An integrated genomic approach to identify predictive biomarkers of response to the Aurora kinase inhibitor PF-03814735

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Abstract

PF-03814735 is a novel, reversible inhibitor of Aurora kinases A and B that finished a phase I clinical trial for the treatment of advanced solid tumors. To find predictive biomarkers, we screened a diverse panel of 87 cancer cell lines for growth inhibition upon PF-03814735 treatment. Small cell lung cancer (SCLC) and to a lesser extent colon cancer lines were very sensitive to PF-03814735. The status of the Myc gene family and retinoblastoma pathway members significantly correlated with the efficacy of PF-03814735. While RB1 inactivation, intact CDKN2A/p16, and normal CCND1/Cyclin D1 status are hallmarks of SCLC, activation or amplification of any of the three Myc genes (MYC, MYCL1, MYCN) clearly differentiated cell line sensitivity within the SCLC panel. By contrast, we found that expression of Aurora A and B were weak predictors of response. We observed a decrease in histone H3 phosphorylation and polyploidization of sensitive lines, consistent with the phenotype of Aurora B inhibition. In vivo experiments with two SCLC xenograft models confirmed the sensitivity of Myc genes driven models to PF-03814735 and a possible schedule dependence of MYC/c-Myc driven tumors. Altogether our results suggest that SCLC and other malignancies driven by the Myc family genes may be suitable indications for treatment by Aurora B kinase inhibitors.
Introduction

Aneuploidy, one of the key features of cancer, is often linked to abnormalities in the regulation of mitotic cell division (1). Targeting dividing cells by inhibiting mitotic processes and their regulation represents a promising approach to cancer treatment (2), and several druggable targets have been identified to be important mitotic regulators (3-4).

The Aurora family of serine/threonine kinases are key regulators of mitosis and are frequently over-expressed in cancer (5). Aurora kinase A (AURKA) plays a key role in centrosome maturation while Aurora kinase B (AURKB) regulates chromatid segregation during mitosis and cytokinesis. On the molecular level, the phenotypic manifestation of Aurora A and B inhibition differs. Aurora kinase A inhibition is characterized by centrosome separation defects, a monopolar spindle, and delayed mitotic entry. The phenotype of Aurora B inhibition, on the other hand, includes decreased histone H3 phosphorylation as well as endomitosis with increased ploidy followed by apoptosis (6-8). The oncogenic role of the remaining Aurora kinase C (AURKC) is less understood.

Over a dozen small molecule Aurora kinase inhibitors have entered clinical development (9). While confirmed responders appear to be rare, several patients with stable disease have been reported (9). Given the relatively low response, reliable predictive biomarkers for patient selection are critical for clinical success.

In this study, we profiled the activity of the Aurora kinase A and B inhibitor PF-03814735 (10) in a panel of diverse carcinoma lines. We found that small cell lung cancer lines in general, and Myc family-driven, RB1/retinoblastoma null models in particular, have increased sensitivity to PF-03814735. The observed decrease in histone H3 phosphorylation and polyploidization of sensitive lines is consistent with an Aurora B inhibition phenotype. Implications for tumor indication and patient selection are discussed.
Materials and Methods

Cell lines, cell cultures and reagents

PF-03814735 was tested in vitro in a panel of 87 cancer lines derived from human lung (small cell and non-small cell), breast, and colorectal tumors. Tumor cells were obtained from ATCC (Manassas, VA), or DSMZ (Braunschweig, Germany). Cells lines were authenticated by the supplier (ATCC and DSMZ) and kept in culture for maximum of 3 months. Cells were grown in appropriate culture media as recommended by the suppliers, including RPMI 1640, MEM, DMEM, and DMEM-F12 (all from Invitrogen, Carlsbad, CA). Supplements included HEPES buffer, sodium pyruvate, non-essential amino acids, Pen-Strep, ITS, glutamine (all from Invitrogen) and FBS, 5-20% (SAFC Biosciences, Lenexa, KS). Some of the SCLC lines were grown in ACL-4 medium as described in ATCC’s online material (supplements from Sigma-Aldrich, St. Louis, MO).

