on the cancer type). AZD4547 has been described as selectively inhibiting FGFR phosphorylation and its related MAPK phosphorylation in a dose-dependent manner. The compound also potently inhibited FRS2 and PLCγ or AKT phosphorylation, but only for some cell lines such as SNU-16 and not, however, in KG-1 nor KMS-11 cell lines [5]. The aim of our work was to perform dose responses of the AZD4547 compound on a panel of representative cancer cell lines with genetic alterations of FGFR1-2 and 3: KG-1, SNU-16, and KMS-11 respectively. The levels of phosphorylated proteins, including in the FGFR signaling pathway, were assessed to establish a differential profiling of phosphorylation levels for the cell line. We then investigated a dose response of AZD4547 on the inhibition of FGFR signaling pathways in the three cell lines using several HTRF assays.



FGFR signaling pathway

Materials and methods

Experimental flowchart



Cell models and reagents

KG-1 cells (ATCC® CCL-246™), human myeloid leukemia cell line, were cultured in Iscove's Modified Dulbecco's Medium supplemented with 20% FBS and 1% antibiotics (penicillin/streptomycin), and plated in 96-well white plates half area at 200,000 cells/well, 1h before treatment.

SNU-16 cells (ATCC® CRL-5974™), human gastric cancer cell line, were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin) and plated in 96-well white plates half area at 100,000 cells/well, 1h before treatment.

KMS-11 cells (JCRB cell bank, JCRB1179), human multiple myeloma cell line, were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin) and plated in 96-well plates at 200,000 cells/well, 24h before treatment.

HTRF cellular assay kits (Revvity) and microplates used for this study were:

- Phospho-FGFR1 (Tyr653/654) # 64FGFR1Y6PEG and Total FGFR1 # 64FGFR1TPEG
- Phospho-FGFR2 (Tyr653/654) # 64FGFR2Y6PEG and Total FGFR2 # 64FGFR2TPEG
- Phospho-FGFR3 (Tyr647/648) # 64FGFR3Y6PEG and Total FGFR3 # 64FGFR3TPEG
- Advanced phospho-ERK (Thr202/Tyr204) # 64AERPEG and Total ERK # 64NRKPEG
- Phospho-MEK1/2 (Ser218/222) # 64ME2PE and Total MEK1 # 64NE1PEG
- Phospho-PLCγ1 (Tyr783) # 64PLCG1Y3PEG and Total PLCγ1 # 64PLCG1TPEG
- Phospho-AKT1/2/3 (Ser473) # 64AKSPEG - Phospho-AKT1/2/3 (Thr308) # 64AKTPEG and Total AKT # 64NKTPEG
- Phospho-STAT5 (Tyr694) # 64AT5PEG
- Alpha-Tubulin Housekeeping # 64ATUBPEG
- Human c-Myc cell-based # 63ADK053PEG
- Proxiplate Plus 384 Lv white plates #6008280

Experimental conditions

Cell treatments and sample generation:

To assess phosphorylation and total levels of FGFR1-3, cells were stimulated for 10 min with the FGF2 agonist at 37 °C, according to the assessment of the most effective dose.

To evaluate phosphorylation and the total levels of various target proteins, cells were incubated with a titration of AZD4547 for 6 h at 37 °C for SNU-16 and KMS-11 cells, and 24 h at 37 °C for KG1 cells. As KG-1 cells exhibit high basal phosphorylation of FGFR1, it was not necessary to stimulate them with FGF2. However, SNU-16 and KMS-11 were stimulated for 10 mins with FGF2 (100 ng/ml and 200 ng/ml respectively) at 37 °C just before lysis.

Following stimulation, cell culture medium of the adherent cells (KMS-11) was removed and replaced with 50 µL of supplemented lysis buffer (Phospho-Total protein lysis buffer 4 # 64KL4FDF for all targets, or with Phospho-Total protein lysis buffer 3 # 64KL3FDF only, to assess Phospho-PLCg1 (Tyr783) and Total PLCg1 for 30 min at room temperature under gentle shaking.

