Phase I Assessment of New Mechanism-Based Pharmacodynamic Biomarkers for MLN8054, a Small-Molecule Inhibitor of Aurora A Kinase

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Running title: Phase I pharmacodynamic evaluation of MLN8054
Abstract

The mitotic kinase Aurora A is an important therapeutic target for cancer therapy. This study evaluated new mechanism-based pharmacodynamic biomarkers in cancer patients in two Phase I studies of MLN8054, a small-molecule inhibitor of Aurora A kinase. Patients with advanced solid tumors received MLN8054 orally for 7 consecutive days in escalating dose cohorts, with skin and tumor biopsies obtained before and after dosing. Skin biopsies were evaluated for increased mitotic cells within the basal epithelium. Tumor biopsies were assessed for accumulation of mitotic cells within proliferative tumor regions. Several patients in the highest dose cohorts showed marked increases in the skin mitotic index after dosing. While some tumors exhibited increases in mitotic cells after dosing, others displayed decreases, a variable outcome consistent with dual mechanisms of mitotic arrest and mitotic slippage induced by antimitotics in tumors. To provide a clearer picture, mitotic cell chromosome alignment and spindle bipolarity, new biomarkers of Aurora A inhibition that act independently of mitotic arrest or slippage, were assessed in the tumor biopsies. Several patients, primarily in the highest dose cohorts, had marked decreases in the percentage of mitotic cells with aligned chromosomes and bipolar spindles after dosing. Evidence existed for an exposure-effect relationship for mitotic cells with defects in chromosome alignment and spindle bipolarity that indicated a biologically active dose range. Outcomes of pharmacodynamic assays from skin and tumor biopsies were concordant in several patients. Together, these new
pharmacodynamic assays provide evidence for Aurora A inhibition by MLN8054 in patient skin and tumor tissues.

Introduction

The mitotic spindle is a validated anti-cancer target, against which many small-molecule inhibitors have been tested. The majority of these inhibitors have targeted β-tubulin, a structural component of the mitotic spindle. As tubulin also plays a necessary role in non-mitotic processes, such as retrograde neuronal transport, tubulin-targeted cytotoxics carry the burden of on-target toxicities such as peripheral neuropathy (1).

Newer antimitotic agents targeting enzymes specifically involved in mitosis represent an alternative approach to antimitotic therapy that promises a narrower spectrum of on-target toxicities and the potential for antitumor activity where traditional antimitotics are ineffective. One such class of antimitotic agents targets the Aurora kinases: Aurora A, Aurora B, or both (reviewed (2-6)). Aurora A and Aurora B are serine/threonine kinases that play essential roles in mitosis.

Aurora A functions in centrosome separation, bipolar spindle assembly, spindle pole integrity, and chromosome segregation during mitosis (reviewed (7-12)). Consistent with the diverse roles of Aurora A kinase, perturbation of Aurora A function in tissue culture or in mice leads to a complex assortment of mitotic defects, including monopolar and multipolar mitotic spindles, failure of chromosome alignment at the metaphase plate, and chromosome
misseggregation during anaphase and telophase (13-17). The spindle defects that arise in cells lacking functional Aurora A lead to mitotic arrest in metaphase, presumably mediated by the spindle assembly checkpoint.

Evidence exists to support two alternative outcomes subsequent to arrest in metaphase. In some settings, mitotic arrest is prolonged and leads to death directly out of prometaphase. In others, the mitotic arrest is transient and is followed by inappropriate passage through mitosis, which is accompanied by chromosome segregation defects and then cell cycle arrest or death (18, 19). Such a diversity of terminal outcomes following mitotic arrest occurs with multiple antimitotic agents such as taxanes and KSP inhibitors (18, 19). In fact, the diversity of terminal outcomes subsequent to the engagement of the spindle assembly checkpoint represents a challenge for incorporating pharmacodynamic readouts in antimitotic drug development. For example, early attempts to place taxane pharmacodynamics on a rational footing by using the mitotic index as a predictive marker were abandoned after preclinical in vivo work showed weak (20) or nonexistent correlations (21) between the degree of mitotic arrest and tumor response. Similar observations were made in patient tumor biopsies (22). If mitotic arrest were the sole outcome induced by taxanes, a correlation between degree of mitotic arrest and tumor response would be expected; the lack of correlation is consistent with the dual mechanisms of mitotic arrest and mitotic slippage induced by antimitotics. Clearly, a more complete picture of the
mechanism of action of newer targeted antimitotic agents would help inform the development and interpretation of pharmacodynamic markers of these agents.