Proliferation assays

Cells were seeded into 96-well tissue culture plates (Costar 3997, Corning, Lowell, MA) and incubated at 37°C in 5% CO₂ in humidified air. Compounds were dissolved in DMSO or PBS, diluted in polypropylene wells (Costar 3359), and added to cell cultures with the aid of a Biomek workstation (Beckman-Coulter, Brea, CA). Compounds were added 24 hr after seeding and plates were further incubated until cultures reached about 80% confluence, usually 3-7 days. Cell growth was monitored with either a fluorescence readout from resazurin (Sigma-Aldrich), 0.01% final concentration, or luminescence from a Cell Titer-Glo kit (Promega, Madison, WI), which were analyzed with an EnVision 2104 Multilabel Reader (Perkin-Elmer, Waltham, MA). Cell counts were adjusted by subtracting the
average of the baseline cell counts from untreated cells assessed one day after cell seeding, and IC$_{50}$ values were computed by fitting the fluorescence and concentration values to non-linear curves as implemented by the drc add-in to the open source language R (11).

**Mutation data, gene amplification and expression data**

The mutation status of the cell lines was determined from the COSMIC database (12) supplemented with custom OncoCarta (Sequenom, San Diego, CA) profiling. Baseline whole-genome expression and copy-number variation in selected cell lines were obtained from public resources and complemented with internal profiling on HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) and SNP 6.0 arrays (Affymetrix) according to the manufacturer's protocols. Publicly available reference profiles of cancer cell lines on the same platforms were obtained from the Welcome Trust Sanger Institute’s CONAN dataset (CoPy Number ANalysis, http://www.sanger.ac.uk/genetics/CGP/CopyNumberMapping/Affy_SNIP6.shtml), GlaxoSmithKline (https://cabig.nci.nih.gov/caArray_GSKdata/), and Genentech (GSE10843). All the new array data were deposited into GEO (GSE27692). For gene expression, we first excluded all arrays with NUSE score $>$ 1.05 and then normalized by GC-RMA (13) using the germa Bioconductor package. When multiple arrays were available, we used the median of normalized values. The final data are available in Supplementary Table 1. SNP 6.0 arrays were processed using the R based aroma.affymetrix methods. (14; http://www.aroma-project.org/). Arrays were ratioed versus a baseline profile of the average of the 128 females from the International HapMap Project. (http://www.affymetrix.com/estore/support/technical/sample_data/genomewide_snp6_data.affx).

Supplementary Table 2 contains the summarized table of DNA alterations used in our analysis.

DNA copy number of Myc family ($MYC$, $MYCN$, $MYCL1$) genes and mRNA expression levels of Myc and $CDKN2A$, $CCND1$, and $RBI$ genes were confirmed via Taqman (Applied Biosystems, Carlsbad, CA).
Manufacturer’s assay codes are listed in Supplementary Table 3. Copy number and expression were assessed using real-time PCR on an ABI Prism 7900 (Applied Biosystems) according to the manufacturer’s protocol. Copy number was calculated from quadruplet reactions using ABI CopyCaller software whereby the cycle threshold (CT) of the target gene was normalized against the CT of an RNaseP reference assay. Gene copy number was calculated relative to non-tumor reference genomic DNA (NA17317, Coriell Institute for Medical Research). mRNA expression was first normalized to GAPDH, and the relative gene expression was calculated by subtracting the average delta CT of triplicate reactions to the median of the average delta CT across each tumor type. The CT values for both copy number and expression data are listed in Supplementary Table 4.

**Data analysis and statistical methods**

DNA changes were summarized as follows: any change on the amino-acid level was defined to be a mutation, while silent mutations were excluded from the analysis. Gene amplifications from whole genome SNP 6.0 arrays were defined to be members of focally amplified chromosomal segments consisting of no more than 10 Mb containing at least 5 probes each with a mean log2 equal or greater than 1. There was no size constraint on the length of the chromosomal deletion: segments were required to contain a minimum of 5 probes each with a mean log2 less than -2.

Amplifications of the Myc family genes were validated by TaqMan PCR. Cell lines with more than 10 copies (as measured by TaqMan PCR) of any Myc family member (MYC, MYCN, MYCL1) were defined to be Myc family amplified. Cell lines were defined to over-express the Myc family if the highest expressing Myc family member had relative expression more than one standard deviation above the median of non-amplified lines.
The nonparametric Wilcoxon or Kruskal–Wallis rank tests as well as Fisher’s exact binomial test as implemented in R were used to correlate in vitro growth inhibition data and categorical predictors such as the presence of genetic changes and tissue of origin. Spearman’s rank correlation was used for continuous RNA expression profiles. The contributions of tissue origin to the observed correlations were tested using ANCOVA and ANOVA tests from the R lm module after factoring the tissue effect.

