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Small Molecule Therapeutics

Molecular Cancer Therapeutics

Barasertib (AZD1152), a Small Molecule Aurora B Inhibitor, Inhibits the Growth of SCLC Cell Lines *In Vitro* and *In Vivo*

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Abstract

Small-cell lung cancer (SCLC) cells have rapid proliferation, universal Rb inactivation, and high rates of *MYC* family amplification, making aurora kinase inhibition a natural target. Preclinical studies have demonstrated activity for Aurora A and pan-Aurora inhibitors with some relationship to MYC family expression. A clinical trial showed activity for an Aurora kinase A inhibitor, but no biomarkers were evaluated. We screened a panel of 23 SCLC lines with and without *MYC* family gene amplification or high *MYC* family gene expression for growth inhibition by the highly potent, selective aurora kinase B inhibitor barasertib. Nine of the SCLC lines were very sensitive to growth inhibition by barasertib, with IC_{50} values of <50 nmol/L and >75% growth inhibition at 100 nmol/L. Growth inhibition correlated with *cMYC* amplification ($P = 0.018$) and *cMYC* gene expression ($P = 0.026$). Sensitive cell lines were also enriched in a published *MYC* gene signature ($P = 0.042$). *In vivo*, barasertib inhibited the growth of xenografts established from an SCLC line that had high *cMYC* gene expression, no *cMYC* amplification, and was positive for the core *MYC* gene signature. Our studies suggest that SCLC tumors with *cMYC* amplification/high gene expression will frequently respond to Aurora B inhibitors and that clinical studies coupled with predictive biomarkers are indicated. *Mol Cancer Ther; 15(10); 2314–22. ©2016 AACR.*

Introduction

Small-cell lung cancer, accounting for approximately 35,000 cases annually, is the sixth most common cause of cancer deaths in the United States (1). Clinically, SCLC tumors have rapid doubling times and a propensity for early development of widespread metastatic disease (2). The overall 5-year survival from diagnosis is less than 5% despite high initial response rates to first-line chemotherapy of 70% to 90% (2, 3).

There have been few therapeutic advances in the treatment of SCLC in recent decades, and SCLC remains a major public health problem. The standard of care for both limited stage (IIB–III) and extensive stage (IV) has been the drug combination of etoposide with cisplatin or carboplatin since the early 1980s (4). Modest improvements in survival have come from the addition of chest radiotherapy in limited stage. Studies evaluating new-generation cytotoxic chemotherapies have not had a significant impact on the standard of care, and topotecan is the only cytotoxic currently approved for the treatment of relapsed SCLC (5, 6). Biological studies have shown that loss of the *RB1* and *TP53* tumor suppressor genes is universally present in SCLC cells (7, 8). In addition, a significant proportion of SCLCs have amplification of various *MYC* family members (9).

A recent biological approach to cancer has been the development of small molecules targeting the key mitotic regulatory serine/threonine kinases Aurora A (AURKA) and Aurora B (AURKB), which are frequently overexpressed in lung cancer (10, 11). During mitosis, AURKA and AURKB coordinate cell-cycle progression through G_2–M. AURKA regulates centrosome maturation and separation, bipolar spindle assembly, and mitotic entry (12). AURKB plays a critical role by regulating chromosome alignment, accurate segregation, and cytokinesis by its movement through the mitotic stages (12). In a human colon carcinoma cell line, AURKB inhibition by barasertib resulted in Rb hypophosphorylation, leading to polyploidy after an aberrant mitosis (13). The phenotypic result of AURKB inhibition is an induction of polyploidy, a hallmark of antitumor activity.

Currently, aurora kinase inhibitors are in clinical trials; however, predictive biomarkers for patient selection are needed (10). In a recent pharmacologic screen of 34 SCLC lines for growth inhibition by the AURKA inhibitor MLN8237 and the dual Aurora A/B inhibitors PHA680632, VX680, and ZM44739, six SCLC lines that had 50% growth-inhibitory concentrations (IC_{50}) of <1 μmol/L to all four drugs were considered sensitive, and response was correlated with amplification of the *cMYC* oncogene (14). However, there were several lines with *cMYC* amplification that did not respond and several other lines without *cMYC* amplification that were sensitive. Furthermore, amplification of

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MYC family members *MYCL1* and *MYCN* did not correlate with sensitivity to dual Aurora A/B inhibitors or the AURKA inhibitor MLN8237 (14). A phase I clinical trial reported activity of MLN8237 in 21% of relapsed SCLC patients; however, *cMYC* expression was not evaluated (15).

In contrast, growth inhibition by the dual Aurora A/B inhibitor PF-03814735 in a panel of 20 SCLC lines correlated with amplification or overexpression of any of the *MYC* family members (*cMYC*, *MYCL1*, and *MYCN*; ref. 16). In this study, 14 SCLC lines with IC_{50}s <100 nmol/L were considered sensitive. Again, there were SCLC lines with no evidence of *MYC* family amplification or overexpression that were sensitive to PF-03814735. Resistance was defined as an IC_{50} of >3 μmol/L, and no *MYC* family amplification was found in these resistant lines. PF-0381475 inhibited the *in vivo* growth of c*MYC* and *MYCN*-amplified cell lines in SCLC tumor xenograft models (16). These studies suggest that there is some link between MYC family members and the Aurora kinases A and B in SCLC, but no studies of specific aurora kinase B inhibitors have been reported.

MYC family gene amplification in conjunction with mutation/deletion of the tumor suppressor genes *TP53* and *RB1* are the most frequently altered genes in SCLC (7–9). Focal amplification of the *MYC* family of transcription factors, including *cMYC*, *MYCL1*, and *MYCN*, has been found in about 30% of SCLC samples, and amplification of *MYCL1* and *MYCN* are found exclusively in neuroendocrine tumors, including SCLC (17). A recent report using chromogenic *in situ* hybridization evaluated *cMYC* amplification in 77 formalin-fixed paraffin-embedded tumor samples from SCLC patients who had a diagnostic biopsy for SCLC (18). *cMYC* amplification was found in 20% of the biopsies and was associated with poor survival. Furthermore, p53 proteins with missense mutations have been shown to transactivate *cMYC* through the C-terminus (19).