This paper describes in detail the Phase I pharmacodynamic activity of MLN8054, a selective small-molecule inhibitor of Aurora A kinase (23). The pharmacodynamic strategy for MLN8054 was challenged by the dual mechanisms of mitotic arrest and mitotic slippage that occur upon Aurora A inhibition. Therefore, a broad panel of pharmacodynamic markers was developed based on the diverse molecular sequelae that occur subsequent to Aurora A inhibition with MLN8054 (14). In addition to measuring mitotic and apoptotic cell accumulation in patient skin and tumor biopsies, the pharmacodynamic marker panel included mitotic cell chromosome alignment and spindle bipolarity. The incorporation of these latter markers was critical, as the degree of chromosome alignment and spindle bipolarity decreases with Aurora A inhibition regardless of whether a cell undergoes prolonged mitotic arrest or a transient mitotic delay followed by mitotic slippage. The goals of this Phase I pharmacodynamic study were two-fold: 1) to gain an understanding of the behavior of a new pharmacodynamic marker panel in a first-in-human clinical setting, and 2) to evaluate modulation of the Aurora A pathway in patient skin and tumor tissues with MLN8054 administration.
Materials and Methods

**Clinical trial design.** Two concurrent Phase I trials were performed in patients with advanced solid tumors to evaluate the dose limiting toxicities and maximum tolerated dose of MLN8054 administered orally for 7-21 consecutive days; clinical findings are described in detail elsewhere (24, 25). As a secondary objective, these trials aimed to evaluate the pharmacodynamic effects of MLN8054 on Aurora A inhibition in skin and tumor biopsies. The C10001 study was performed at 3 clinical sites in the United States, and the C10002 study was performed at 2 sites in Spain. Dose escalation followed a standard phase I 3+3 design. The information in this report was derived from patients dosed with MLN8054 four times daily (QID), with the largest dose in the evening hours.

**Blood Samples.** Blood samples for pharmacokinetic analyses were drawn once at baseline and serially for the second dose on day 7. Plasma was extracted from blood immediately and stored at -70°C until analysis. Analysis of plasma samples used a validated LC/MS/MS assay. The lower and upper limits of quantification for the undiluted MLN8054 samples were 5 ng/mL and 2500 ng/mL, respectively. Calculation of PK parameters was performed using WinNonlin Professional Version 5.2.

**Skin and tumor biopsies.** Skin and tumor biopsies were obtained from patients as previously described (26, 27). Institutional review board approval was obtained at all participating centers, and informed consent for pharmacodynamic analysis was received from patients who underwent biopsies. All samples were
fixed in 10% neutral buffered formalin overnight and stored in 70% ethyl alcohol before embedding in paraffin.

**Histopathology assessment.** 5-μm skin sections of formalin-fixed, paraffin-embedded skin punch biopsies were deparaffinized and stained by standard methods with hematoxylin and eosin using a Leica Autostainer XL (Meyer Instruments, Inc.). Stained sections were evaluated for the presence of mitotic and apoptotic cells within the basal epithelial layer.

**Skin mitotic index.** Immunohistochemistry was performed on 5-μm sections of formalin-fixed, paraffin-embedded skin punch biopsies. Sections were stained using the Discovery® XT automated slide staining instrument (Ventana Medical Systems). After sections were deparaffinized with EZ Prep™ solution (Ventana Medical Systems), antigen retrieval was completed with Cell Conditioning 1 solution (CC1, Ventana Medical Systems). Sections were stained with mouse anti-phospho-Ser/Thr-Pro, MPM-2 antibody (1:100; Upstate Biotechnology) and rabbit anti-Phospho-histone H3 polyclonal antibody (1:25; Upstate Biotechnology) for 60 minutes. The ARK Peroxidase (DAKO Animal Research Kit; Dako) method was used to prepare a working dilution of the MPM-2 antibody. Secondary antibodies included Alexa Fluor 488-conjugated Streptavidin (1:100; Molecular Probes) and Rhodamine-Red-X-AffiniPure goat anti-rabbit IgG (1:25; Jackson Immunoresearch). To reduce tissue autofluorescence, the slides were stained with 0.5% Sudan Black B (Sigma Research).
Aldrich) and mounted with DAPI Vectashield Hard Set Mounting Medium (Vector Laboratories).

