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Real-Time Nanoscale Proteomic Analysis of the Novel Multi-Kinase Pathway Inhibitor Rigosertib to Measure the Response to Treatment of Cancer

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Abstract

Introduction—Rigosertib (ON01910.Na), is a targeted therapeutic that inhibits multiple kinases, including PI3K and Plk1. Rigosertib has been found to induce the proliferative arrest and apoptosis of myeloblasts but not of other normal hematopoietic cells. Rigosertib has significant clinical activity as a therapy for patients with high-risk myelodysplastic syndrome (MDS) who are otherwise refractory to DNA methyltransferase (DNMT) inhibitors. Moreover, rigosertib has potential clinical activity in a multitude of solid tumors.

Areas covered—The objective of this review is to evaluate the mechanism of activity, efficacy and dosing of rigosertib. Furthermore, we discuss the challenge in the clinical development of rigosertib, to identify the specific patients that are most likely to benefit from this therapeutic agent. A PubMed search was performed using the following key words: rigosertib and ON01910.Na.

Expert opinion—We describe the application of a novel nanoscale proteomic assay, the nanoimmunoassay (NIA), a tractable approach for measuring the activity and predicting the efficacy of rigosertib, in real-time, using limited human clinical specimens. Our strategy suggests a possible paradigm where proteomic analysis during the pre-clinical and clinical development of a therapy can be used to uncover biomarkers for the analysis and prediction of efficacy in human patients.

Keywords

Nanoimmunoassay; myelodysplastic syndromes; polo-like kinase; phosphatidylinositol 3-kinase; Rigosertib; ON 01910.Na; DNA methyl transferase inhibitors

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This manuscript is dedicated to the memory of Shei-Yeh Chen.

1. INTRODUCTION

Cancers are frequently addicted to specific oncogenic signaling pathways (1–14). The targeted inactivation of kinases has become a general paradigm for the treatment of cancer. Tyrosine kinase inhibitors have been shown to have activity in hematopoietic and solid tumor malignancies (15–19). However, these therapies although initially effective in the treatment of some cancers, generally eventually fail. Hence, novel therapeutic approaches are required.

Rigosertib is a novel therapeutic agent with multi-kinase inhibitory activity with unique features that make it a potentially exciting therapy for human cancer. Rigosertib is the sodium salt of (E)-2,4,6-trimethoxystyryl-3-carboxymethylamino-4-methoxybenzyl sulfone, a member of a broader class of unsaturated sulfone kinase inhibitors (20). Notably different from other kinase inhibitors, this class of molecules does not compete for the ATP-binding site of kinases but rather, allosterically inhibits substrate binding (Figure 1). As a result, this novel mode of therapeutic action offers a potentially complementary approach for inhibiting kinase activation.

The development of targeted cancer therapeutics presents many novel challenges. To interrogate and predict therapeutic efficacy, it would be ideal to be able to directly measure biological activity not only in pre-clinical models but also in human patients. We have developed a novel nanoimmunoassay (NIA) for interrogating the efficacy of therapeutics (21–23). NIA is performed on a highly sensitive, automated, microcapillary electrophoresis-based platform (Nanopro1000, Protein Simple) that can separate proteins by charge, followed by antibody detection (21–29,30, 31–34). Proteins and ampholytes are loaded into the capillary, an electric current is passed through the capillary to separate proteins by charge. Since different phosphorylation states have discrete isoelectric points, separation by charge is able to resolve unphosphorylated, single- and multi- phosphorylated isoforms of individual proteins within the capillary. Next, the proteins are immobilized to the capillary wall. The protein of interest is identified and quantified using antibody detection, with a chemiluminescent signal. This approach is quantitative for both protein abundance and phosphorylation state. Results for 12 capillaries are obtained in 4 hours, enabling same day results. Up to 96 measurements can be made in a single overnight run. This technology enables the real-time analysis of even limited clinical specimens from blood, bone marrow biopsies and fine needle aspirates (FNA's) (21, 22). As few as 25 cells, 5 pgs and 20 nl of material can be examined. NIA overcomes many of the barriers of conventional proteomic methods for analysis of small amounts of clinical material.