In both of the above reports, the associated phenotypic drug induced changes by the aurora kinase inhibitors, including increased G_2–M arrest, polyploidy, and a decrease in histone H3 phosphorylation, were thought to be primarily due to inhibition of AURKB (14, 16). We therefore evaluated barasertib, an AURKB-specific inhibitor, in a panel of 23 SCLC lines with and without *MYC* family amplification.

Materials and Methods

Reagents

Barasertib and barasertib-HQPA were provided by AstraZeneca Pharmaceuticals. The structure of barasertib was previously published (20). Barasertib is rapidly converted by plasma phosphatases to the active barasertib-HQPA metabolite. Therefore, *in vitro* studies were conducted with barasertib-HQPA and *in vivo* experiments with barasertib (20). The barasertib-HQPA IC_{50} for AURKB is 0.37 nmol/L versus 1,369 nmol/L for AURKA (20).

SCLC cell lines

The SCLC lines H82, H146, H187, H211, H345, H378, H446, H524, H526, H748, H774, H841, H889, H1092, H1694, H1963, H2029, H2081, H2141, H2171, and N417 were obtained from Drs. Adi Gazdar and John Minna (UT Southwestern, Dallas, TX) and deposited in the UCCC Tissue Culture Core. Authentication of the cell lines by short tandem repeat DNA profiling was through the Tissue Culture Core and the UCCC DNA Sequencing and Analysis Core. The DMS114 and H69 lines were obtained from the ATCC. All cell lines were used within 6 months of resuscitation of a frozen vial from the authenticated passage. The clinical characteristics of the patients from whom the cell lines were derived are shown in Table 1 (21). All cell lines were mycoplasma free and maintained in RPMI1640 + 5% or 10% FBS at 37°C in 5% CO_2. The cell lines were obtained in 1993 (H146, H187, H211, H345, H378, H446, H524, H526, H748, H774, and H841), 2011 (DMS114), and 2014 (H69, H82, H889, H1092, H1694, H1963, H2029, H2081, H2141, H2171, and N417).

MYC family gene amplification, gene expression, and *MYC* core gene signature

Cell line *MYC* family amplification (14, 16), *MYC* family gene expression (Cancer Cell Line Encyclopedia, accession number GSG36133; broadinstitute.org), and the *MYC* core gene signature (Gene Expression Omnibus, accession number GSE15523; ncbi.nlm.nih.gov; ref. 22) were used to determine whether they related to cell line sensitivity to barasertib.

Aurora A/B gene and Aurora B protein expression by Western blotting

AURKA and *AURKB* gene expression in our panel of 23 SCLC lines is shown in Supplementary Fig. S1A and S1B. Gene expression data was from the CCLE (accession number GSE36133) and was significantly higher than in 17 normal epithelial airway samples (accession number GSE24337; $P < 0.001$). We confirmed AURKB protein expression by Western blotting in a subset of SCLC lines (Supplementary Fig. S1C). Cell lysates were prepared in Triton X-100 lysis buffer, sonicated, and protein concentration determined by DC Protein Assay (Bio-Rad). Protein (50 μg) was resolved by SDS-PAGE. Gels were electroblotted onto PVDF membranes and probed for AURKB (Cell Signaling Technology). Protein bands were visualized by chemiluminescence using SuperSignal West Femto (Thermo Scientific). β-Actin (Cell Signaling Technology) served as a loading control. AURKB protein expression was similar across the SCLC lines.

Modified tetrazolium growth assays

Inhibition of cell growth was assessed using MTS (CellTiter Aqueous One Solution, Promega; ref. 23). Briefly, 2×10^3 to 1×10^4 viable cells were plated in growth medium in 96-well plates (Corning) and incubated overnight at 37°C. Barasertib-HQPA, 0 to 100 nmol/L, was added, and plates were incubated for 5 days, after which the MTS was added and the absorbency of each well was measured at 490 nm using an automated plate reader (Molecular Devices).

Immunofluorescence staining phosphorylated histone H3

Phosphorylated histone H3 (Ser10) expression was determined by FACS analysis. Briefly, cells were fixed with 4% formaldehyde at 37°C for 10 minutes and then chilled on ice for 1 minute. Fixed cells were permeabilized on ice for 30 minutes in ice-cold 90% ethanol and then blocked in 0.5% BSA for 10 minutes at room temperature. The fixed permeabilized block cells were stained for 60 minutes at room temperature, followed by three washes. Primary antibody was a rabbit mAb to phosphorylated histone H3 (Ser10) directly conjugated with Alexa Fluor 488 (Cell Signaling Technology), and the isotype control was a rabbit IgG directly conjugated with Alexa Fluor 488 (Cell Signaling Technology). Cell fluorescence was measured by FACS

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Table 1. Cell line characteristics

Cell line	Stage[a]	Gender[a]	PY[a]	Amplification[b]	*cMyc* GE[c]	*MYCL1* GE[c]	*MYCN* GE[c]
H82	E	M	Unk	MYC	13.7	8.8	5.9
H211	E	F	35	MYC	13.2	5.9	6.7
H446	L	M	80	MYC	12.7	7.5	5.1
N417	Unk	F	Unk	MYC	13.7	6.1	5.5
H524	L	M	30	MYC	13.9	6.4	5.3
H2171	E	M	150	MYC	13.4	8	4.9
H378	E	F	50	MYCL1	3.8	13.1	5.1
H748	E	M	50	MYCL1	4.5	13.4	5.4
H1092	E	M	120	MYCL1	5	12.4	4.9
H1694	E	M	Unk	MYCL1	6.2	13.4	6.3
H1963	L	M	70	MYCL1	4.2	12.8	4.9
H2029	E	F	52	MYCL1	4.9	12.2	4.8
H2141	E	M	50	MYCL1	3.8	13.1	3.8
H69	E	M	Unk	MYCN	6	6.1	13.1
H526	E	M	30	MYCN	5.1	7.7	12.9
H146	L	M	100	None	11.8	5.7	5
H187	E	M	50	None	4.6	8.7	9.9
H345	L	M	60	None	7.2	9.9	6.5
H774	E	M	30	None	5.6	11.2	5.4
H841	L	M	70	None	10.6	7.4	5.1
H2081	E	F	25	None	12.9	6.3	4.8
DMS53	Unk	M	Unk	None	11.6	9.6	5
DMS114	Unk	M	Unk	None	10.8	7.1	5.1

Abbreviations: E, extensive stage; F, female; GE, gene expression; L, limited stage; M, male; PY, pack year; Unk, unknown.
[a]Data obtained from ref. 20.
[b]*Myc* family amplification status obtained from refs. 15 and 17.
[c]*MYC* family gene expression was obtained from the Cancer Cell Line Encyclopedia (broadinstitute.org).