The number of mitotic cells within the basal epithelial layer was counted manually in a blinded fashion. The length of the basal epithelial layer (BEL) was determined by acquiring skin biopsy images at low magnification (2x objective) using an automated microscope (Nikon Instruments) with filter wheel (Sutter Instruments), XYZ stage (Prior Scientific), CCD camera (Hamamatsu), and automated slide loader (Prior Scientific) controlled by MetaMorph software (Molecular Devices). Measurement of the BEL was performed using a touch pen and MetaMorph software to manually draw a region on the image; calibrated lengths of each sample were determined. The skin mitotic index was calculated by determining the number of mitotic cells per millimeter of BEL.

**Tumor mitotic index.** Formalin-fixed, paraffin-embedded tumor biopsies were sectioned at 5 μm. Sections were deparaffinized with xylene and hydrated with ethanol washes; antigen retrieval was performed with 0.01 M citrate buffer solution (pH 6.0) with a pressure-cooker heating source. Sections were stained with mouse anti-Ki67, K-2 mouse monoclonal antibody (1:1; Ventana) and rabbit anti-phospho-histone H3 polyclonal antibody (1:25) for 60 minutes at room temperature. Secondary antibodies included Alexa-488 Goat anti-mouse (A11001, 1:100; Molecular Probes) and Rhodamine-Red-X-AffiniPure goat anti-rabbit IgG (1:25; Jackson Immunoresearch). Slides were mounted with DAPI Vectashield Hard Set Mounting Medium (H-1500; Vector Laboratories). Images
were captured using the previously described microscope system (10x objective) and the mitotic index was determined from the percentage of phospho-histone H3 (pHistH3) immunopositive cells within the Ki67-positive area using Metamorph Software.

**Mitotic cell spindle bipolarity and chromosome alignment.** Formalin-fixed, paraffin-embedded tumor biopsies were sectioned at 5 μm and stained using the Discovery® XT automated slide-staining instrument. Sections were deparaffinized on the instrument with EZ prep™ solution and antigen retrieval was completed with CC1. Immunofluorescence staining was performed using mouse anti-α-tubulin clone DM1A FITC conjugated antibody (1:100; Cell Signaling) for 60 minutes. The previously described microscope system (40x objective) was used to acquire image stacks (26 focal planes spaced 0.2 μm apart). The resulting image stacks were deconvolved to remove out-of-focus information, the mitotic cells were identified, and 3D reconstructions of the cells were generated using MetaMorph image analysis software. Five or six scorers reviewed 3-dimensional rotations of each mitotic cell in a blinded, randomized order to characterize chromosome alignment (aligned versus not aligned) and spindle bipolarity (bipolar versus not bipolar) according to pre-established criteria. The majority call for each spindle was used for further analyses; spindles for which no majority call existed were not included. The concordance of all scorers (defined as the percentage of identical calls made by all possible pairs of scorers) was >85% for both the chromosome alignment and spindle bipolarity...
and assessments. Misaligned chromosomes and the absence of properly formed bipolar mitotic spindles are both hallmark phenotypes of Aurora A inhibition (14, 16).

**Exposure-response assessment of tumor pharmacodynamics.**

Plasma concentrations of MLN8054 were measured from patient blood samples to calculate steady-state areas under the plasma concentration-time curve over the dosing interval (AUC$_{0-\tau}$) using noncompartmental methods, as described previously (24, 25). For purposes of exposure-response assessments, steady-state average concentration ($C_{ss,avg}$, calculated as the area under the concentration-time curve [AUC$_{0-\tau}$] divided by the length of the dosing interval) was used as the independent variable in each patient with tumor pharmacodynamic data. The dependent variables were percentage of pre-treatment percentage of mitotic cells with aligned chromosomes and percentage of pre-treatment percentage of mitotic cells with bipolar spindles. The distribution of $C_{ss,avg}$ of MLN8054 over the clinically tested dose range (10-80 mg per day) was forecasted using SigmaPlot Version 11, based on the observed dose-normalized $C_{ss,avg}$ data in patients with steady-state PK measurements. In order to infer a biologically active dose range for MLN8054, the exposure-response relationship was viewed in the context of the dose-exposure relationship.
Results

All patients enrolled in both the C10001 and C10002 trials underwent ~3 millimeter skin punch biopsies for pharmacodynamic analysis. Patients underwent 3 skin biopsies; on day 1 prior to the first daily dose, on day 7, 2-4 hours before the second daily dose, and on day 7, 2-4 hours after the second daily dose (Supplementary Figure 1). The purpose of these biopsies was to detect inhibition of Aurora A by MLN8054 in proliferating basal epithelial cells. Assessment of Aurora A inhibition in the skin biopsies relied on detecting an accumulation of mitotic cells within the basal epithelial layer by immunofluorescent staining of 5 μm skin sections for the mitotic markers pSer\textsuperscript{10} Histone H3 (pHistH3) and MPM2, as well as for DNA. A mitotic index in skin was determined by counting the number of mitotic cells per millimeter length of the basal epithelial layer. In addition, the hematoxylin-eosin stained skin biopsy sections were evaluated histopathologically.