The first report of NIA in clinical specimens measured levels of oncogenic signaling molecules in diagnostic samples and clinical trial correlative study samples. Fan et al. examined the levels of MYC and BCL2 oncogenes in 49 diagnostic lymphoma specimens to distinguish Burkitt's lymphoma from follicular lymphoma and benign hyperplasia. In a study of daily atorvastatin for patients with lymphoma, decreases in phospho-STAT3/5 from baseline were quantified in tumor cells sampled from patients after 8 days of treatment. In a study of patients with chronic myeloid leukemia (CML) treated with tyrosine kinase inhibitors, a decrease in a mono-phosphorylated ERK isoform distinguished patients who

responded to treatment from those who did not (22). In another CML study, Goff et al. used NIA to quantify decreases in phospho-CRKL in leukemia stem cells from patients in blast crisis treated with dasatinib (31). Kentsis et al. demonstrated that acute myeloid leukemia cells are dependent on autocrine activation of phospho-MET isoforms detected by NIA (27). NIA analyses have also been used in solid tumors. Fan et al. analyzed over 200 FNA's obtained from solid tumors using NIA, comparing tumor cells with paired adjacent non-tumor tissue. NIA has shown that differences in MAPK signaling between tumor cells and non adjacent tissue can distinguish the presence of tumor in specific malignancies (35). NIA is currently being applied in a study of renal cell carcinoma (RCC) to profile changes in hypoxia signaling in cells serially sampled from patients treated with tyrosine kinase inhibitors directed at the VEGF pathway (36). Hence, NIA is a powerful approach to evaluate the activity of kinase inhibitors and has been integrated into the clinical investigation of rigosertib.

2. Rigosertib's Mechanism of Action and Anti-Cancer Activity

Rigosertib inhibits multiple kinase activities both *in vitro* and *in vivo* (Figure 2). Rigosertib appears to exert its clinical activity through the inhibition of dephosphorylation of gene products that regulate mitosis (37, 38) (Figure 2). Rigosertib treatment of cancer cells induces both marked mitotic spindle abnormalities and abnormal centrosome localization. This induces the misalignment of chromosomes, activates the G₂-M checkpoint, and results in mitotic catastrophe and cell death (39, 40). The activity of rigosertib phenocopies the inhibition of the mitotic kinase, polo-like kinase1 (Plk-1) (41–44) (Figure 2). Further, rigosertib inhibits PI3K activity in mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL) (40, 45). In addition, rigosertib activates apoptosis associated with the induction of pro-apoptotic gene products from the Bcl-2 family and the inhibition of the activation of anti-apoptotic proteins such as Mcl-1 (45). Hence, rigosertib activity is demonstrated through the inhibition of both the PI3K and Plk-1 pathways (Figure 2).

In vitro, rigosertib induces cytotoxicity against a broad range of human tumor-derived cell lines, with IC₅₀s ranging from 50 to 250 nM (39, 46). Rigosertib treatment alone inhibits tumor growth in xenograft models (39, 46, 47). In fact, the addition of rigosertib improves the activity of oxaliplatin, doxorubicin, gemcitabine, paclitaxel, and vincristine. In a study conducted by Gumireddy et al, xenograft mice treated with a combination of rigosertib and oxapliplatin or doxorubicin experienced a total reduction in tumor presence. In the same study mice treated with a combination of gemcitabine and rigosertib also experienced a significant decrease in tumor size (39). Notably in mice, rigosertib appears to provide some protective effect against doxorubicin-induced weight loss, cardiotoxicity, and death (48). Moreover, *in vitro* studies show that rigosertib does not exhibit cytotoxicity in the blood, liver or brain. Thus, rigosertib appears to have synergistic anti-cancer activity with existing cytotoxic agents and moreover, does not induce cytotoxicity in non-neoplastic human cells. Rigosertib demonstrated strong synergy with numerous chemotherapeutic agents frequently causing the complete regression of tumors. It acts as an inhibitor of Plk1 activity, thus inducing mitotic arrest of tumor cells. (39).

The relative selective toxicity of rigosertib to malignant but not to nonmalignant normal hematopoietic cells was demonstrated dramatically in patients with MDS (49). The *in vitro* treatment with rigosertib of malignant blasts from patients with MDS decreased their numbers as measured by CD34⁺ and CD33⁺ bone marrow precursors, as well as measurement of Trisomy 8 (49). In contrast, normal human bone marrow progenitors exhibited markedly reduced toxicity to rigosertib (50). Rigosertib had specific effects on cell cycle related proteins in the CD34⁺ blast cells from patients with Trisomy 8. Notably, blast cells expressed high levels of oncoproteins cyclin D1 and *c-myc*, and anti-apoptotic protein survivin (51, 52). However, we note that the distinction of malignant from “normal” CD34⁺ cells based on the presence or absence of trisomy 8 may not be definitive, since all myeloid cells in MDS are likely clonal (53). Rigosertib may be an effective agent for hematopoietic malignancies including MDS, as described further below.