(Beckman FC500, Beckman Coulter). The percentage of positive phosphorylated histone H3 (Ser10) cells was determined using the Coulter software.

Ploidy analysis

Briefly, 3×10^5 cells were exposed to barasertib-HQPA 30 or 50 nmol/L for 24 and 48 hours and then stained with saponin, propidium iodine (PI), and RNAse solution overnight. Alterations in DNA ploidy were determined by flow cytometry. Doublet cells were gated out of the analysis. Remaining cells were analyzed on a log2 scale to determine the percentage of the population that was tetraploid (DNA content 4N) and the subsequent accumulation of polyploid cells (DNA content $\geq$8N).

Nude mouse xenograft tumor model

Athymic nude mice (4- to 6-week-old females) obtained from the NCI (Bethesda, MD) were maintained in the University of Colorado Denver Animal Resources Center (Denver, CO) with an approved protocol by IACUC. The SCLC line H841 was mixed with an equal volume of cold Matrigel and implanted at 5×10^6 cells in 100 μL in the rear flank. Treatment began on day 20 after tumor cell implantation. Mice were randomized into 8 mice per group, and 5 to 7 of these that developed tumors were treated with vehicle, barasertib at 50 or 100 mg/kg per day for 5 days, rested over the weekend, and injected for 5 days the following week for a total of 10 days of treatment. Tumor volumes were measured three times a week with a digital caliper.

Statistical analysis

Fisher exact test was used to determine the correlation between gene amplification of *MYC* family members and sensitivity to growth inhibition by barasertib-HQPA. Fisher exact test was also used to determine the correlation between a published *MYC* gene signature and growth inhibition (22). A two-group *t* test (gene expression as a continuous variable) and an OR estimate (dichotomized gene expression) were used to determine a correlation between *MYC* family gene expression and growth inhibition. Linear mixed regression models were used to analyze the association between log tumor volumes and barasertib treatment over time.

Results

In vitro growth inhibition by barasertib-HQPA

We evaluated the growth-inhibitory effects of barasertib-HQPA on 6 *cMYC* amplified, 7 *MYCL1* amplified, 2 *MYCN* amplified, and 8 SCLC lines with no *MYC* family amplification (Table 1). Barasertib-HQPA concentrations evaluated were 0 to 100 nmol/L, ensuring that AURKA was not inhibited. The barasertib-HQPA concentrations were similar to those used in other preclinical studies and exceeded the trough levels achieved in human trials (24, 25).

As shown in Fig. 1, we found three patterns of growth inhibition. Pattern A (Fig. 1A) included 9 SCLC lines that were the most sensitive to barasertib-HQPA. The growth inhibition IC_{50} concentration for all 9 lines was <50 nmol/L, and at 100 nmol/L, barasertib-HQPA growth inhibition was >75%. *cMYC* was amplified in 5 of these lines, 2 lines were *MYCL1* gene amplified, and 2 lines had no *MYC* family amplification.

The 10 SCLC lines shown in Fig. 1B were classified as having intermediate sensitivity to barasertib-HQPA, as growth inhibition at 100 nmol/L was 32% to 50%. Increasing the barasertib-HQPA concentration to 1 μmol/L did not increase growth inhibition (data not shown). One cell line with intermediate sensitivity was *cMYC* amplified, 4 lines were *MYCL1* amplified, 2 lines had *MYCN* gene amplification, and 3 lines had no *MYC* family amplification.

The 4 SCLC lines shown in Fig. 1C were classified as resistant to barasertib-HQPA, as growth inhibition at 100 nmol/L was <20%, and increasing the concentration to 1 μmol/L did not

