AZD4573 Is a Highly Selective CDK9 Inhibitor That Suppresses MCL-1 and Induces Apoptosis in Hematologic Cancer Cells

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ABSTRACT

Purpose: Cyclin-dependent kinase 9 (CDK9) is a transcriptional regulator and potential therapeutic target for many cancers. Multiple nonselective CDK9 inhibitors have progressed clinically but were limited by a narrow therapeutic window. This work describes a novel, potent, and highly selective CDK9 inhibitor, AZD4573.

Experimental Design: The antitumor activity of AZD4573 was determined across broad cancer cell line panels in vitro as well as cell line- and patient-derived xenograft models in vivo. Multiple approaches, including integrated transcriptomic and proteomic analyses, loss-of-function pathway interrogation, and pharmacologic comparisons, were employed to further understand the major mechanism driving AZD4573 activity and to establish an exposure/effect relationship.

Results: AZD4573 is a highly selective and potent CDK9 inhibitor. It demonstrated rapid induction of apoptosis and subsequent cell death broadly across hematologic cancer models in vitro, and MCL-1 depletion in a dose- and time-dependent manner was identified as a major mechanism through which AZD4573 induces cell death in tumor cells. This pharmacodynamic (PD) response was also observed in vivo, which led to regressions in both subcutaneous tumor xenografts and disseminated models at tolerated doses both as monotherapy or in combination with venetoclax. This understanding of the mechanism, exposure, and antitumor activity of AZD4573 facilitated development of a robust pharmacokinetic/PD/efficacy model used to inform the clinical trial design.

Conclusions: Selective targeting of CDK9 enables the indirect inhibition of MCL-1, providing a therapeutic option for MCL-1-dependent diseases. Accordingly, AZD4573 is currently being evaluated in a phase I clinical trial for patients with hematologic malignancies (clinicaltrials.gov identifier: NCT03263637).

Introduction

Cyclin-dependent kinases (CDK) are a family of closely related serine/threonine kinases known for playing crucial roles in regulating either cell-cycle progression (CDK1, 2, 4, 6) or gene transcription (CDK7, 8, 9, 12, 13; ref. 1). As integral nodes in these regulatory networks, CDKs have been studied extensively as possible targets for cancer therapy, resulting in the development of nonselective CDK inhibitors (2) that show signs of clinical activity (3–6). Despite pharmacologically inhibiting multiple CDKs, the observed clinical activity of nonselective CDK inhibitors has been primarily attributed to their CDK9 activity (7–9).

CDK9 is crucial for the proper regulation and progression of transcription. Through phosphorylation of serine 2 (pSer2) in the heptapeptide repeats within the C-terminal domain of RNA polymerase II (RNAP2), CDK9 releases RNAP2 from its paused state to enable transcription elongation (10). Acute inhibition of CDK9, therefore, results in transient transcriptional suppression and preferential depletion of short-lived transcripts and proteins (11), providing a therapeutic approach to indirectly target key driver oncoproteins with short half-lives. Nonselective CDK9 inhibitors, such as dinaciclib and flavopiridol (alvocidib), have been repurposed to explore this opportunity but have not progressed clinically as monotherapies due to narrow therapeutic indices (6, 12). However, clinical trials are ongoing to evaluate these molecules in combination with other therapeutics. Despite clinical development of multiple inhibitors with CDK9 activity, the precise mode of action driving antitumor effects has yet to be fully elucidated, although their preclinical activity has been attributed predominantly to depletion of MCL-1 protein and subsequent induction of tumor cell death (13–15). MCL-1 is an antiapoptotic, BCL-2 family protein whose high expression has been associated with increased cancer cell survival that translates to chemotherapy resistance and poor patient prognosis (16–18).