For the suspension cells (SNU-16 and KG-1), 10 µL of supplemented lysis buffer were added for 30 min at room temperature under gentle shaking.

HTRF cellular assays:

HTRF cellular assays are no-wash sandwich immunoassays based on TR-FRET, where the fluorescent signal intensity is proportional to the analyte concentration in the sample. The detection is achieved using analyte-specific antibodies conjugated to donor and acceptor fluorophores. In the particular case of a phospho-protein, one of the antibodies specifically recognizes the phosphorylated residue(s) of interest.

To perform most of the HTRF assays, 16 µL of sample (cell lysate) were transferred into a 384-well low volume white plate, and 4 µL of pre-mixed HTRF detection reagents were added. For the Alpha-Tubulin Housekeeping kit, 4 µL of sample were transferred into the same plate, then 12 µL of kit diluent were added followed by the dispensing of 4 µL of premixed detection antibodies. Finally, for the human c-Myc kit, 10 µL of cell lysate were transferred into the same plate and 10 µL of the HTRF c-Myc detection reagents were added.

After incubation according to the manufacturer's instructions, the signal was recorded on an HTRF compatible microplate reader.

To ensure operation within the linear range of each assay, samples were measured neat and diluted. The results presented hereafter correspond to the optimal detection conditions.

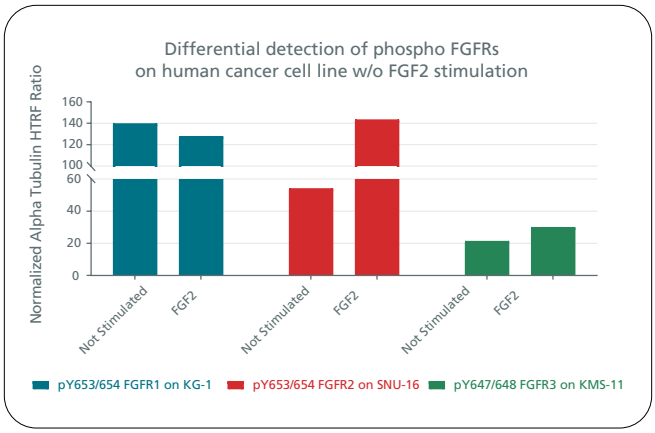
HTRF data handling:

The measurement of intracellular phospho- and total proteins is a relative quantification where the HTRF signal intensity (HTRF Ratio) is directly proportional to the concentration of the phosphorylated or total protein in the cell lysate. More details about HTRF signal treatment and analysis are available at https://fr.cisbio.eu/content/signal-treatment-and-analysis. More details about HTRF cell-based phospho-protein data normalization are available at https://fr.cisbio.eu/alpha-tubulin-housekeeping-cellular-kit-40556.

Results

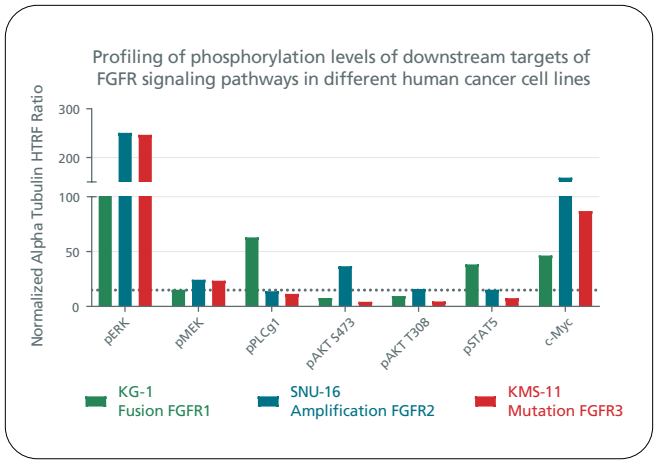
Prior to testing the AZD4547 inhibitor compound on the 3 respective cell lines, a preliminary study was performed to analyze basal levels of FGFR phosphorylation with and without stimulation, using the FGF2 pan-FGFR reference agonist.