3. Clinical Studies of Rigosertib in Solid Tumors

Rigosertib has been evaluated as a single agent as well as in combination therapy for patients with breast, colon, pancreas, ovarian, lung-, head- and neck cancer and lymphoma (Table 1). In the first-in-man Phase I study, 20 patients with solid tumors refractory to standard therapy were enrolled to determine the dose-limiting toxicities of the drug (DLT's) (54). Rigosertib was administered as a 2-hour infusion on days 1, 4, 8, 11, 15 and 18, in 28-day cycles. Dose levels of 80, 160, 320, 480, 800, 1280, 2080, 3120 and 4370 mg were given in single-patient cohorts. Dose limiting toxicities were identified at the 4370 mg dose, therefore 3120 mg was felt to be the maximum tolerated dose. In one out of seven patients treated at the dose level of 3120 mg, the dose limiting toxicity (DLT) was found to be grade 3 abdominal pain. Other toxicities included fatigue, pain (skeletal, abdominal and tumor areas), nausea, and urge to defecate. Pharmacokinetics revealed the distribution half-life to be 1 hour, and the elimination half-life to be 27 hours. Of note, one patient with hepatocellular carcinoma (HCC) had stable disease but withdrew consent after 2 cycles due to fatigue, and one patient with refractory ovarian cancer had an objective response after 4 cycles and remained progression free for 24 months. From these results, the 3120 mg dose was selected for further studies.

Rigosertib has been studied in combination with other chemotherapeutic agents in solid tumors. Jimeno et al. used molecular profiling of a novel xenograft system of primary pancreatic cancer to discover that Plk-1 mediates gemcitabine resistance (47, 55, 56). Treating tumors derived from fine needle aspirate (FNA) of gemcitabine-refractory xenografts with both rigosertib and gemcitabine resulted in tumor regression.

A phase I study combined rigosertib with gemcitabine in patients with solid tumors who failed standard therapy or were candidates for gemcitabine-based therapy (57). Forty patients were enrolled to determine the best dose for a Phase II study of the combination of rigosertib with gemcitabine, 19 in the dose-escalation phase and 21 in the expansion cohort. 19 of the 40 patients had pancreatic ductal adenocarcinoma (PDA). One DLT occurred at the highest dose tested. Significant grade II/III toxicities included fatigue, nausea, neutropenia, anemia and thrombocytopenia. Three patients, one with thymic cancer, one with Hodgkin's lymphoma, and one with gemcitabine-refractory PDA, had partial responses to treatment.

The study established that 1800mg/m² a dosing schedule that is currently being used in a randomized phase II-III trial of gemcitabine ± rigosertib in advanced pancreatic cancer.

An oral formulation of rigosertib is being evaluated in patients with MDS and with advanced solid tumors. Results have been reported from a Phase I study of single-agent oral rigosertib in 48 patients with solid tumors including colorectal carcinoma, head and neck squamous cell carcinoma and ovarian carcinoma (64). Patients were treated twice daily with rigosertib for 21 day cycles, with doses ranging from 70–700 mg. Dose limiting toxicity was urinary toxicity. A complete response was observed in one patient with head and neck cancer, a partial response in another patient with head and neck cancer, and 8 patients with stable disease for greater than 12 weeks.

4. Clinical Studies of Rigosertib in MDS

Rigosertib has been shown to have activity in MDS (Table 2). Multiple studies have been conducted in patients with high risk MDS (49), including 4 phase I–II clinical trials with a total of 77 enrolled patients. Most patients (n=55, 71%) had been previously treated with DNA methyltransferase (DNMT) inhibitors, including 39 patients with higher-risk disease, (RAEB-1, -2, or -t). Enrolled patients were deemed to have a poor prognosis because they progressed, were refractory or failed treatment with DNMT inhibitors. Out of the 30 evaluable patients with excess bone marrow blasts (RAEB-1, RAEB-2, RAEB-t) who had previously failed DNMT inhibitor treatment, 12 (40%) achieved a 50% reduction in bone marrow blasts as their best response, including 5 (17%) patients with a complete bone marrow remission (mCR) per 2006 IWG criteria (58). Fifteen (50%) patients exhibited stabilization of bone marrow blasts (<50% change from pre-study values), while only three (10%) patients exhibited an increase in the fraction of blasts. A measurable clinical response in the number of bone marrow blasts was observed across all World Health Organization (WHO)/French-American-British (FAB) disease categories, including 4 (40%) patients with RAEB-1, 5 (38%) patients with RAEB-2, and 3 (43%) patients with RAEB-t. The subset of 13 patients who received rigosertib 1800 mg/24hr × 3 days every other week (the dosing schedule currently being used in the pivotal phase III trial) included 5 patients with stable disease, 2 patients who achieved a partial bone marrow response, and all 5 patients who achieved a mCR.

In all 4 Phase I/II studies, patients were observed to achieve cytogenetic and hematologic responses. Nine patients were evaluable for cytogenetic response. One patient with complex cytogenetics achieved a major cytogenetic response. Another patient with Trisomy 8 MDS achieved a minor cytogenetic response and two additional patients achieved reductions in abnormal metaphases by 23% and 40%, respectively. In the remaining 5 evaluable patients, the number of abnormal metaphases did not change. Out of 39 patients previously treated with DNMT inhibitors, hematological improvements (58, 59) were seen in a total of 5 (13%), including 1 erythroid response, 3 neutrophil responses and 3 improvements in platelet counts. Of these five patients, 3 achieved bone marrow blast stabilization, one achieved a partial bone marrow response, and one patient achieved a complete bone marrow response.