The presence of a predictive signature was tested using BRB-ArrayTools v4.1 (15). We split the samples into two categories: sensitive, defined as having IC50 < 100nM, and resistant, with IC50 >3μM, and removed the lines with intermediate values of IC50. We filtered out control probesets, non-informative probesets whose expression values deviate at least 1.5-fold in either direction from the median value in fewer than 20% of the cell lines, and those probesets with more than 50% of their data missing. Probesets with univariate mis-classification rate below 0.2 were used for class prediction using the diagonal linear discriminant analysis model. The leave-one-out cross-validation method was used to estimate the mis-classification rate.

**Western immunoblots**

For Western immunoblots, cells were first washed in PBS and then lysed/protein extracted for 1 hr at 0°C in cell extraction buffer (Invitrogen) supplemented with phosphatase inhibitors 2 and 3, PMSF, (Sigma-Aldrich), and Complete protease inhibitor cocktail (Roche, Indianapolis, IN), before centrifugation at 14,000xg for 10 min. Supernates were assayed for protein content with BCA reagents (Pierce, Rockford, IL) to ensure uniform gel loading. Polyacrylamide gel samples were prepared in NuPAGE sample buffer supplemented with reducing agent (Invitrogen) and run on 4-12% Bis-tris NuPage gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and immunoblotted for PARP (Cell Signaling 9542, Danvers, MA), MYC/C-Myc (Santa Cruz sc-40, Santa Cruz, CA), MYCN/N-Myc (Cell Signaling 9405), or the loading control protein ezrin (Cell Signaling 3145), and imaged with SuperSignal West Pico...
(Pierce) with the aid of an Alpha Imager (Cell Biosciences, Santa Clara, CA). For the “washout” phase of the time course PF-03814735 was removed after 24 hr by centrifugation at 200xg followed by PBS washes before resuspension in fresh medium and further incubation. For the MYC protein decay curves, NCI H82 cells were left untreated or were treated with 500 nM PF-3814735 for four hr before removal of duplicate samples immediately prior to addition of 50 μg/ml cycloheximide, and at several time points thereafter. MYC protein levels were estimated by densitometric analysis of myc bands on immunoblots using the image analysis software AlphaEaseFC (Cell Biosciences).

siRNA Transfection

siRNAs were transfected into NCI-H82 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s reverse transfection protocol. Briefly, 18 pmol of siRNA (Dharmacon ON-TARGET plus Human MYC, Thermo Fisher, Waltham, MA) and 1.5μl of Lipofectamine RNAiMAX were used to prepare RNAi duplex-Lipofectamine RNAiMAX complexes in 100 μl of Opti-MEM I medium (Invitrogen) in 24-well plates. After 20 min, 8x10⁴ cells in 500 μl of complete culture medium were added to each well. Cells were either transferred to 96-well plates 24 hr after transfection for proliferation assays (as previously described), or were pooled in groups of three, 90 hr after transfection, and lysed for Western immunoblots to monitor MYC knockdown.

Flow cytometry

Cell samples for flow cytometry were washed in PBS, fixed by dropwise addition of cold methanol, incubated 20 min at 0°, and stored for up to a week in 50% methanol/50% PBS. Cells were stained for phospho-histone H3 (Alexa 488 conjugated anti-histone H3 antibody, Cell Signaling 9708) in 50 μl wash buffer consisting of 2% BSA, 0.5% Tween 20, and 1 mM EDTA (all Sigma-Aldrich) in PBS. The cells were then washed and resuspended in saline containing 50 μg/ml propidium iodide and 30 u/ml RNase A
(both Sigma-Aldrich). The cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) prior to population discrimination with WinList and ModFit software (Verity, Topsham, ME).

**Human tumor xenograft studies**

We selected two SCLC lines for further testing *in vivo*. For xenograft studies, 2x10⁶ tumor cells in 50% matrigel were implanted in the right flank of female nu/nu mice (Charles River, Wilmington, MA) housed in microisolator cages and provided with food and water ad libitum. All procedures were conducted in compliance with Pfizer institutional animal care and use committee guidelines. Tumors were allowed to grow to a size of about 200 mm³ before randomization into treatment groups of 5-10 animals each. Animals were treated po with PF-03814735 prepared in 12.5% ethanol, 12.5% cremophor (Sigma-Aldrich) in saline at doses and schedules shown below. Tumors were measured with calipers in two dimensions and their volumes were calculated according to the formula (length x width²) x 0.5. Tumor measurements and body weights were taken twice per week until the tumors reached a size of about 2000 mm³, at which point the mice were sacrificed. Tumor growth delay and cell kill analyses were preformed as described previously (16).