In the C10002 trial, approximately 2 of every 3 patients in each dose cohort underwent tumor biopsies. Biopsies were obtained on day 1 prior to the first daily dose and on day 7, 2-4 hours after the second daily dose (Supplementary Figure 1). Aurora A inhibition in the tumor biopsies was assessed by evaluating an accumulation of mitotic cells within proliferative tumor regions by immunofluorescent staining of tumor sections for pHistH3, KI67, and DNA. The mitotic index was evaluated in tumor biopsies by calculating the percentage of total cells (nuclei count) that were mitotic (pHistH3
immunopositive) within the proliferative tumor regions (Ki67 immunopositive) using automated image processing. In addition, mitotic cells within the tumor sections were assessed for mis-aligned chromosomes and the absence of properly formed bipolar mitotic spindles, which are both hallmark phenotypes of Aurora A inhibition (14, 16). Preclinical studies in HCT-116 colon tumor bearing immunocompromised mice demonstrated an exposure related decrease in tumor mitotic cell chromosome alignment and spindle bipolarity (Supplementary Figure 2).

In order to assess tumor mitotic cells for chromosome alignment and spindle bipolarity, sections underwent immunofluorescent staining for α-tubulin and DNA (Figure 1A). Examples of mitotic cells from patient tumor biopsies with aligned chromosomes / bipolar spindles, unaligned chromosomes / bipolar spindles, and unaligned chromosomes / non-bipolar spindles are shown (Figure 1B).

The pharmacodynamic activity of MLN8054 was evaluated in patient (Pt) 147 to exemplify the assays used in these studies. This patient was diagnosed with colorectal cancer that metastasized to liver and lung, and was treated with 70 mg/day of MLN8054 with QID dosing. The percentage of cells with aligned chromosomes was examined in the pre-dose and day 7 biopsies (Figure 2A). Sixty-eight percent of the mitotic cells had aligned chromosomes in the pre-dose biopsy, whereas 25% of the mitotic cells had aligned chromosomes in the day 7 biopsy. A similar decrease was observed when evaluating the percentage of
bipolar spindles: 67% of the mitotic cells in the pre-dose biopsy had bipolar spindles, and this decreased to 23% in the day 7 biopsy (Figure 2B). These tumor biopsies were also evaluated for mitotic index, which increased from 6.8% in the pre-dose biopsy to 9.7% in the post-dose biopsy (Figure 2C). This decrease in aligned chromosomes and bipolar spindles in mitotic cells and increase in mitotic index in the tumor biopsies after treatment with MLN8054 are outcomes consistent with inhibition of Aurora A. Skin biopsies from this patient also demonstrated an increase in the number of mitotic cells within the basal epithelial layer (Figure 2D and 2E). The mitotic index increased from 0.13 in the pre-dose biopsy to 3.72 and 3.04, respectively, in the two biopsies on day 7. In addition, when evaluating hematoxylin and eosin stained sections, many mitotic figures could readily be discerned in the day 7 biopsies, whereas these were rarely observed in the pre-dose biopsy (Figure 2F). Moreover, several of these cells were histopathologically characterized as being either pre-apoptotic or frankly apoptotic according to morphological features. The increase in the mitotic index along with the appearance of apoptotic cells in the skin biopsies after treatment with MLN8054 were outcomes consistent with inhibition of Aurora A.

The skin mitotic index was assessed in all patients who had evaluable pre-dose and day 7 biopsies from the C10001 and C10002 studies (n = 67; Figure 3A and 3B, respectively; Supplementary Table 1). For this evaluation, the mitotic index of the pre-dose biopsy was subtracted from the average mitotic index of the day 7 post-dose biopsies 1 and 2. Positive values reflect an increased mitotic
index in the post-dose biopsies relative to the pre-dose biopsies (baseline). On average, the pre-dose skin biopsies contained 0.20 mitotic cells per millimeter of basal epithelial layer. When examining patients across both studies at all doses within the QID dose cohort, there was a significant difference in the pre-dose biopsy mitotic index compared to the mean of the day 7 post-dose biopsies 1 and 2, which rose to 0.50 mitotic cells per millimeter of basal epithelial layer ($p < 0.0001$; Supplementary Table 2). Moreover, 48 of the 67 patients with evaluable pre-dose and post-dose skin biopsies showed an increased mitotic index. Nevertheless, the magnitude of this increase varied greatly, and in certain cases the increase may simply have been due to baseline variation. Notably, in several patients in the two highest dose cohorts of the C10002 study (70 and 80 mg), there were robust increases in the mitotic index in the day 7 biopsies relative to baseline. It is unclear why similar effects were not observed in the C10001 study in patients dosed in the 70 and 80 mg cohorts.