The median overall survival of the 39 patients with RAEB-1, -2, -t previously treated with DNMT inhibitors was 36 weeks. Median overall survival was 85 weeks for the 7 patients alive at the time of analysis. In 15 patients who received rigosertib 1800 mg/24hr $\times$ 3 days every other week (the dosing schedule currently being used in the pivotal phase III trial), median survival was 37 weeks. The best bone marrow blast response appears to be predictive of overall survival in these studies. It is important to note however, that while early blast clearance is a predictor of survival of patients with AML (60), this has not yet been shown to be a predictor of survival in MDS and holds relatively moderate prognostic value, especially in the recently revised IPSS (61). Thus bone marrow blast response will be studied as a secondary endpoint in the ongoing phase III pivotal trial for patients with MDS.

Based on the results of the 4 phase I–II rigosertib studies in MDS, a randomized phase III trial of IV rigosertib is being conducted for patients with MDS (RAEB-1, RAEB-2, RAEB-t) or chronic myelomonocytic leukemia (CMML). Patients are eligible if they have progressed on, or are refractory to DNMT inhibitors. The study is randomized 1:2 to best supportive care that can include low-dose cytosine arabinoside versus best supportive care plus rigosertib (1800 mg/24hr $\times$ 3 days every 2 weeks) for 16 weeks, followed by treatment every 4 weeks until progression or death. The accrual goal of 270 patients has been met, with overall survival as the primary endpoint, and bone marrow blast response, cytogenetic response, hematologic improvement and CR/PR, secondary infections, and quality of life, as secondary endpoints.

5. Nanoscale Proteomic Analysis of Patient Cells to Predict Outcome of Rigosertib

The ability to directly measure the activity of a targeted drug before and after administration to a patient may provide a strategy to individualize treatment. Recently, we have described a NIA that provides a tractable approach for rapid measurement of drug action. We have developed methods that enable the examination of limiting amounts of clinical material from blood, FNA, biopsy, bone marrow. Conditions for processing, storing and transporting specimens have been defined. Measurements are highly reproducible (22). For solid tumors, NIA is especially well suited for proteomic studies, since samples do not require laborious processing either into single cell suspensions or the fixation of the tissues. For NIA analysis, samples of solid tumors can be snap frozen, stored at -80°F and then shipped on dry ice. NIA profiling of serial tumor core biopsy samples has been incorporated into a Phase II study of oral rigosertib for the treatment of head and neck cancer. In this study, investigators will obtain a core biopsy of the tumor that will be immediately snap frozen and subsequently examined. NIA that can analyze nanoscale amounts of tumor lysate can greatly facilitate proteomic profiling of clinical material.

Hence, NIA can be used to serially sample tumor cells from patients including FNA's and FACS sorted cells such as CD34+ cells from bone marrow (Figure 3) (21–23). In these samples, we have measured changes in signaling before and during treatment with kinase inhibitors in MAPK, PI3K, STAT, proliferative and apoptotic pathways. NIA represents a tractable approach to measure biologic activity in clinical studies.

NIA has been used to examine the biologic activity of rigosertib in MDS patients (21). NIA measured changes in intracellular phosphorylation of AKT2 and its isoforms in CD34⁺ bone marrow cells sampled from 5 patients before and after 1 cycle of treatment. In 3 clinical responders, AKT2 phosphorylation decreased by 12% to 19% following cycle 1. In contrast, in 2 patients who progressed on treatment, AKT2 phosphorylation did not decrease, or increased by 19% post therapy, respectively. NIA provided high resolution of different phosphorylated and unphosphorylated states of AKT2, to reveal that the decrease in overall phosphorylation was predominantly due to a decrease in phosphorylated AKT2 isoform 3 (21). NIA was also used to demonstrate that rigosertib treatment can be associated with changes in the MAPK pathway: patients who responded to treatment had a 20% mean decrease in MEK1 phosphorylation (35). In order to develop therapeutics that target cancer signaling pathways, it is essential to quantify changes in protein activity within a clinical setting. The development of NIA enables quantification of unphosphorylated, single- and multi- phosphorylated isoforms of proteins within patient samples. NIA can provide rapid, high-resolution analysis of biologic response to novel therapeutics.

Notably, other proteomic approaches have been used to examine rigosertib activity. Flow cytometry has been used to study rigosertib signaling. Olnes used flow cytometry to measure cyclin D1 levels in CD34⁺ cells from peripheral blood of 3 patients (62) at serial time points after rigosertib infusion. 3 out of 3 patients tested exhibiting hematologic improvements had decreased the fraction of CD34⁺ cells expressing cyclin-D1 by approximately 50% from baseline. In contrast, patients who did not have hematologic improvements failed to have decreases in Cyclin D1. Hence, standard flow cytometry can be used to measure changes in cell cycle proteins in patients receiving rigosertib.