**Results**

*PF-03814735 activity in vitro*

We tested PF-03814735 activity *in vitro* in a diverse panel of 87 lung, colon, and breast tumors lines. Activity ranged from sensitive cell lines with low double-digit nM IC₅₀ to resistant lines with IC₅₀ greater than 10μM (Fig. 1A). Significant tissue-specific differences in sensitivity were observed (Kruskal–Wallis
p < 0.0001): small cell lung carcinoma (SCLC) and colon lines were on average more sensitive to PF-03814735 than non-small cell lung carcinoma (NSCLC) and breast models (Fig. 1B). Because the sensitivity profile was not bimodal, we carried out initial predictive biomarker analyses using nonparametric rank tests without dividing models into sensitive and resistant categories.

First, we compared the compound activity measured by IC₅₀ to the genetic status of selected key cancer drivers (Fig. 1C) as measured by whole-genome copy-number arrays and somatic mutation data (see Material and Methods). No correlation was observed between the rare amplifications of Aurora kinase genes and sensitivity. We noticed increasing sensitivity of lines with amplified MYCL1/L-Myc (Kruskal–Wallis p = 0.024) and a trend for MYC/c-Myc (not significant), and confirmed these results by TaqMan PCR data (Fig. 1D). We also found correlations between the antiproliferative activity of PF-03814735 and several RB1 pathway genes. RB1/retinoblastoma mutations/deletions (p = 0.023) were positively correlated with response; amplifications of CCND1/cyclin D1 (p = 0.041) and mutations/deletions of CDKN2A/p16INK4A (p = 0.027) were associated with resistance.

Figure 1E shows mRNA expression of the Aurora kinase family, Myc family, and key RB1 pathway genes measured by TaqMan PCR. We found only weak correlations with Aurora kinase: the weak positive correlation between AURKA mRNA expression and IC₅₀ (Spearman’s rank R = 0.27; p-value = 0.012) indicates increased expression of this gene in cell lines resistant to the inhibitor. By contrast, AURKB expression was weakly (Spearman’s rank R = -0.19; p-value = 0.072) linked to increased sensitivity. High mRNA expression of MYC (R = -0.26; p-value = 0.012), MYCLI (R = -0.44; p-value < 0.0001), as well as MYCN (R = -0.22; p-value = 0.035) were significantly associated with sensitivity. Initial genetic correlations between CCND1 and CDKN2A/p16INK4A and IC₅₀ values were confirmed on the RNA level: cell lines with low CCND1 (R = 0.28; p-value = 0.008) and high CDKN2A/p16INK4A (R = -0.31; p-value = 0.003) were on average more sensitive. Contrary to the DNA data, however, no association was found between the RB1 mRNA level and PF-03814735 efficacy.
Given the significant association of PF-03814735 activity with the cell line origin (see above), one of the potentially confounding factors in our analysis is nonrandom alterations of genes in different tissues. Indeed, amplification of Myc loci and RB1 inactivation are hallmarks of SCLC (17-20). When controlled for the tumor type in ANCOVA (see Material and Methods), the status of the Myc family genes and RB1 the pathway genes were no longer significant.

To further elucidate the confounding role of cell line origin, we specifically analyzed Myc family amplification and expression on sensitivity of SCLC cell lines (Table 1). We found that no resistant models had Myc family amplifications. The difference in IC$_{50}$ values was significant between the lines with high amplification and those with normal/low copy number (p = 0.001; Wilcoxon rank test). Interestingly, even low-level amplification of any Myc family member is sufficient to sensitize some cell lines to PF-03814735 inhibition in vitro. Taken together, we conclude that within the SCLC line panel, Myc gene activation sensitized lines to PF-03814735. We were unable to test the role of the RB1 pathway status in SCLC lines, since all lines were RB1-null.