The tumor mitotic index was also evaluated for all patients that had evaluable pre- and post-dose tumor samples for this assay ($n = 15$; Figure 4A). In the pre-dose biopsies, the mitotic index ranged between 0% and 9.4% (Supplementary Table 3). Several patients demonstrated increased levels of mitotic cells after MLN8054 dosing; however, the magnitude of these increases varied considerably. In some patients, the mitotic index also decreased after dosing. Moreover, when examining the tumor mitotic index across all patients,
there was no difference in the pre-dose biopsy mitotic index when compared to the day 7 post-dose biopsies ($p = 0.5614$; Wilcoxon signed rank test).

Mitotic cell chromosome alignment and spindle bipolarity were examined in all patients that had evaluable pre- and post-dose samples of sufficient quality for this immunofluorescent based analysis ($n = 9$ of 15 available paired pre and post-dose biopsies). Unlike the changes in the tumor mitotic index assay, unaligned chromosomes and the non-bipolar spindles occur immediately after Aurora A inhibition in mitotic cells and are therefore refractory to the complexities that affect interpretation of the tumor mitotic index. In the pre-dose tumor biopsies, the percentage of mitotic cells with aligned chromosomes varied considerably, and ranged between 16% and 68% (Figure 4B; Supplementary Table 4). The percentage of mitotic cells with bipolar spindles ranged between 25% and 67% (Figure 4C; Supplementary Table 5). Regardless of the baseline levels for mitotic cell aligned chromosomes and bipolar spindles, several patients (5 of 9 examined), notably those in the highest dose cohorts (60, 70 and 80 mg), had marked decreases in the percentage of mitotic cells with aligned chromosomes and bipolar spindles after 7 days of MLN8054 dosing.

For patients with evaluable pre- and post-dose skin and tumor biopsies for the multiple pharmacodynamic readouts used in this study ($n = 9$), chromosome alignment and spindle bipolarity showed a strong concordance with each other (Pearson $r = 0.95$, $p < 0.0001$) (Table 1). Tumor mitotic index also correlated with the percent of mitotic cells with aligned chromosomes (Pearson $r = 0.69$, $p =$
0.039), and spindle bipolarity (Pearson r = 0.80, p = 0.0100). The correlation between tumor and skin mitotic index is not significant (Pearson r = 0.46, p = 0.21). It is interesting to note, however, that patients in the lowest dose cohorts had no pronounced effect in either the skin or tumor assays and the two patients with large increases in mitotic index in skin also showed large increases in tumor mitotic index.

Exploratory analysis of exposure-response relationships of tumor pharmacodynamic effects was consistent with a steady-state exposure-related decrease in the percentages of tumor mitotic cells with aligned chromosomes (Figure 5A) and bipolar spindles (Figure 5B). Based on the visual assessment of underlying exposure-response relationships, a steep relationship between systemic exposure to MLN8054 and tumor pharmacodynamic effects was observed. Near-maximal pharmacodynamic effects were observed at C_{ss,avg} of approximately 1500 nM or higher. Pharmacokinetic analysis in all patients treated in the C10001 and C10002 trials (n = 85) permitted projection of the distribution of C_{ss,avg} over the clinically tested dose range of 10-80 mg/day (Figure 5C). When the dose-exposure relationship was viewed in the context of the pharmacokinetic and pharmacodynamic assessments (Figure 5A and 5B), it appeared that doses of 50 mg/day or higher provided steady-state exposures associated with pharmacodynamic effects in tumor tissue in >50% of patients. Approximately 75% of patients treated in the 70-80 mg/day cohorts would be expected to demonstrate tumor pharmacodynamic effects following MLN8054 treatment.
However, the dose limiting toxicities of somnolence and transaminitis encountered in these trials (24, 25) precluded achieving these pharmacodynamically active doses for multiple cycles of treatment.
Discussion

This is the first description of the biologic effects of Aurora A kinase inhibition in human tumor and skin specimens obtained from patients with advanced solid tumors. A broad panel of mechanistic biomarkers was used to demonstrate Aurora A kinase modulation in patients treated with MLN8054. The pharmacodynamic marker panel comprised the mitotic index, a histopathologic assessment in skin biopsies; and the mitotic index, chromosome alignment and spindle bipolarity analyses in tumor biopsies. In several patients who had evaluable pre- and post-dose skin and tumor biopsies, there was a concordant response between all of these assays, which was consistent with Aurora A kinase inhibition.