In addition, Multi-color flow cytometry can be used to perform single cell network profiling (SCNP), in which functional signaling profiles of protein networks are analyzed after *ex vivo* stimulation of cells with various cytokines. Cesano used SCNP to evaluate CD34⁺ functional signaling profiles of PI3K and STAT pathways in patients receiving rigosertib on Phase II study 04–17 (63). Cells collected at baseline and during treatment were analyzed after cytokine stimulation. SCNP revealed CD34⁺ cells from a patient with stable disease on rigosertib exhibited slightly decreased PI3K axis responsiveness to stimulation, and a patient who progressed exhibited an increased responsiveness. Data from these two patients suggests the feasibility of measuring intracellular signaling responses using SCNP to predict the biological activity of rigosertib. This is a promising technology that deserves future clinical examination through the analysis of additional MDS specimens.

Taken together, preliminary results from multiple investigators suggest that short-term treatment with rigosertib is associated with a significant clinical response in the malignant population of MDS cells associated with a reduction in cyclin D1, phospho-AKT2 levels, and PI3K signaling. Early clinical suggest that in solid tumors, rigosertib induces a biological response. Ongoing proteomic studies of patients receiving rigosertib are incorporating NIA to measure changes in signaling during treatment of MDS as well as solid tumors including head and neck cancer. These studies should be able to define biomarkers of drug action *in vivo* that could predict the response to rigosertib.

6. Conclusion: Nanoscale Proteomics as a General Paradigm for Measuring Drug Activity

The development of rigosertib as a therapeutic has incorporated novel methodologies to both determine mechanism of activity and define the optimal combination with specific chemotherapeutic agents during both preclinical development and as part of ongoing clinical studies (21, 62, 64). These studies have suggested that proteomic measurements may be a powerful approach to predicting the clinical activity of rigosertib. Most pointedly, NIA has the unique advantage of enabling the detailed proteomic analysis of even rare populations of tumor cells.

The first suggestion of the value of proteomics in interrogating rigosertib activity came from preclinical studies. Rigosertib induced changes in biological activity in tumor cells, for example, in samples by FNA's from a novel pancreatic cancer xenograft model, provided a rationale for combining rigosertib with gemcitabine chemotherapy (47). In early clinical studies, proteomic methods including NIA, flow cytometry and SCNP have been used to measure changes in signaling after administering the drug to patients. Proteins in cell cycle and PI3K pathways, including a specific phospho-AKT2 isoform, have emerged as potential biomarkers of drug activity of rigosertib (21, 62, 63). Ongoing studies will further interrogate whether these biomarkers predict clinical response.

Then, in early clinical studies of solid tumors, rigosertib was found to have activity in head and neck tumors and pancreatic cancer that was associated with PI3K inhibition. The clinical responses seen in a small number of patients are suggestive of therapeutic activity, but remain to be validated in larger studies. Although the mechanism of activity appears to involve PI3K inhibition, this remains to be confirmed. Thus, the Jimeno group has shown that rigosertib inhibits the PI3K pathway as measured using a proteomic analysis of FNAs from a pancreatic cancer xenograft model (47). However, in patients, genetic alterations of PIK3CA and PTEN were seen in both patients who had, as well as in patients who did not have clinical benefit (64). This apparent discordance may relate to the fact that genetic profiling is not necessarily predictive of perturbations in the activity of pathways, since they can be altered through many genetic and nongenetic mechanisms. Hence, the direct proteomic assessment of the activity of the PI3K pathway may be a more sensitive way to actually measure the drug response. A multi-institutional Phase II study is underway that includes NIA assessments of the PI3K pathway before and during treatment. This study should be able to confirm the relevance of the PI3K pathway.

Most recently, in hematologic disorders, rigosertib has been shown to be clinically active against higher-risk MDS refractory to DNMT inhibitors (21, 54, 57, 62, 65–76). Rigosertib was associated with significant reduction or stabilization in the number of bone marrow blasts that has been associated with specific proteomic changes. An ongoing pivotal phase III randomized clinical trial of IV rigosertib for patients with higher risk MDS resistant to DNMT inhibitors recently has met its accrual goal. Notably, in MDS, rigosertib induced reduction in the measured protein levels of both cyclin D1 as well as in the detection of the karyotypic abnormality of Trisomy 8. Pointedly, proteomic measurements suggest that a

reduction in phospho-AKT2 in the MDS blasts appears to be biomarkers of drug activity and treatment response.,

Indeed, proteomic measurements, using NIA have made it uniquely possible to directly quantify protein signaling changes even in specific rare populations of cells such as CD34+ bone marrow stem cells. This approach is part of the generally emerging paradigm that direct analysis of signaling pathways in the relevant subpopulations of tumors cells can be used to measure the activity of a drug in patients to individualize therapy (Figure 3). Notably, NIA also has been used to analyze nanoscale amounts of tumor lysate in solid tumors in even limited clinical specimens such as FNA's before and after therapeutic treatment in real time (22, 35, 36). Now, NIA assays have been optimized for proteins in numerous pathways including oncogene (MET, PI3K, STAT), proliferation (MYC, ERK, MEK), apoptosis (BCL2, cleaved caspase 3, JNK), metabolic and hypoxia pathways (22, 27, 31). Nanoscale protein profiling has potential to be applied to interrogate protein signaling across a broad range of malignancies.