Activation of individual Myc family genes in our cell line panel is mutually exclusive (Fig. 1C-E, Table 1). Therefore, we combined Myc gene activity into a summarized value in an effort to improve the predictive power. Indeed, as shown in Fig. 2A-B, the differences between lines with amplification or over-expression of at least one Myc gene and the remaining lines became highly significant (Wilcoxon p-value = 0.0018 and 0.000008 for amplified and over-expressed line, respectively). However, there are several sensitive lines without any obvious Myc family activation and thus the combined status of Myc family genes do not fully explain the sensitivity profile.

Figures 2C-D compare the predictive power of all the major binary predictors identified in this work (see the figure legend and Material and Methods for details). MYC and MYCL1 over-expression, MYCN and
MYCL1 amplifications, RB1 deletions/mutations, SCLC origin, and activation of any Myc gene by amplification and over-expression were the best predictors of sensitivity measured by odds ratio and average IC₅₀. CCND1 amplifications and inactivations of p16⁠^{INK4A}/CDKN2A were associated with cell line resistance. At the statistical significance level, the best predictors were activation of Myc family by over-expression, as well as a new PF-03814735 gene expression response signature described next.

We developed a gene expression classifier from whole genome expression data. We split the samples into two categories: sensitive, defined as IC₅₀ < 100nM and resistant, with IC₅₀ > 3μM, and removed lines with intermediate response. 247 probesets with univariate misclassification rate below 0.2 were used for class prediction using diagonal linear discriminant analysis. Leave-one-out cross-validation of the derived signature in our dataset correctly predicted 86% of all cell lines with 0.844 specificity and 0.875 sensitivity. The observed sensitivity signature requires validation in an independent set of samples. The full prediction model including the genes, weights, and threshold is described in the supplementary files.

MYC down-regulation by siRNAs decreases cell sensitivity to PF-03814735

To further characterize the role of Myc genes in PF-03814735 sensitivity, we modulated MYC RNA in MYC amplified NCI-H82 cells and compared with control cells (Fig. 3). We used two different MYC-targeted siRNAs, one of which down-regulated MYC expression. A strong knockdown of MYC resulted in complete inhibition of NCI-H82 proliferation (not shown) and thus we used only partially inhibited MYC. Even a modest down-regulation of MYC expression by 31% was sufficient for the loss of sensitivity to PF-03814735, unlike the other siRNAs or controls that did not modulate MYC expression. Thus MYC expression both correlates with PF-03814735 sensitivity in a diverse panel of cell lines and is required for sustained inhibition by this compound in MYC-driven lines.

Phenotypic effects of PF-03814735 treatment
Since Aurora A has been shown to modulate MYCN protein stability and longevity in neuroblastoma cells (21), we performed a series of experiments with three SCLC lines, NCI-H82 (amplified MYC), NCI-H69 (amplified MYCN), and NCI-H345 (normal Myc genes), to assess Myc protein levels by Western blot as a function of exposure to the Aurora kinase inhibitory activity of PF-03814735 at two concentrations. In addition, we immunoblotted for Poly-ADP ribose polymerase (PARP) and its cleavage product as a measure of apoptosis (Supplementary Fig. 1S A-C). Over a time course of 1-48 hr, plus a series of time points following the washout of inhibitor, MYC levels were unaffected by treatment with PF-03814735. There was little evidence of PARP cleavage during the first 48 hr of exposure to PF-03814735. We observed no difference in MYC protein stability or mRNA expression levels after PF-03814735 treatment (Supplementary Fig. 2S-3S), or by siRNA AURKA or AURKB inhibition (not shown).

Cells from these same time course experiments were stained for phospho-histone H3 (a substrate of Aurora B kinase) and DNA content, and analyzed by flow cytometry. Fig 4A depicts an example dot plot illustrating the gating strategy employed to estimate the fraction of phospho-histone H3 positive cells. Cells just above the major G2M population toward the right side of the cell distribution have phosphorylated histone H3. A region was drawn around the majority of cells with highly phosphorylated histone H3 for the untreated control cells at each time point, and cells in this region were classified as phospho-histone H3 positive in the treated groups. The proportion of phospho-histone H3 positive G2M cells is plotted in Fig. 4B as a function of time after inhibitor addition. Addition of PF-03814735 at 500 nM immediately suppressed H3 histone phosphorylation in all three cell lines compared with untreated controls, with NCI-H345 showing slightly less sensitivity than the other two.