This report also marks the first use of the chromosome alignment and spindle bipolarity biomarkers as a clinical pharmacodynamic readout for antimitotics. In part due to the challenging nature of the mitotic index, and in part due to the historic use of the term cytotoxic to describe antimitotics, myelosuppression has consistently been the pharmacodynamic readout described in clinical trials with this class of drugs. A number of antimitotic agents have been developed in the past decade with no pharmacodynamic readout other than myelosuppression. While myelosuppression has been demonstrated to be associated with clinical efficacy (28, 29), studies of alternate dosing schedules (for example with weekly taxanes in breast cancer) have demonstrated the therapeutic value of schedules that reduce myelosuppression.
without reducing clinical response rates (reviewed in (30)). This suggests that in at least some settings the pharmacokinetic/toxicity relationship for myelosuppression may not always track with the pharmacokinetic/response relationship. Thus, the development of the chromosome alignment and spindle bipolarity assays in patient tumor samples provides a significant addition to the toolbox for evaluating antimitotic drugs.

Caution was required in the interpretation of the results from the assays used in this study, as the chromosome alignment and spindle bipolarity assays were simultaneously developed and deployed in this first-in-human trial for a first-in-class molecule. Two factors in particular provided a measure of confidence around the interpretation of the data. First, the semi-quantitative nature of these assays (morphological assays with a binary readout) enabled us to minimize issues of signal-to-noise and immunofluorescence staining variability. The scoring of the individual spindles was performed in a randomized and blinded manner and was based on concordance between individual scorers. Second, the weight of evidence approach enabled a composite picture of the behavior of multiple markers simultaneously. For the majority of patients with tumor biopsies, there were no disagreements in the direction of change among all four markers (Table 1).

Tumor cells undergoing a mitotic delay as a result of Aurora A kinase inhibition would be expected to show defects in chromosome alignment and spindle bipolarity (14, 16). However, defects in spindle bipolarity and
chromosome alignment may not necessarily be accompanied by a mitotic delay. As was seen in the tumor mitotic index in patients treated with MLN8054, considerable variability in mitotic index may occur depending on the response of individual tumors to antimitotic agents as well as the duration of target inhibition. Given these complexities, the results of the tumor mitotic index in clinical studies with patients with different tumor types were difficult to interpret. However, a significant increase in the mitotic index of some sequential tumor biopsies obtained from patients treated with MLN8054 was observed, and this likely occurred through Aurora A inhibition. The marker used for determining the mitotic index in both skin and tumor biopsies was histone H3 phosphorylated on serine 10 (pHistH3). In cells, this phosphorylation is catalyzed by Aurora B. If MLN8054 were inhibiting Aurora B, a decrease in pHistH3 positive cells would occur, rather than an increase. Therefore, at the exposures achieved in these phase 1 studies, MLN8054 has proven to be selective for Aurora A relative to Aurora B.

Unlike the changes in the tumor mitotic index assay, unaligned chromosomes and non-bipolar spindles occur immediately after Aurora A inhibition in mitotic cells and are not influenced by the complexities that affect interpretation of the tumor mitotic index. Therefore, the incorporation of the mitotic cell chromosome alignment and spindle bipolarity assays in this study improved our ability to gauge Aurora A kinase inhibition in the tumor biopsies, as these readouts are only assessed in mitotic cells and are therefore independent
of the size of the mitotic fraction. Importantly, the quantitative endpoints in these assays permitted exploration of potential exposure-response relationships for the tumor pharmacodynamic effects, thus enabling conclusions regarding the biologically active dose range. Based on these analyses, pharmacodynamic activity of MLN8054 is expected to occur at doses at or above 70 or 80 mg/day.

Preclinical work performed \textit{in vitro} suggests that changes in the mitotic index, spindle bipolarity, and chromosome alignment are necessary for the death or terminal arrest of cells lacking functional Aurora A (14, 23). However, detection of these outcomes demonstrating Aurora A inhibition may not be sufficient for death or terminal arrest of cells. As such, the magnitude and duration of the pharmacodynamic modulation presented in this study cannot be interpreted as predicting clinical efficacy for this molecule because of the necessary but not sufficient nature of these pharmacodynamic biomarkers. In fact, there was limited evidence for antitumor clinical activity in the C10001 and C10002 trials (24, 25).