As shown in Figure 1, a 10 min stimulation with the FGF2 ligand did not increase the detection of phosphorylation levels of FGFR1 in KG-1 cells, as the basal level in this cell line is already very high. However, FGF2 treatment induced a strong increase in FGFR2 phosphorylation in SNU-16 cells and a moderate FGFR3 phosphorylation in KMS-11 cells. Data are normalized against alpha-tubulin levels to take into account intracellular non-phosphoprotein targets and the fact that FGFR total proteins are not the same in the 3 cell lines studied here. Upregulation of the FGF/FGFR signaling pathway has been widely studied in several cancer types. In these 3 cell lines, the phosphorylation levels increased differently in the presence of ligand, as shown in Figure 1.



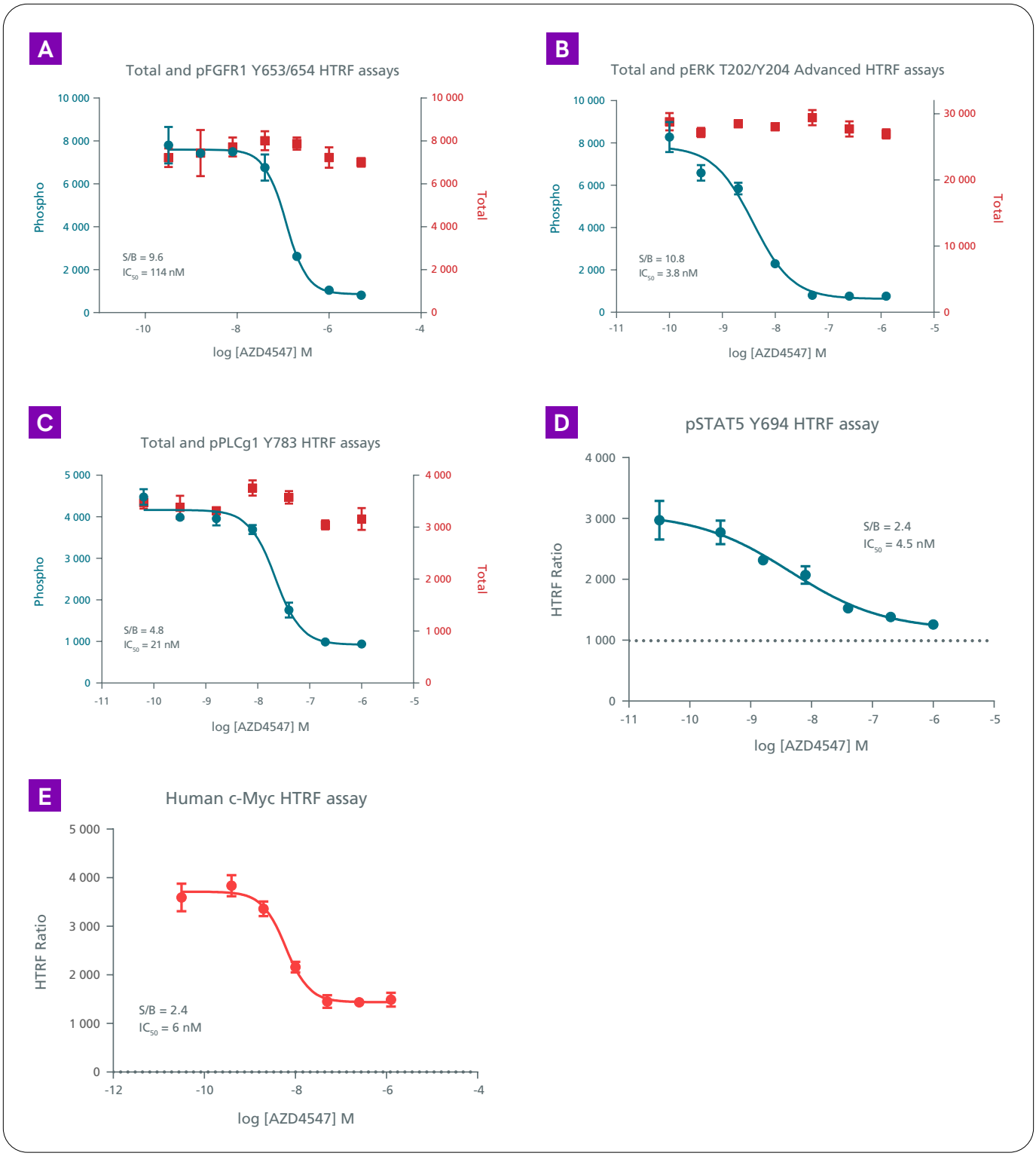
HTRF analysis of FGFR1-2-3 phosphorylation levels in three cancer cell lines upon treatment with FGF2.