After washing the inhibitor from the cells, histone H3 phosphorylation levels returned to control values immediately (data not shown). Despite the return to pre-treatment phosphorylation levels, NCI-H82 and NCI-H69 cells had protracted disruption of their cell cycles, with significant arrest in G2M and
accumulation of polyploid (>4N DNA content) cells (Fig. 4C). The MYC- and MYCN-driven cell lines NCI-H82 and NCI-H69, respectively, had higher S-phase fractions (with or without Aurora kinase inhibitor treatment) than the cell line NCI-H345 with no Myc gene activation.

**PF-03814735 in vivo activity in Myc driven NCI-H82 SCLC xenografts**

We selected two SCLC models sensitive *in vitro* to PF-03814735 inhibition, NCI-H82 and NCI-H69, for further *in vivo* experiments in mice; the tumorigenicity of the normal Myc expressing cell line NCI-H345 was too inconsistent for xenograft antitumor studies. Since there was lasting disruption of cell cycle after washout *in vitro*, we compared intermittent dosing with daily administration *in vivo*. Figures 5A and 5B show the effect of PF-03814735 on MYC-driven NCI-H82 xenografts when administered on two different treatment schedules: bid once weekly x 3, and bid daily x 10, respectively. PF-03814735 was much more effective in NCI-H82 xenografts when administered on a weekly dosing schedule at 80 mg/kg compared with a daily schedule at 15 mg/kg. PF-03814735 delayed growth by 23.5 days on the weekly schedule, which corresponds to 0.9 logs of net cell kill during the course of treatment. The antitumor efficacy against the NCI-H69 model was modest and was not schedule dependent (Fig. 5C). The maximum tolerated dose was lower in this study (50 mg/kg vs 80 mg/kg in the H69 and H82 studies, respectively).
Discussion

We tested PF-03814735 activity in vitro in a diverse panel of lung, colon, and breast tumor lines. We found weak correlations between sensitivity and high Aurora B expression as well as low Aurora A expression. Surprisingly, small cell lung carcinoma (SCLC) lines were most sensitive. The status of several key cancer driver genes including the Myc family and RB1 pathway members correlated with the efficacy of PF-03814735.

One of the key effector proteins regulated by Aurora B kinase is the RB1 protein (22-24). Nair et al. (24) observed that Aurora B kinase directly phosphorylates RB1 independently of cyclin-dependent kinase inhibition and CDKN2A/p16 expression, and proposed that Aurora B inhibitors activity may differ depending on the RB1 status. This functional link offers a possible explanation for the observed correlation between RB1 pathway genes and PF-03814735 sensitivity. However, in our multivariate analysis the correlations with the RB1 pathway genes are no longer significant when the origin of cell lines is taken into account. The results of multivariate analysis are consistent with previous reports that RB1 status alone does not seem to correlate with sensitivity of pre-clinical models to Aurora kinase inhibitors (22, 25). Notably, no correlation was found with the p53 status confirming previous findings with other Aurora kinase inhibitors (8, 22, 25).

In the SCLC panel, activation via gene amplification and/or upregulated expression of Myc family genes was associated with sensitivity that is reversible upon siRNA knockdown. No obvious difference was seen between individual Myc family members, indicating a common mechanism of action, despite the fact that the downstream gene expression signatures of Myc family amplifications have little overlap (26). Indeed, analyses of MYC and MYCN driven models indicated common downstream consequences of Aurora B inhibition including a decrease in phosphohistone H3 and increased cell ploidy. These findings
are consistent with the molecular phenotype of PF-03814735 as well as other Aurora kinase B inhibitors (6-7, 10, 23).

In a MYC driven model NCI-H82, PF-03814735 was more effective when administered on a weekly dosing schedule at 80 mg/kg compared with a daily schedule at 15 mg/kg. One explanation is that more complete target inhibition causes lasting damage to cell cycle progression as seen in washout experiments in vitro. This contrasts with the results obtained with the MYCN xenografts and with previous xenograft data that showed no schedule dependence (10). One implication might be the possibility to reduce the dosing frequency in patients with Myc-driven malignancies. It is not clear why the in vivo sensitivity of the MYCN expressor NCI-H69 did not match its relative sensitivity observed in vitro.