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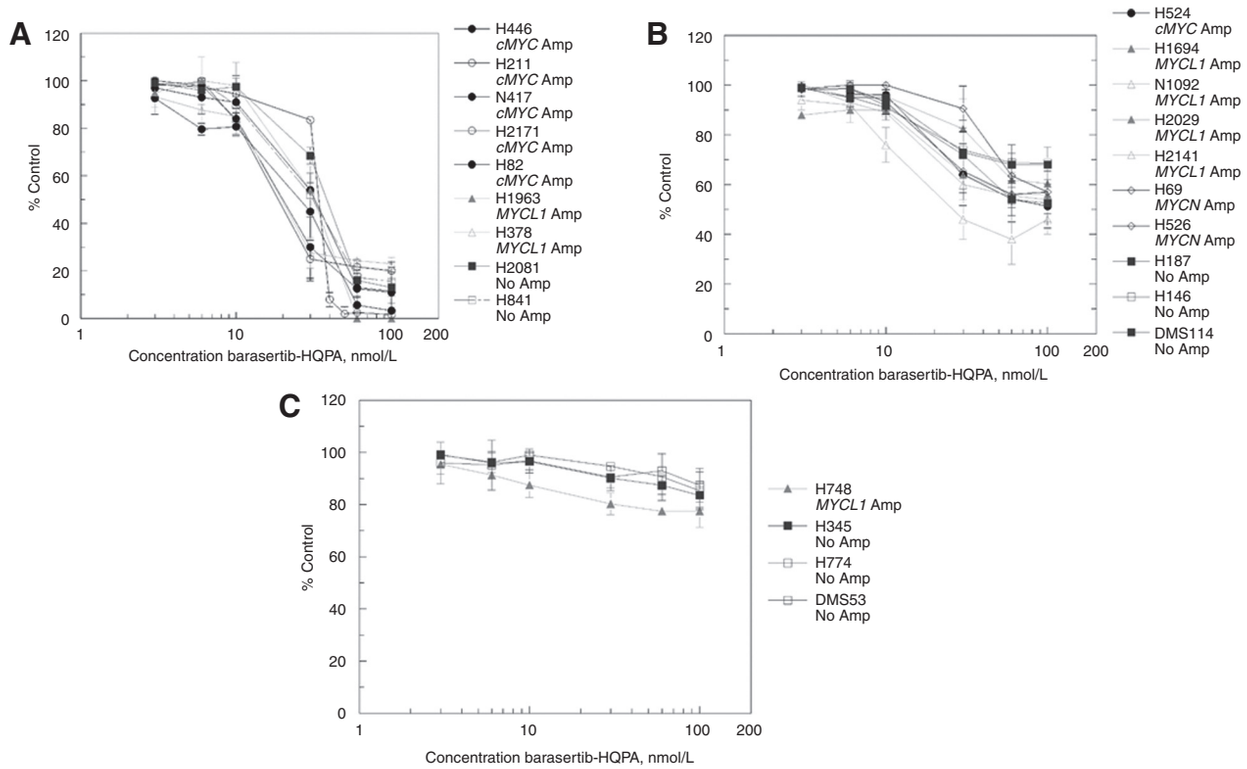

Figure 1.

Growth inhibition by barasertib-HQPA in SCLC lines. The percentage growth inhibition compared with control was determined in MTS assays at 120 hours. **A,** these cell lines were classified as sensitive as the IC_{50} concentration was <50 nmol/L and growth inhibition at 100 nmol/L was >75%. **B,** these lines were classified as moderately resistant as growth inhibition at 100 nmol/L was 32% to 50%. **C,** these cell lines were classified as resistant as growth inhibition at 100 nmol/L was <20%.

alter the growth inhibition (data not shown). Three resistant lines had no *MYC* family amplification and 1 line was *MYCL1* gene amplified.

Relationship of barasertib-HQPA growth inhibition to *MYC* family gene amplification

As shown in Fig. 1A–C, 6 cell lines were *cMYC* amplified, of which 5 were sensitive. Seventeen cell lines were not *cMYC* amplified, of which 4 were sensitive. Thus, there was a significant association between sensitivity to growth inhibition by barasertib-HQPA and *cMYC* amplification ($P = 0.018$). The odds of being sensitive were 16 times higher [95% confidence interval (CI), 1.4, 183] for *cMYC*-amplified SCLC lines compared with non-*cMYC*–amplified cell lines. The association between *MYCL1* gene amplification and growth inhibition was not significant ($P = 0.2$). As there were only 2 *MYCN*-amplified lines and both had intermediate sensitivity, we did not evaluate this as a separate group. There was also no significant association between sensitivity to barasertib-HQPA and having any *MYC* family gene amplification ($P = 0.4$).

Relationship of barasertib-HQPA growth inhibition to *cMYC* gene expression

We next determined whether there was an association between *cMYC* gene expression and sensitivity to growth inhibition by barasertib-HQPA (Table 1; Fig. 2A). The mean *cMYC* gene expression in sensitive lines (10.9 ± 4) was higher than the mean *cMYC* gene expression of the intermediate/resistant lines (7.2 ± 3.3). The *cMYC* gene expression was high (>10) in 11 cell lines, of which 7 were sensitive, and the *cMYC* gene expression was low (<7.5) in 12 cell lines, of which 2 were sensitive to barasertib-HQPA. This difference was significant ($P = 0.026$).

There was no significant association between the mean *MYCL1* gene expression in the sensitive cell lines (8.4 ± 2.7) and the intermediate/resistant lines (9.8 ± 2.8; $P = 0.28$). There was also no significant association between the mean *MYCN* gene expression in the sensitive cell lines (5.3 ± 0.67) and the intermediate/resistant lines (6.7 ± 3; $P = 0.13$).

We then separated the cell lines into two groups based on *cMYC* gene expression modeled as a categorical variable based on quartile cut-off values of the initial distribution of being sensitive, intermediate, or resistant to growth inhibition by barasertib-HQPA. Categories were combined if similar coefficients were observed. The best functional form of *cMYC* gene expression was the dichotomized variable, with two categories at the cut-off point of 12.9 (<12.9 vs. $\geq$12.9), which corresponded to the 75th percentile of the *cMYC* gene. On the basis of these criteria, the odds of being sensitive were 11 times higher (95% CI, 1.2, 103) for cell lines with *cMYC* gene expression $\geq$12.9 compared with cell lines with *cMYC* gene expression <12.9.

As shown in Fig. 2B, we analyzed our cell line panel for the expression of a core *MYC* gene signature (22). The *cMYC* signature was present in 9 cell lines, of which 6 were sensitive to barasertib-HQPA. The *cMYC* gene signature was absent in 14 cell lines, of