We next sought to determine the phosphorylation status of other proteins downstream from the FGFR signaling pathway in the chosen panel of three cell lines. A screening of phosphoproteins was performed using optimal detection conditions for phospho-FGFR1-2-3 respectively in KG-1 (unstimulated), and in SNU-16 and KMS-11 cells (FGF2 stimulated). The results indicate different phosphorylation patterns depending on the cell line. For example, phosphorylation of PLCγ1 on Y783 residue and STAT5 on residue Y694 were detected in KG-1 cells only and pAKT on residue S473 was detected in SNU-16 cells, whereas pERK phosphorylation on T202/Y204 was observed in the three cell lines (Fig. 2). These results suggest that there are different profiles for FGFR pathway activation through the phosphorylation of the proteins involved, depending on the cell line and cancer type.



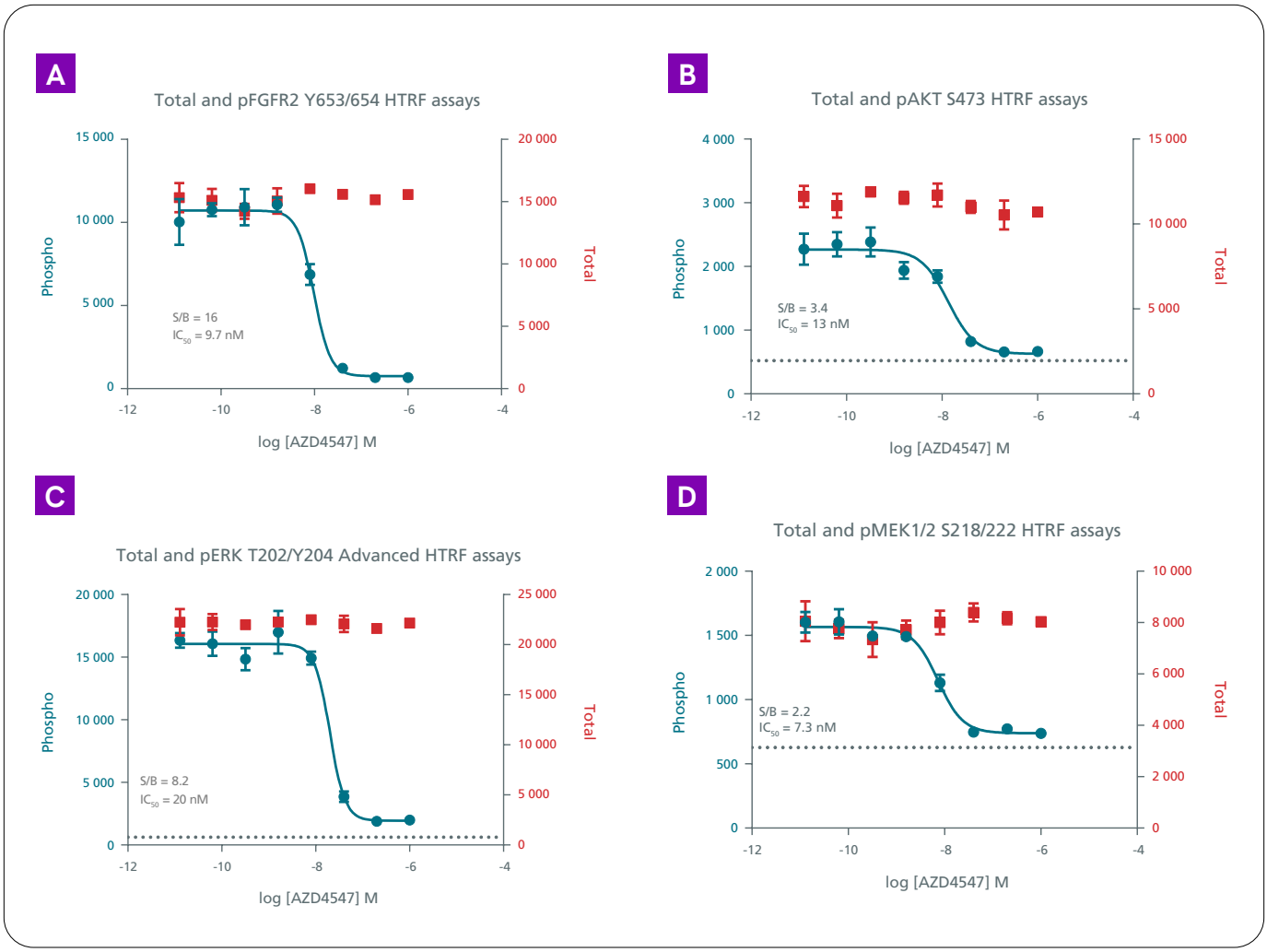
HTRF analysis of the phosphorylation levels of various key proteins involved in FGFR signaling pathways in three cancer cell lines.