What is the mechanism of selective sensitivity of Myc family-driven tumors to PF-03814735? One possibility is direct transcriptional regulation of Aurora gene expression by Myc-Max complexes. Indeed, AURKA and AURKB transcripts and protein levels are highly elevated in Myc-driven B-cell lymphomas in both mice and humans (8). The interplay between AURKA and MYCN is more complex in neuroblastoma: AURKA expression is correlated with MYCN amplification in neuroblastoma (27) and AURKA protects the MYCN protein from degradation (21). Recently, Faisal et al. (28) provided additional evidence that AURKA regulates MYCN protein levels in neuroblastoma cells through an inhibitory phosphorylation of GSK3β. AURKA inhibition decreased activity of pro-proliferative signaling pathways including MYC signaling in medulloblastoma lines (29). These findings suggest direct links between Myc and Aurora proteins. However, neither PF-03814735 inhibition nor siRNA knockdown of both AURKA and AURKB had any effect on MYC expression in the MYC amplified SCLC line NCI-H82, suggesting that tissue-specific differences exist. Moreover, when we compared mRNA expression levels of all Aurora family with PF-03814735 activity, we found no significant correlation for AURKB and a weak negative correlation between AURKA mRNA expression and sensitivity.
Interestingly, Myc family activation and Aurora B inhibition share common characteristics in their propensity to endomitosis and increased cell ploidy. MYC overexpression uncouples DNA replication from mitosis and leads to polyploidy (30). Aurora kinase B inhibition is accompanied by increased ploidy via direct phosphorylation of the RB1 protein (23-24, 31). Thus the propensity to increase copy number may represent the Achilles heel of Myc-driven tumors and represent a therapeutic opportunity for Aurora B inhibitors.

Recently, Yang et al. (25) showed that the Aurora kinase B inhibitor VX-680 selectively kills cells that overexpress MYC. This synthetic lethal interaction is linked to disabling of the chromosomal passenger protein complex and DNA replication in the absence of cell division. The resulting polyploid cells undergo sequential apoptosis and autophagy. These results as well as those published by den Hollander et al. (8) along with those presented here suggest that Myc driven malignancies may be a suitable target for treatment with Aurora kinase B inhibitors. Potential indications include neuroblastoma, B-cell lymphomas, small cell lung carcinoma, as well as other tumor indications where patients with activation of Myc genes can be selected.

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References


### Tables

#### Table 1. *In vitro* antiproliferative activity of PF-03814735 in a panel of SCLC cell lines.

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*PCR

#RT-PCR, GAPDH-normalized, median-centered
Figure legend

**Figure 1** PF-03814735 activity in a cell panel correlated with the status of pathway genes and major cancer drivers. A. Ordered PF-03814735 *in vitro* antiproliferative activity measured as IC50 in a panel of breast, colon, lung small cell and non-small cell tumor cell lines. B. Comparison of IC50 values with the tissue or origin. C. Comparison with the genetic status of Aurora kinases and major cancer drivers. The red color shows protein-coding mutations as obtained from COSMIC and Oncocarta profiling, green amplification (see Material and Methods), blue mutations or deletions in tumor suppressors, and yellow indicates likely wild-type status. On the right are the p-values from the Kruskal-Wallis test. D. PCR-measured DNA copy number of the Myc gene family. MYCL1 copy-number is significantly correlated with sensitivity in the Spearman’s rank correlation test (highlighted in red). E. Comparison with PCR-measured RNA expression of Aurora genes, Myc gene family and key RB pathway genes. Significant correlations are highlighted in colors; red mean positive correlation between high expression and IC50 (and thus sensitivity), blue signifies correlation between high expression and resistance (Spearman’s rank correlation).

**Figure 2** Molecular predictors of sensitivity to PF-03814735. A. Correlation between Myc family amplifications and PF-03814735 activity (measured as IC50). The cell line was defined to be Myc family-amplified if one or more Myc genes was present in more 10 copies as measured by TaqMan PCR (see methods). The tissue of origin of different lines is indicated by colors. B. Correlation between Myc family over-expression and PF-03814735 activity (measured as IC50). We first calculated the median mRNA expression of Myc genes from TaqMan PCR in non-amplified lines (<10 copies for all Myc genes). Cell lines were labeled Myc over-expressed if any of the Myc genes had expression greater than 1 standard deviation above the median. C. IC50 effects of sensitivity predictors. The average IC50 of all cell lines with the specific predictor was plotted with the Wilcoxon rank test p-values (log scale). Predictors #1-3 were obtained from somatic mutation and whole-genome copy-number data.
Amplification and expression values (#4-9) are from TaqMan PCR. Values #10-11 were obtained as described in the panels A and B. The last two predictors represent the diagonal linear discriminant analysis (DLDA) expression classifier (#12) and SCLC origin of cell lines versus all other tissues (#13).