Nevertheless, the pharmacodynamic markers implemented in this study have been used to answer the specific question, ‘Is Aurora A activity modulated in skin and tumor tissue from patients treated with MLN8054?’ In that respect, the biomarker panel used in this trial provided clear evidence that Aurora A was inhibited in patients treated with MLN8054. These markers are now being implemented for the clinical evaluation of a second generation Aurora A kinase inhibitor MLN8237, which is being tested in multiple phase 1 and 2 clinical trials in cancer patients.
Acknowledgements

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References


19. Shi J, Orth JD, Mitchison T. Cell type variation in responses to antimitotic


FIGURE LEGENDS

Figure 1. Method for scoring chromosome alignment and spindle bipolarity in mitotic cells. (A) Tumor sections were immunofluorescence stained for α-tubulin and DNA. Stained sections were imaged by multiplane acquisition and the resultant Z-stack images were deconvolved and used to generate three-dimensional reconstructions of mitotic cells. These three-dimensional images were presented to multiple scorers in a random, blinded manner for scoring chromosome alignment and spindle bipolarity; the majority call was used for the final determination. (B) Tumor sections from MLN8054-treated patients were stained for α-tubulin (green) and DNA (blue). Representative examples of mitotic cells with aligned chromosomes / bipolar spindles, unaligned chromosomes / bipolar spindles, or unaligned chromosomes / non-bipolar spindles are shown.

Figure 2. Pharmacodynamic activity of MLN8054 in Pt 147. Tumor sections from Pt 147 were evaluated for the percentage of mitotic cells with aligned chromosomes (A), the percentage of mitotic cells with bipolar spindles (B), and the tumor mitotic index (C) in the pre-dose and day 7 biopsies. P-values demonstrate a statistical difference between the pre-dose and day 7 biopsies. (D) Sections of the pre-dose biopsy and day 7 biopsy were stained for the mitotic markers pHistH3 (red) and MPM2 (green) as well as DNA (blue). Adjacent high-resolution images were stitched to view the entire basal epithelial layer of the biopsies. Arrows point to pHistH3 and MPM2 double positive mitotic cells. (E) The average number of mitotic cells counted in 4 sections from skin biopsies taken in the pre-dose biopsy, the day 7 biopsy 1 and the day 7 biopsy 2. The P-value demonstrates a statistical difference between the pre-dose and either day 7 biopsy. (F) Hematoxylin and eosin stained skin in the pre-dose, the day 7 biopsy 1 and the day 7 biopsy 2. Arrows point to mitotic cells, arrow heads point to mitotic / pre-apoptotic cells and the asterisk is adjacent to an apoptotic cell.
Figure 3. Skin mitotic index in patients with evaluable pre-dose and day 7 biopsies. Mitotic cells (pHistH3 immunopositive) were counted in two to four 5 μM sections of each skin biopsy taken from patients enrolled in the C10001 (A) and C10002 (B) MLN8054 trials. The average number of mitotic cells in the pre-dose biopsy was subtracted from that of the day 7 biopsies (biopsy 1 and biopsy 2). Positive values represent an increase in the mitotic cells in the day 7 biopsies relative to the pre-dose biopsy, a phenotype consistent with Aurora A inhibition. The dose cohort in which each patient belonged is shown.

Figure 4. Tumor mitotic index and mitotic cell chromosome alignment and spindle bipolarity in patients with evaluable pre-dose and day 7 tumor biopsies. (A) The mitotic index was determined from the percentage of pHistH3 immunopositive cells that were also Ki67 positive. The percent pHistH3 positive cells in the pre-dose biopsy were subtracted from that of the day 7 biopsy. Positive values represent an increase in the mitotic index in the day 7 biopsy relative to the pre-dose biopsy; a phenotype consistent with Aurora A inhibition. The percentage of mitotic cells with aligned chromosomes (B) and the percentage of mitotic cells with bipolar spindles (C) in the day 7 biopsies were subtracted from those of the pre-dose biopsy. Positive values represent a decrease in the percent of mitotic cells with aligned chromosomes or bipolar spindles in the day 7 biopsy relative to the pre-dose biopsy; a phenotype consistent with Aurora A inhibition. The dose cohort in which each patient belonged is shown.