There are several advantages of utilizing nanoscale proteomic technologies for clinical investigation. The gold standard for protein quantification, the Western Blot, requires approximately 1 million cells per analysis (using microgram quantities of protein input from cellular lysate), requires numerous manual steps to perform, and requires use of phosphospecific antibodies to detect phosphorylated isoforms. Standard flow cytometry requires 10,000 cells per analysis, is able to stain intact cells in a single cell suspension, and provide information on protein levels in single cells and different populations of cells within the specimen. However, flow cytometry also requires numerous manual steps to perform, and requires use of phospho-specific antibodies. SCNP, a flow-cytometry based technique, has all the same advantages and limitations of standard flow cytometry, but provides additional valuable information on the ability of cell signaling pathways to respond to *ex vivo* cytokine stimulation. In contrast, NIA can analyze as few as 25 cells per measurement (using nanogram quantities of protein input from cellular lysate), is performed on a fully automated instrument, and has a charge-based separation step that resolves different phosphorylated states into distinct peaks which can all be detected with a single antibody that recognizes a non-phosphorylated epitope, thus phospho-specific antibodies are not required for phospho-protein quantification. In particular, for solid tumors, NIA is especially well suited for proteomic studies, since samples do not require laborious processing into single cell suspensions or fixation. NIA and SCNP have been studied in large cohorts of patients in other contexts; however, for therapeutic monitoring of rigosertib, these promising methods need to be validated in larger numbers of patients. In the future, serial nanoscale proteomic analysis will be highly useful throughout drug development, including preclinical studies and clinical investigation.

7. Expert Opinion

Rigosertib is a member of a novel therapeutic class of multikinase inhibitors that function through the allosteric antagonism of substrate binding. Pre-clinical studies suggest activity in many tumor types, both as a single agent and in combination with approved agents. Rigosertib has been demonstrated to have encouraging clinical activity in patients with

myelodysplasia and head and neck cancer in phase I–III studies. Definitive clinical trials are in progress.

A key issue in the clinical development of novel therapeutics is to understand the mechanism of activity in patients. Though preclinical studies can indicate what pathways a drug targets and validate that cells can be “addicted” to that target, results are not necessarily the same when the agent is administered to patients. In clinical trials, many agents have failed because the mechanism of activity in patients could not be studied in real time. Only in retrospect have some agents been discovered not to reach their intended targets. Ideally, for kinase inhibitors, investigators would have the ability to measure activity of target protein pathways in malignant cells of individual patients prior to treatment, and also to monitor the kinetics and degree of target protein inhibition following treatment. The main barrier to achieving this using traditional methods such as Western Blot, has been the large amount of tissue required to make protein measurements, rendering it difficult to obtain enough cells for analysis through serial sampling. Now, with the availability of novel technologies such as NIA, even rare CD34+ cells from serial bone marrow specimens can be analyzed for changes in protein profiles.

Based on preclinical studies, rigosertib inhibits both the Plk1 and PI3K pathways. In these studies, activity of rigosertib is measurable through proteomic analysis using conventional methods, such as Western analysis and flow cytometry. Through the emerging technology of NIA, it appears that it may be possible to identify specific proteomic biomarkers associated with rigosertib in patients. As proof-of-principle, NIA has been used to show decreases in PI3K activity in patients treated with rigosertib. Conventional flow cytometry and SCNP have also been used to show inhibition of the Plk1 and PI3K pathways, respectively. We note that the studies of NIA and SCNP for rigosertib reported to date have only analyzed small numbers of specimens. Ongoing, larger studies will determine if serial nanoproteomic analysis before and after therapeutic treatment with rigosertib predicts clinical activity. If successful, novel proteomic studies could have the potential to 1) confirm inhibition of the putative target in each patient, assuring that the drug has reached the malignant cells, 2) measure how long the target pathway is suppressed, 3) analyze if compensatory pathways are upregulated 4) determine biologic response to the drug in the cells of interest [by assessing cell cycle arrest, differentiation, apoptosis, etc.], and 5) develop biomarkers to stratify patients for treatment, measure proteomic response and understand therapeutic resistance.