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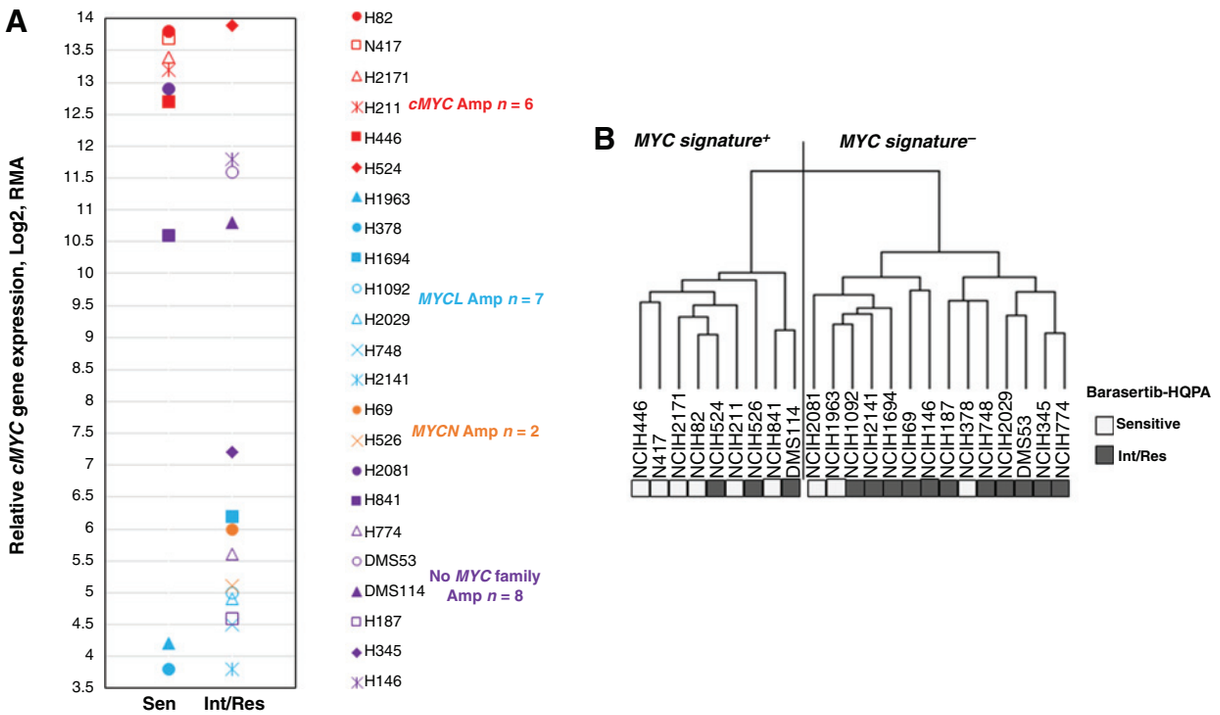

Figure 2.

cMYC gene expression and a core *MYC* gene signature correlated with growth inhibition by barasertib-HQPA in SCLC lines. **A,** the mean *cMYC* gene expression in the 9 SCLCs classified as sensitive (Sen) was 10.9 (SD 4) versus 7.6 (SD 3.3) in the intermediate (Int)/resistant (Res) lines. RMA, robust multiarray average. By a two-tailed *t* test, the difference of 3.7 between sensitive and intermediate/resistant lines was significant ($P = 0.026$). *cMYC* gene expression was from the CCLE. **B,** there was a correlation between being positive for the *MYC* core gene signature and growth inhibition by barasertib-HQPA ($P = 0.042$).

which 3 were sensitive to barasertib-HQPA. All 5 *cMYC* gene amplified lines that were sensitive to growth inhibition by barasertib-HQPA expressed the core *MYC* gene signature, as did the H524 *cMYC*-amplified line, which was intermediate in growth inhibition. None of the 7 *MYCL1*-amplified lines expressed the core *MYC* gene signature, including the H378 and H1963 lines, which were sensitive to growth inhibition. The 2 *MYCN*-amplified lines were both intermediate to growth inhibition, and H526 was positive for the core signature and H69 was negative. Two of the 8 SCLC lines that did not have *MYC* family gene amplification were positive for the *MYC* core gene signature, and the H841 line was sensitive to growth inhibition and DMS114 was intermediate. The remaining 6 SCLC lines without *MYC* family amplification were negative for the core *MYC* gene signature. The H2081 line was sensitive to growth inhibition by barasertib-HQPA, and the remaining 5 lines were intermediate or resistant to growth inhibition. There was a statistically significant correlation between expression of the *MYC* core gene signature and sensitivity to growth inhibition by barsertib-HQPA ($P = 0.042$), although this correlation was not superior to the use of *cMYC* gene expression ($P = 0.026$) or amplification ($P = 0.018$).

Barasertib-HQPA inhibited phosphorylation of histone H3

To ensure that resistance to growth inhibition by barasertib-HQPA was not due to lack of drug uptake by the cell lines, we evaluated phosphorylated histone H3 (Ser10) by flow cytometry. Phosphorylation on histone H3 (Ser10) by AURKB is required for chromosome condensation during mitosis (26). The fraction of detectable cells with phosphorylated H3 (Ser10) was very low in untreated SCLC lines (data not shown). Therefore, we treated the SCLC lines with paclitaxel for 24 hours to arrest the cells in the G_2–M phase of the cell cycle, increasing the fraction of cells with phospho-H3 (Ser10). As shown in Fig. 3, a concurrent 24-hour treatment with paclitaxel and barasertib-HQPA reduced the fraction of phosphorylated H3 (Ser10)-positive cells induced by paclitaxel alone. This demonstrated that barasertib-HQPA actively inhibited the kinase activity of AURKB in cell lines both sensitive (Fig. 3A, H446) and resistant (Fig. 3B and C, H345 and H748) to barasertib-HQPA–induced growth inhibition, suggesting that drug uptake does not explain the difference. Although it is possible that barasertib-HQPA could be less stable in the resistant lines during a longer exposure, it has been shown to be stable for >72 days at pH 7.4 and the extrapolated half-life at pH1 is >11.4 days (personal communication on July 12, 2016, Dr. Sue Ashton, AstraZeneca).