This preliminary screen paved the way for the selection of pertinent activated phosphoprotein targets to be able to perform in-depth pharmacological profiles using the anti-cancer FGFR inhibitor AZD4547. Possible toxic effects were ruled out by monitoring the housekeeping protein Alpha-tubulin with the respective HTRF assays. No significant variations of alpha-tubulin protein were observed in any of the compound doses tested (data not shown).



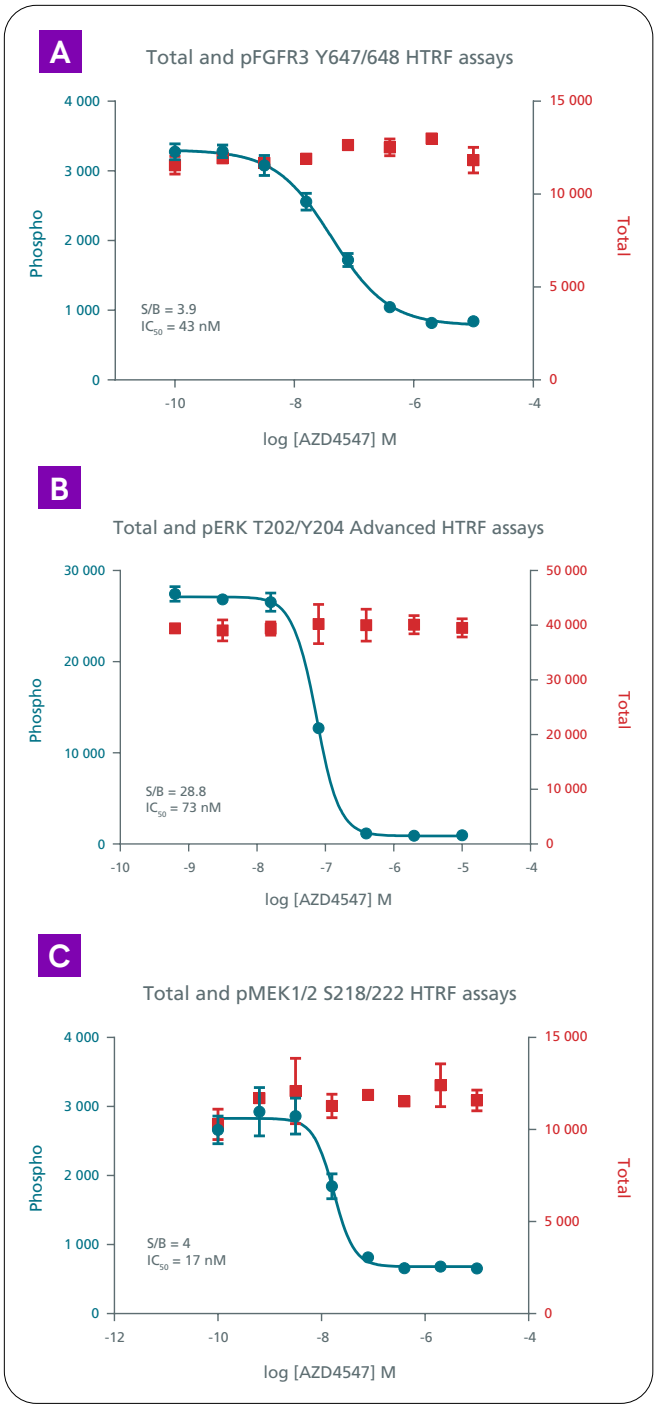
HTRF analysis of phospho (blue) and total (red) FGFR1, ERK, PLCγ1, phospho STAT5, and c-Myc levels in the KG-1 cell line.