Currently, very little is known about mechanisms of resistance to the unsaturated sulfone kinase inhibitors in patients. As the rigosertib clinical studies mature, proteomic approaches will likely help determine the mechanism of therapeutic resistance to rigosertib. If the mechanism of rigosertib resistance is found to be distinct from the more typical kinase inhibitors that inhibit ATP binding, rigosertib could be potentially combined with other kinase inhibitors for increased therapeutic efficacy and decreased resistance over time. Rigosertib is a new drug with many unique features: it is an allosteric kinase inhibitor, it targets both Plk1 and PI3K, and nanoproteomic studies show it may inhibit a specific phospho-isoform of AKT2. Ongoing studies will define which patients with hematologic and solid malignancies will benefit from this promising new therapeutic agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Rigosertib is allosteric inhibitor of protein kinase activity. Rigosertib exhibits a novel mechanism of action. Rather than targeting the kinase active site, rigosertib binds to the target protein thereby blocking the ability to interact with its substrate. This has potentially important implications for it would enable one to consider combining Rigosertib with other kinase inhibitors

Figure 2.

Rigosertib is a multi-kinase inhibitor that can influence signaling of multiple pathways important for cellular proliferation and survival. Rigosertib inhibits both Plk-1 and PI3K. This could explain rigosertib's influence on AKT activity, thereby regulating protein synthesis, cell survival, proliferation and metabolism. Rigosertib is also likely to influence the activity of other gene products.

Figure 3. Rigosertib activity can be measured through the serial analysis of specific proteins from clinical materials

Serial samples of CD34+ cells from bone marrow before and after rigosertib treatment can be analyzed for changes in protein activity. Recent results suggest that phosphorylated AKT2 decreases in response to treatment.

Table 1

Ongoing and completed clinical trials of rigosertib

Study No.	Phase	Study Design	Route of Administration	Study Population	(Planned) Enrollment	Outcome
04-01	I	Open-label, single center, dose-escalating	IV	Adult patients with advanced malignant solid tumors	20	A refractory ovarian cancer patient had a PR after 4 cycles and remained progression free for 24 months (3)
04-02	I	Open-label, single center, dose-escalating	IV	Adult patients with advanced cancer	28	Of 22 evaluable patients, 9 (41%) achieved SD (4)
04-03	I	Open-label, single center, dose-escalating	IV	Adult patients with advanced cancer	40	Of 36 evaluable patients, 12 (33.3%) achieved SD (5)
04-04	I	Open-label, multicenter, dose escalating	IV	Adult patients with advanced solid tumors	25	Of 14 evaluable patients, 5 (35.7%) achieved SD
04-12	II	Open-label, single-center, single-arm, single dose	IV	Adult female patients with recurring platinum-resistant ovarian cancer	1	Study discontinued for slow enrollment
04-12A	II	Open-label, multicenter, single-arm, single dose	IV	Adult female patients with recurring platinum-resistant ovarian cancer	17	Pending
04-06	I	Open-label, single-center, dose escalating (RIG only), 2-arm (RIG + IRI or RIG + OXA)	IV	Adult patients with advanced solid tumors	18	In the RIG+OXA cohort, 1 patient with metastatic ovarian cancer had a PR (17.4 weeks). Four patients had SD (6).
04-08	I	Open-label, single-center, dose escalating (RIG only), 2-arm (RIG + IRI or RIG + OXA)	IV	Adult patients with hepatoma and other advanced solid tumors	16	PR was observed in 1 patient with chemotherapy-refractory ovarian cancer (11 wks) and in another patient with metastatic breast cancer (13 wks). SD was observed in 4 patients with colon cancer (42 wks and 12 wks), ovarian and renal cancer (12 wks) (7).
04-09	I	Open-label, single-center, dose escalating (RIG only) (RIG + GEM)	IV	Adult patients with advanced or metastatic solid tumors	40	PRs were observed in 1 patient with gemcitabine-pretreated Hodgkin lymphoma, 1 with thymic cancer, and 1 with gemcitabine-pretreated pancreatic cancer. Another patient with gemcitabine-naïve pancreatic cancer had an unconfirmed response. One patient with non-small cell lung cancer achieved SD (48 weeks). (8)
04-10	I	Open-label, single-center, dose escalating (RIG only)(RIG + GEM)	IV	Adult patients with advanced or metastatic solid tumors	10	Study discontinued for safety reasons.
04-22	II/III	Multicenter randomized controlled (GEM versus GEM + RIG)	IV	Adult patients with previously untreated metastatic pancreatic cancer	150 in Phase II portion. Sample size recalculation after 100 deaths.	Ongoing