Barasertib-HQPA induced polyploidy

The induction of polyploidy (DNA content ≥8N) is the hallmark of phenotypic changes induced by AURKB inhibition as the cells reenter the S-phase of the cell cycle without going through cytokinesis (27). We evaluated the degree of polyploidy at 24 and 48 hours posttreatment with 30 or 50 nmol/L barasertib-HQPA in 10 SCLC lines, representing lines with *cMYC*, *MYCL1*, *MYCN*

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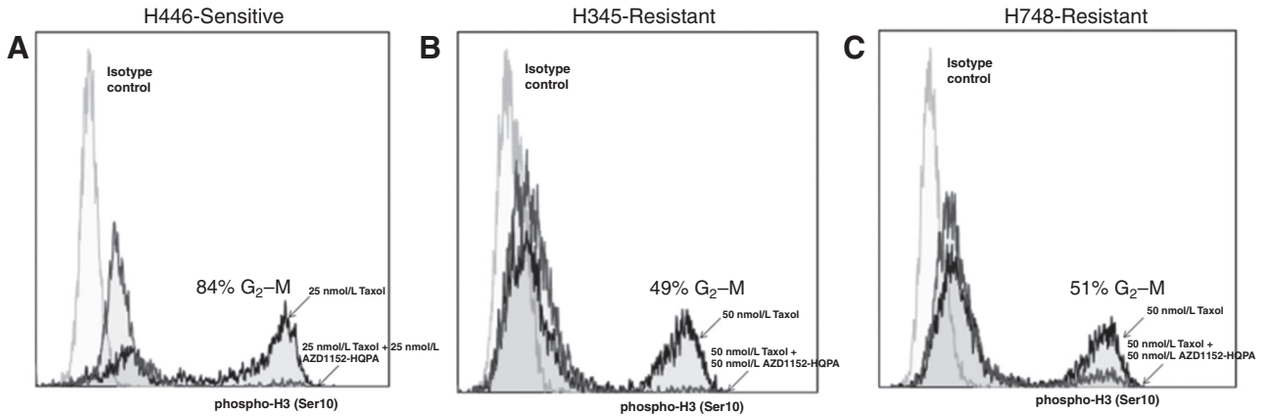

Figure 3.

A–C, barasertib-HQPA inhibits the kinase activity of Aurora B in SCLC lines that are sensitive (H446; **A**) and resistant [H345 (**B**) and H748 (**C**)] to growth inhibition. Cell lines were treated for 24 hours with paclitaxel to arrest the cells in G_2–M, increasing the fraction of phosphorylated histone H3 (Ser10) cells. Concurrent treatment with equal concentrations of paclitaxel and barasertib-HQPA reduced the fraction of phosphorylated histone H3. Analysis was done by intracellular staining with α-phosphorylated histone H3 (Ser10) and by flow cytometry.

amplification, and no *MYC* family amplification, that were sensitive, intermediate, or resistant to growth inhibition by barasertib-HQPA (Table 2).

After exposure to 30 nmol/L barasertib-HQPA, the sensitive cell lines H378, H841, H211, and H446 had a significant increase in the fraction of 4N cells at 24 hours and the fraction of 8N cells at 48 hours (Table 2). Similarly, in the intermediate lines H69, H146, H187, and H524 the fraction of 4N cells was significantly increased at 24 hours, and the fraction of 8N cells increased at 48 hours.

While the time course for the appearance of a tetraploid peak followed by a polyploid peak was consistent across the above cell lines, this was not the scenario in the more resistant lines. The H345 cell line was initially treated with 30 nmol/L barasertib-HQPA, and as shown in Table 2, despite an increase in the fraction of 4N cells, there was no increase in the 8N cell fraction. Subsequently, we treated H345 and the other resistant cells lines DMS53, H774, and H748 with 50 nmol/L barasertib-HQPA; as shown in Table 2, the cell lines remained 4N following 48 hours of treatment. In summary, cytokinesis failure in the cell lines sensitive and intermediate to growth inhibition by barasertib-HQPA resulted in DNA endoreduplication and the appearance of polyploid cells (DNA content 8N). In contrast, the resistant cell lines H345, DMS53, H774, and H748 remained in a tetraploid state, did not reenter the S-phase, and did not undergo endoreduplication.

Barasertib inhibited tumor xenografts

We evaluated the *in vivo* efficacy of barasertib on the SCLC line H841 (no *cMYC* family amplification, high *cMYC* gene expression, and positive for the *MYC* gene expression signature). In the *in vitro* growth inhibition assays described above, we demonstrated that 100 nmol/L barasertib-HQPA inhibited the growth of H841 by 85%. The H841 cells were implanted subcutaneously in nude mice and treated with barasertib at 50 or 100 mg/kg/day Monday to Friday for 2 weeks. The barasertib doses were within the range previously published for *in vivo* studies of solid tumors and provided trough barasertib-HQPA serum concentrations above the concentrations used *in vitro* (28, 29).

As shown in Fig. 4A, the H841 xenografts were significantly growth inhibited during the 2-week barasertib treatment (days 20–31) compared with control mice. On day 34, the control mice were sacrificed due to tumor size. On day 34, the control tumors had a mean tumor volume of $2{,}774 \pm 2{,}106$ mm^3 compared with 232 ± 186 mm^3 in the 50 mg/kg treatment group ($P = 0.011$). The treatment groups were followed for an additional 30 days following cessation of treatment. At day 61, the mean tumor volume in the 50 mg/kg treatment group was $2{,}828 \pm 3{,}670$ mm^3, comparable with the growth of the control tumors at sacrifice.

The 100 mg/kg barasertib dose caused H841 tumor xenograft regression, and the tumors remained regressed through day 61 when the experiment was terminated. There was no loss of weight throughout the course of treatment in treated animals compared with control mice (Fig. 4B).

Discussion

In this study, we show that the specific AURKB inhibitor barasertib-HQPA has growth-inhibitory effects in some SCLC lines and that *cMYC* amplification or high gene expression or *MYC* gene signature is a useful predictive biomarker. There has been little advance in the therapy of SCLC over the past three decades, and new therapeutic options are urgently needed. The rapid growth and frequent overexpression of aurora kinases, which are key regulators of mitosis, has made aurora kinase inhibitors attractive targets for SCLC therapy. AURKB directly phosphorylates Rb at serine 780, playing a critical role in regulating postmitotic checkpoints to prevent polyploidy after an aberrant mitosis (13). In SCLC, loss of Rb and p53 function is essentially universal, leading to suppression of postmitotic checkpoints that prevent polyploidy after aberrant mitosis by eliciting a pseudo G_1 arrest. Barasertib-HQPA was previously reported to induce polyploidy in tumor cells, providing rationale for the study of barasertib-HQPA in SCLC (13). We report that polyploidy is induced in sensitive line but not in resistant lines.