Figure 5. Exposure-response assessments of relationships between MLN8054 steady-state average plasma concentrations and cell chromosome alignment (A) or spindle bipolarity (B) in mitotic tumor cells. The percent of mitotic cells with aligned chromosomes or bipolar spindles were calculated for the pre-dose and post-dose biopsies, and the percent of the post-dose percentage relative to the
pre-dose percentage is shown. (C) Projected distributions of steady-state average concentrations over the 10-80 mg/day dose range. The boxes are defined by the medians and the 25th and 75th percentiles; whiskers are placed at the 10th and 90th percentiles; and filled circles at the 5th and 95th percentiles of the distributions.
Table 1. Summary of responses in patients evaluated with multiple pharmacodynamic readouts in tumor and skin biopsies

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<th>% Day 7</th>
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<td>-12</td>
<td>-1.2</td>
<td>0.06</td>
</tr>
<tr>
<td>130</td>
<td>Colorectal</td>
<td>70mg</td>
<td>38</td>
<td>34</td>
<td>0.4</td>
<td>3.25</td>
</tr>
<tr>
<td>131</td>
<td>NSCLC</td>
<td>70mg</td>
<td>38</td>
<td>30</td>
<td>-0.9</td>
<td>-0.23</td>
</tr>
<tr>
<td>147</td>
<td>Colorectal</td>
<td>70mg</td>
<td>43</td>
<td>44</td>
<td>2.4</td>
<td>0.95</td>
</tr>
<tr>
<td>153</td>
<td>Colorectal</td>
<td>80mg</td>
<td>55</td>
<td>53</td>
<td>2.9</td>
<td>3.73</td>
</tr>
</tbody>
</table>

Abbreviation: NSCLC, non-small cell lung cancer

- **Patient identification**
- **Dose cohort**
- **Positive values are in a direction consistent with Aurora A inhibition**
- **Negative values are in a direction not consistent with Aurora A inhibition**
Immunofluorescence staining

Multiplane image acquisition

Deconvolution and 3D reconstruction

Image randomization

Blind scoring by multiple scorers

A

B

Aligned chromosomes / Bipolar spindles
Unaligned chromosomes / Bipolar spindles
Unaligned chromosomes / Non-bipolar spindles
Figure 2

A

% cells with aligned chromosomes

<table>
<thead>
<tr>
<th></th>
<th>Pre-dose biopsy</th>
<th>Day 7 biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>% cells</td>
<td>68</td>
<td>25</td>
</tr>
<tr>
<td>p</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>

% cells with bipolar spindles

<table>
<thead>
<tr>
<th></th>
<th>Pre-dose biopsy</th>
<th>Day 7 biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>% cells</td>
<td>67</td>
<td>23</td>
</tr>
<tr>
<td>p</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>

Tumor mitotic index

<table>
<thead>
<tr>
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<th>Pre-dose biopsy</th>
<th>Day 7 biopsy</th>
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<tr>
<td></td>
<td>9.7</td>
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</tbody>
</table>

B

Pre-dose biopsy

Day 7 biopsy 2

C

Average mitotic cells per section

<table>
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<tr>
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<th>Pre-dose biopsy</th>
<th>Day 7 biopsy 1</th>
<th>Day 7 biopsy 2</th>
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</thead>
<tbody>
<tr>
<td>Average mitotic cells per section</td>
<td>0.13</td>
<td>3.72</td>
<td>3.04</td>
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<tr>
<td>P</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3

A

Mitotic index C10001
(Day 7 biopsy average minus pre-dose biopsy)

B

Mitotic index C10002
(Day 7 biopsy average minus pre-dose biopsy)
Figure 4

A  Tumor mitotic index
(Day 7 biopsy minus pre-dose biopsy)

B  Aligned chromosomes
(Pre-dose biopsy minus day 7 biopsy)

C  Bipolar spindles
(Pre-dose biopsy minus day 7 biopsy)
Figure 5

A: Percentage of pre-treatment percentage of mitotic cells with aligned chromosomes vs. MLN8054 Steady-state Average Concentration (nM)

B: Percent of pre-treatment percentage of mitotic cells with bipolar spindles vs. MLN8054 Steady-state Average Concentration (nM)

C: Dose (mg/day) vs. MLN8054 Steady-state Average Concentration (nM)
Phase I Assessment of New Mechanism-Based Pharmacodynamic Biomarkers for MLN8054, a Small-Molecule Inhibitor of Aurora A Kinase

Arijit Chakravarty, Vaishali Shinde, Josep Tabernero, et al.

Cancer Res Published OnlineFirst December 10, 2010.

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