D. Odds ratio and p-value comparison for significant predictors of sensitivity. For each predictor, the genomic information was condensed into a binary absent-present status in each line. Then we divided cell lines into sensitive (IC₅₀ < 100nM) and resistant (IC₅₀ ≥ 3μM) and obtained a 2x2 table with frequencies. From the frequency tables odds ratios and Fisher’s binomial test p-values were calculated. The dashed vertical line corresponds to odds ratio 1 (equal frequency in resistant and sensitive lines). Odds ratios greater than one indicate alterations positively correlated with response; ratios less than one characterize changes found in resistant lines. Changes found only in resistant or only in sensitive cell lines are plotted outside the odds ratio scale (grey vertical lines) on the left and right, respectively.

Figure 3 Effect of MYC down-regulation on sensitivity to PF-03814735. MYC amplified NCI-H82 cells were transfected with three siRNAs. #23 and #26 correspond to siRNA against the MYC mRNA. #2: off-target siRNA, CTL: cell transfected lipid only, CC: untreated cell control. The upper barplot shows PF-03814735 activity in proliferation assays begun 24 hr after transfection (mean ± SD, n = 2). The Western immunoblots below show MYC knockdown after transfection. Only the MYC-targeted siRNA #26 modulated MYC expression (by 31%) and cell transfected with siRNA lost their sensitivity to PF-03814735 with IC₅₀ > 1000 nM compared to 290-520 nM IC₅₀ in cell transfected with other siRNAs or controls that did not modulate MYC expression.

Figure 4 PF-03814735 effect on histone H3 phosphorylation, mitotic arrest, and accumulation of polyploid cells. A. Phospho-histone H3 positive G2M cells. Dot plot with the region defining phospho-histone H3 positive G2M cells from the MYC over-expressing line NCI-H82 outlined with an oval. Regions of Phospho-H3+ cells were defined for the untreated control population at each time point. B.
The fractions of cells with highly phosphorylated histone H3 during time course of treatment with 500 nM PF-03814735 plotted as per cent of control values. C. Mitotic arrest with PF-03814735 treatment and accumulation of polyploid cells. Representative DNA histograms showing mitotic arrest with 500 nM PF-03814735 treatment and subsequent accumulation of polyploid cells after removing the inhibitor following an initial 24 hr exposure period in MYC over-expressing NCI-H82 cells, MYCN over-expressing NCI-H69 cells and NCI-H345 cells with normal Myc family expression.

**Figure 5** Effect of PF-03814735 on growth of NCI-H82 and NCI-H69 SCLC xenografts. A. MYC-driven NCI-H82 treated po with two injections spaced about 8 hr apart on a once weekly x 3 schedule (bid qwkx3). B. MYC-driven NCI-H82 treated po twice daily for 10 days (bid qdx10). C. Effect of PF-03814735 on growth of MYCN-driven NCI-H69 xenografts when administered in two po injections spaced about 8 hr apart on a once weekly x 2 schedule (bid qwkx2) or po twice daily for 10 days (bid qdx10). Error bars: 60th, 40th percentiles; the center point corresponds to median values. Arrows indicate treatment days or duration of treatment.
Figure 1

A

\[-\log_{10} IC_{50} \text{ [nM]}\]

-8
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8

B

Color keys

A: IC_{50}  
<100 nM  
100-500 nM  
500-2500 nM  
> 2500 nM

B: Tissue

breast

colon

NSCLC

SCLC

C: Alteration

wildtype

mutation

amplification

mutation/deletion

D-E: CNV & Expression

high

low

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

IC$_{50}$ [nM]

0 200 400 600 800 1000

#23 #26 #2 CTL CC

MYC

Ezrin
Figure 4

A

DNA content (relative fluorescence) vs. Phospho-histone H3 (relative fluorescence)

B

Time post addition of PF-03814735 (h) vs. Phospho-H3 positive cells (% of control)

C

Control 24 h 24 h washout

DNA content (relative fluorescence)
Figure 5

(A) NCI-H82

(B) NCI-H82

(C) NCI-H69
Molecular Cancer Therapeutics

An integrated genomic approach to identify predictive biomarkers of response to the Aurora kinase inhibitor PF-03814735

Kenneth E Hook, Scott J Garza, Maruja E Lira, et al.

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