Study No.	Phase	Study Design	Route of Administration	Study Population	(Planned) Enrollment	Outcome
04-05	I	Open-label, single-center, dose escalating	IV	Adults with refractory leukemia or poor risk MDS	21	Four patients achieved bone marrow CR, 2 bone marrow PR, hematologic improvement (1 erythroid, 1 platelet), 3 patients SD (9).
04-15	I/II	Open-label, single center, single arm	IV	Adult patients with MDS and AML	36	Two of 6 patients had significant decreases in bone marrow blast counts compared to pre-treatment values. Two patients had hematological improvements (neutrophil and erythroid) (10).
04-17	II	Open-label, single center, single arm	IV	Adult MDS patients with Tri 8, or Int-1, Int-2, or high-risk MDS	13	Four patients with bone marrow CR among 8 with SD (2)
07-H-0225	I	Open-label, single center, dose escalating	IV	Adult RAEB-1 and RAEB-2 MDS and AML with Tri 8	14	Three patients had a bone marrow PR and 3 patients had hematologic improvements (1)
09-H-0094	I	Open-label, single center, dose escalating	IV	Adult patients with relapsed MCL, MM, CLL and related lymphoid malignancies	16	Six patients achieved SD (11).
04-19	I/II	Open-label, single center, single arm	IV followed by PO	Adult patients with relapsed or refractory AML or ALL or transformed myeloproliferative neoplasms	26	Six patients had SD, 4 patients had transient hematological improvement of platelets (12).
04-21	III	Open-label, multicenter, randomized, controlled. BSC + RIG versus BSC alone (2:1)	IV	Adult MDS patients with excess blasts relapsing after, or refractory to, or intolerant to AZA or DEC	270	Completed enrollment. Results pending.
09-03	I	Open-label, multicenter single and multiple dose escalating	PO	Adult patients with advanced cancer	6	Closed to enrollment
09-04	I	Open-label, single center, multiple-dose escalating	PO	Adult patients with advanced cancer	48	Activity was seen in head and neck SCCs (1 CR, 1 PR) and SD for 12 weeks was observed in 8 additional patients (13).
09-09	II	Open-label, multicenter	PO	Adult patients with relapsed or metastatic SCC who previously received platinum-based chemotherapy and/or chemoradiation therapy	80	Ongoing
09-01	I	Open-label, multicenter, dose escalating	PO	Adult patients with low, Int-1, Int-2 or high-risk MDS	33	Two bone marrow CRs in RAEB-1 patients previously treated with azacitidine. In addition, 4 patients each achieved transfusion independence and hematological improvements (14, 15).
09-02	I	Open-label, multicenter, single and multiple dose escalation	PO	Adult patients with refractory or progressive Int-1, Int-2, or high risk MDS	6	Ongoing
09-05	II	Open-label, multicenter, randomized (1:1), 2-arm, intermittent versus continuous dosing	PO	Adult, transfusion-dependent low or Int-1 MDS patients based on IPSS classification	60	Ongoing. Fifteen patients have been treated with intermittent dosing for at least 8 weeks. Seven (47%) patients achieved transfusion independence, which lasted 8 to 27+ weeks (16).

IV = intravenous; PO: oral; CR: Complete response/remission; PR: Partial response/remission; SD: Stable disease; RIG = rigoseritib, IRI = irinotecan; OXA = oxaliplatin; GEM = gemcitabine; AML = acute myeloid leukemia; ALL = acute lymphocytic leukemia; AZA = azacitidine; DEC = decitabine; Tri 8 = trisomy 8; RAEB = refractory anemia with excess blasts; MCL = mantle cell lymphoma, MM = multiple myeloma; CLL = chronic lymphocytic lymphoma; BSC: Best supportive care; SCC: Squamous cell carcinoma;

Table 2

Clinical trials of intravenous rigosertib in MDS

Study Number Cancer.gov registration number (reference)	07-H-0225 NCT00533416 (1)	04-05 NCT00854646	04-15 NCT00854945	04-17 NCT00906334 (2)
Institution	NHLBI	Icahn School of Medicine at Mount Sinai	Columbia University	Stanford University
Patient Population	Trisomy 8, refractory AML or MDS	MDS/AML refractory to DNMT inhibitors	Any MDS/AML	Trisomy 8 and Int-2 to High-risk refractory MDS
Rigosertib Dosing Regimen	800 mg/m ² × 3–5 days Q2W	650–1700 mg/m ² × 3–6 days Q2W	800–1500 mg/m ² × 2 days QW for 3 weeks of a 4- week cycle, amended to 1800 mg/24hr × 3 days Q2W	800 mg/m ² × 2 days QW for 3 weeks of a 4-week cycle, amended to 1800 mg/ 24hr × 3 days Q2W
Total Patients	14	14	36	13
Prior DNMT inhibitors	6	7	13	13
Responses	2 BmPR, 2 SD	2 BmCR, 2 BmPR	1 BmPR, 4 SD	3 BmCR, 3 BmPR, 5 SD

BmCR: Bone marrow complete response; BmPR: Bone marrow partial response; SD: Stable disease