Prior studies with dual aurora kinase A/B inhibitors and with specific AURKA kinase inhibitors demonstrated considerable activity in a small number of SCLC cell lines (14, 16). Growth

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Table 2. Percent ploidy induced by barasertib-HQPA

Cell line	Conc. nmol/L	Time	4N	≥8N	%GI 30 nmol/L at 120 h
H446		0 h	31	6	
cMYC	30	24 h	75	10	55
Amp		48 h	11	71	
H211		0 h	24	1	
cMYC	30	24 h	80	5	75
Amp		48 h	19	41	
H841		0 h	25	0	
No Amp	30	24 h	61	19	48
		48 h	40	29	
H378		0 h	28	0	
MYCL1	30	24 h	44	0	72
Amp		48 h	55	11	
H524		0 h	22	0	
cMYC	30	24 h	53	2	36
Amp		48 h	55	24	
H69		0 h	44	3	
MYCN	30	24 h	82	7	35
Amp		48 h	38	58	
H187		0 h	42	9	
No Amp	30	24 h	85	10	28
		48 h	53	45	
H146		0 h	40	7	
No Amp	30	24 h	72	9	26
		48 h	45	45	
H345		0 h	35	6	
No Amp	30	24 h	61	7	10
		48 h	72	13	
H345		0 h	41	3	
No Amp	50	24 h	40	4	10
		48 h	65	6	
DMS53		0 h	21	2	
No Amp	50	24 h	62	1	5
		48 h	76	6	
H774		0 h	22	2	
No Amp	50	24 h	31	2	10
		48 h	58	3	
H748		0 h	20	1	
MYCL1	50	24 h	44	3	10
Amp		48 h	55	8	

Abbreviations: 4N, tetraploid DNA content; ≥8N, polyploid DNA content; h, hour; Amp, amplification; Conc., concentration; GI, growth inhibition; ND, not done.

inhibition by dual aurora A/B inhibitors and with AURKB knockdown has been correlated with *cMYC* amplification alone (14) and by other investigators with any *MYC* family amplification or high *MYC* family gene expression (16). Recently, it has also been shown through activity-based protein profiling that AURKB is a critical kinase in *cMYC*-amplified SCLC cell lines but not in SCLC lines that lack *cMYC* amplification (30). Amplifications and overexpression of *MYC* family oncogenes has been reported in 15% to 30% of SCLCs (17, 18).

We report that 39% of the cell lines tested were sensitive to growth inhibition by barasertib-HQPA at concentrations that can be achieved in humans and do not inhibit AURKA. We found that *cMYC* amplification, *cMYC* gene expression, and a *cMYC* gene signature were significant predictors of growth inhibition to barasertib-HQPA. The strongest association between growth inhibition was *cMYC* gene amplification ($P = 0.018$), but *cMYC* gene expression ($P = 0.026$) and *cMYC* gene signature ($P = 0.042$) also correlated with growth inhibition. In addition, by an OR estimate, *cMYC*-amplified cell lines were 16 times more likely to be sensitive to growth inhibition by barasertib-HQPA than non-*cMYC*–amplified cell lines. For *cMYC* gene expression, the odds of being sensitive to growth inhibition were 11 times higher for cell lines with cMYC gene expression >12.9. Although the correlations between *cMYC* amplification, *cMyc* gene expression and *cMYC* gene signature, and response to barasertib-HQPA are significant, the sample size is small and should be confirmed in clinical trials where *cMYC* amplification status is assessed in all patients. Although there was some relationship to expression/amplification of other *MYC* family members and sensitivity to barasertib-HQPA, these correlations were not significant. However, in both our study and the studies of Sos and colleagues, there were *cMYC*-amplified cell lines that were not sensitive to growth inhibition by AURKB or dual aurora kinase A/B inhibitors (14).

In contrast, Hook and colleagues found significant association between IC_{50} values for the dual AURKA/AURKB inhibitor PF-3814735 and activation/amplification of any *MYC* family member ($P = 0.001$; ref. 16). We did not observe this with *MYCL1* amplification ($P = 0.2$). There was also no significant association between sensitivity and being amplified for any *MYC* family ($P = 0.4$). We also did not find a statistically significant association between high *MYCL1* ($P = 0.28$) or *MYCN* ($P = 0.12$) gene expression and sensitivity to growth inhibition.

Further support of the potential combined predictive power of *cMYC* amplification/high gene expression and the *MYC* core gene signature is evidenced by our *in vivo* experiments. We implanted the SCLC line H841, which has high *cMYC* gene expression, no *MYC* family amplification, but was positive for the core *MYC* gene signature. This line was sensitive *in vitro* to growth inhibition by barasertib-HQPA, $IC_{50} < 50$ nmol/L. Mice were dosed with either 50 or 100 mg/kg of the barasertib. The 50 mg/kg dose inhibited tumor growth; however, when treatment was stopped, the tumors began growing. At the 100 mg/kg dose, the H841 tumor regressed and remained regressed until the termination of the experiment.

Previous investigators evaluated the dual aurora A/B inhibitor PF-03814735 in mice-bearing xenografts of the *cMYC*-amplified SCLC line H82 and the *MYCN*-amplified SCLC line H69 (16). Growth inhibition in the *cMYC*-amplified H82 xenograft model was greater than in the *MYCN* H69-amplified xenografts. In our *in vitro* investigation of the efficacy of barasertib-HQPA, the *cMYC*-amplified line H82 was sensitive to growth inhibition ($IC_{50} < 30$ nmol/L), and the H69 *MYCN*-amplified line had only intermediate sensitivity, as at 100 nmol/L barasertib-HQPA, growth inhibition was 43%. The *cMYC*-amplified line H82 was positive for the core *MYC* gene signature and the *MYCN*-amplified H69 line was not.