For the KG-1 cell model, investigations were carried out on FGFR1, ERK, PLCγ1, and STAT5 phosphorylation levels as well as on c-Myc total expression. Total levels of phosphorylated targets were unchanged in KG-1 cells treated with increasing doses of AZD4547 (Fig. 3, A to E, in red). As expected, the compound induced an inhibition of the pathway by inhibiting phosphorylation throughout the pathway until the repression of c-Myc transcription factor protein expression.



HTRF analysis of phospho (blue) and total (red) FGFR2, ERK, AKT, and MEK1/2 levels in the SNU-16 cell line.

In the case of the SNU-16 cell line with FGFR2 amplification, a different pathway was triggered downstream of the FGF2 activated FGFR2 receptor (Fig. 2). Based on this preliminary screen, FGFR2, ERK, AKT, and MEK phosphorylation levels as well as c-Myc in cell lysates were selected as readouts for pharmacological profiles with the AZD4547 compound (Fig. 4, A to D). While the compound did not change total levels of these proteins, it induced a strong phosphorylation inhibition for these proteins included in the pathway. Despite these inhibitions, c-Myc transcription factor was detected but not modulated here in this cell line under these conditions (c-Myc data not shown).



HTRF analysis of phospho (blue) and total (red) FGFR3, ERK, and MEK1/2 levels in KMS-11 cell line.

Finally, the third cell line, KMS-11, also exhibited its own signaling pathway profile upon activation by the stimulation of FGFR3 receptor with the ligand FGF2 (Fig. 2). The modulation of phosphorylation for FGFR3, ERK, and MEK levels as well as c-Myc in cell lysates were performed. As expected, the total levels of these proteins remained constant whereas phosphorylation of FGFR3 Y647/648, ERK T202/Y204, and MEK1/2 S218/222 were suppressed by increasing concentrations of the pan-FGFR inhibitor. As in the SNU-16 cells, c-Myc was detected but not modulated in this cell line (data not shown).

Conclusion

Using a panel of three cell lines representing different cancer types, we observed that FGFR expression and activation levels differ depending on mutations, which demonstrates the importance of selecting the appropriate cell model for cancer research studies.

Our data showed a differential activation profile for the FGFR signaling pathways through the phosphorylation of associated downstream proteins. From this initial characterization, we then used a pan-FGFR inhibitor to inhibit these previously identified pathways. AZD4547, an inhibitor currently in clinical trials, inhibited FGFR activation and downstream signaling with IC_{50} values lower than 50nM, depending on the cellular background. The IC_{50} values reported with the HTRF kits are in line with the IC_{50} data reported in the literature for numerous studies [5]. The protein readouts used in this study are similar to those selected in reference articles describing FGFR pathways.

HTRF technology provides a fast, reliable, and sensitive quantification method for both total and phospho-protein expression levels in cell lysates, requiring less sample and time than conventional Western Blotting. Revvity offers a comprehensive portfolio of over 200 ready-to-use assay kits to monitor cellular signal transduction across all types of cancer cell models.

Bibliographic references

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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com