There are some clinical data suggesting a role for aurora kinase inhibitors in SCLC. A phase II study of alisertib (AURKA inhibitor) given twice daily for 7 days every 21 days showed a response rate of 21% among 48 previously treated SCLC patients (15). Unfortunately, no biomarker correlates, such as *cMYC* amplification or gene expression, were determined. This alisertib phase II study was followed by a randomized global phase II trial of alisertib plus weekly paclitaxel versus placebo plus weekly paclitaxel in the second-line setting. This trial has completed accrual, but the results have not been reported. Unfortunately, *cMYC* amplification was not evaluated in this study. Clinical studies of alisertib

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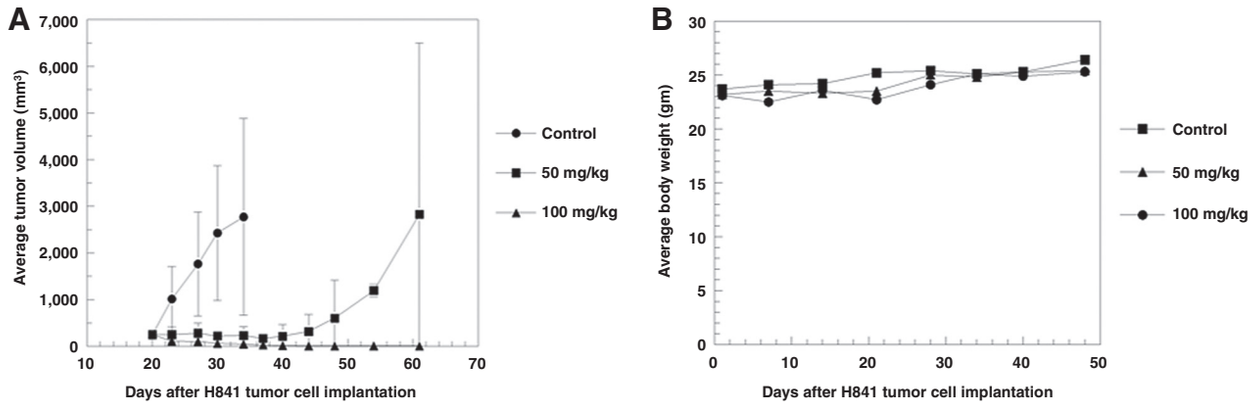

Figure 4.

A, antitumor effects of barasertib on H841 (no *MYC* family amplification, high *cMYC* gene expression, and for *MYC* gene expression signature) tumor xenografts heterotransplanted into athymic nude mice. The mice were treated with 50 or 100 mg/kg for 5/7 days for two weeks. **A,** significant tumor growth delay compared with untreated control mice at day 34 after tumor transplantation (time point at which control mice were sacrificed) was observed in the *in vitro* sensitive SCLC line H841 at the 50 mg/kg dose of barasertib ($P = 0.011$). However, regrowth occurred once treatment was discontinued. Tumor regression was observed at the 100 mg/kg dose. **B,** barasertib at 50 or 100 mg/kg had no deleterious effects on body weight.

and other aurora kinase inhibitors have generally used a continuous dosing schedule largely based on preclinical studies with leukemia models. The early clinical studies using continuous dosing schedules reported myelosuppression as the dose-limiting toxicities (15, 24, 25). In our studies, SCLC cell lines were growth inhibited with a single exposure *in vitro* and an intermittent exposure *in vivo*.

Recently, AstraZeneca developed nanoparticles containing AZD2811 formerly known as barasertib-HQPA that increases biodistribution to tumor sites with minimal impact on bone marrow pathology, resulting in lower toxicity and increased efficacy in multiple tumor models at half the dose intensity of barasertib (31). Thus, we believe that SCLC clinical trials with aurora kinase inhibitors, such as AZD2811, using a panel of MYC biomarkers and intermittent drug scheduling studies should be done where a high dose is given intermittently, as this is maybe more effective and would allow recovery of the bone marrow. Support of an intermittent dosing schedule was also supported by an *in vivo* xenograft study using the *cMYC*-amplified H82 SCLC line, where tumor growth control was more effective with a once weekly high dose 80 mg/kg (2× daily once for 3 weeks) of the dual A/B PF-03814735 inhibitor compared with a low-dose daily schedule using 15 mg/kg (2× daily for 10 conservative days; ref. 16). Furthermore, a high-dose short course of barasertib therapy in a xenograft flank model using a *MYCN*-overexpressing medulloblastoma model was efficacious at reducing tumor volume. Mice were dosed with barasertib at 50 mg/kg 2× daily for 2 days, and tumor volumes were significantly different from 4 days after treatment until the end of the experiment ($P < 0.05$; ref. 32). We believe that an AZD2811 clinical trial should be considered in SCLC patients who progress after etoposide/platinum doublet therapy with assessment of *cMYC* amplification and gene expression in all patients to determine the response rate in SCLC patients with and without *cMYC* amplification/gene expression. Alternatively, a "window of opportunity" maintenance trial could be done after 4 cycles of etoposide/cisplatin to determine time to progression in patients with and without *cMYC* amplification.

In conclusion, the AURKB inhibitor, barasertib-HQPA, inhibited the growth of SCLC lines both *in vitro* and *in vivo*. Growth inhibition was greater in cell lines where barasertib-HQPA induced endoreduplication. Our studies suggest that SCLC tumors with *cMYC* amplification/high gene expression will frequently respond to Aurora B inhibitors and that clinical studies of the novel AZD2811 nanoparticle coupled with predictive biomarkers are indicated especially using an intermittent dosing schedule.

Disclosure of Potential Conflicts of Interest

P.A. Bunn reports receiving a commercial research grant from and is a consultant/advisory board member for AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: B.A. Helfrich, D.C. Chan, P.A. Bunn Jr
Development of methodology: B.A. Helfrich
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.A. Helfrich, D.C. Chan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.A. Helfrich, J. Kim, D. Gao, D.C. Chan, A.-C. Tan
Writing, review, and/or revision of the manuscript: B.A. Helfrich, J. Kim, D. Gao, A.-C. Tan, P.A. Bunn Jr
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