This work describes the mechanism of action and preclinical activity of the novel and highly selective CDK9 inhibitor, AZD4573. An agnostic approach utilizing integrated transcriptomic and proteomic analyses revealed MCL1 as one of the top oncoproteins most significantly and robustly downregulated at the mRNA and protein level upon acute AZD4573 treatment. Subsequent pharmacologic studies further supported an MCL1-mediated mechanism, including derivation of a pharmacokinetic (PK)/pharmacodynamic (PD)/efficacy model linked to CDK9 inhibition, MCL-1 depletion, and induction of apoptosis in...
Translational Relevance

Multiple cyclin-dependent kinase 9 (CDK9) inhibitors, both nonselective and selective, have been evaluated in clinical trials. Despite signs of clinical activity and ongoing combination studies, monotherapy development was ultimately not pursued due to narrow safety margins. Contributing factors could include a lack of compound selectivity for CDK9, the absence of a clear patient selection strategy, and suboptimal choice of dose and/or schedule. The development and full preclinical mechanistic, pharmacokinetic, and pharmacodynamic understanding of a highly selective and potent CDK9 inhibitor, like AZD4573, could mitigate those limitations. Accordingly, pharmacologic evaluation of AZD4573 revealed broad antitumor activity across preclinical hematologic cancer models. This work established a quantitative relationship between extent and duration of CDK9 inhibition, depletion of MCL-1, and rate of apoptosis induction that was used to estimate the efficacious dose range and hopefully maximize the therapeutic window in clinical studies.

Response to AZD4573. Application of this mechanistic understanding using short-term treatment and intermittent dosing with AZD4573 was purchased from MedKoo. For chemical models. Collectively, this work supports the clinical evaluation of AZD4573 and AZD5991 were synthesized at AstraZeneca. Venetoclax (ABT-199) was purchased from MedKoo. For in vitro assays, compounds were solubilized to 10 mmol/L in DMSO. Compounds were dosed to yield a final DMSO concentration of ≤0.3%. For in vivo studies, AZD4573 was prepared in a 2%/30%/68% mix of N,N-dimethylacetamide, PEG-400, and 1% (v/v) Tween-80, AZD5991 was formulated in 30% 2-hydroxypropyl-beta-cyclodextrin (HPBCD) at pH 9, and venetoclax was solubilized in a 10%/30%/60% mix of ethanol, PEG-400, and Phosal 50 PG.

Materials and Methods

Chemicals
AZD4573 and AZD5991 were synthesized at AstraZeneca. Venetoclax (ABT-199) was purchased from MedKoo. For in vitro assays, compounds were solubilized to 10 mmol/L in DMSO. Compounds were dosed to yield a final DMSO concentration of ≤0.3%. For in vivo studies, AZD4573 was prepared in a 2%/30%/68% mix of N,N-dimethylacetamide, PEG-400, and 1% (v/v) Tween-80, AZD5991 was formulated in 30% 2-hydroxypropyl-beta-cyclodextrin (HPBCD) at pH 9, and venetoclax was solubilized in a 10%/30%/60% mix of ethanol, PEG-400, and Phosal 50 PG.

Enzyme assays
AZD4573 was screened at 0.1 μmol/L against 468 kinases and relevant variants using the KINOMEScan assay (DiscoveRx), and kinase selectivity images were generated using TREESpot Software Tool. SignalChemiompound selectivity profiling was utilized to screen AZD4573 against select CDKs and non-CDK kinases at 5 mmol/L (high) ATP concentration for IC₅₀ determination.

Immunoprecipitation and immunoblotting
Cell and tumor lysates and immunoprecipitation (IP) samples were prepared and subjected to immunoblotting as described previously using antibodies listed in Supplementary Table S1 (19).

In vitro caspase and viability assays
Assays were conducted as previously described (19) and according to the manufacturer’s protocols for Caspase-Glo 3/7 and CellTiter-Glo (Promega). For cell panel screens, cells were plated in 384-well plates and dosed the following day with 10-point, 1/2 log dilutions of compound, whereas combination treatments used 5-point, 1/2 log dilutions. Automated dosing was performed using an Echo 555 acoustic liquid dispenser (Labcyte). To calculate percent caspase activation, luminescence values were normalized against maximum (100%, mixture of MCL-1, BCL-2, and BCL-XL inhibitors) and minimum (0%, DMSO) controls. Percent growth inhibition was normalized to untreated cells at the time of dosing, whereas percent viability was normalized against maximum (100%, DMSO) and minimum (0%, medium only) controls. Dose–response curves were generated and half-maximal effective (EC₅₀) and growth inhibitory (GI₅₀) concentrations calculated using GeneData Screener and GraphPad Prism.

Washout assay
Cells were seeded the day before treatment with the indicated AZD4573 dose. At the indicated times, cells were washed twice in complete media and plated at 1 × 10⁵ cells/well in 96-well plates. Cells were incubated until 12 or 24 hours after the time of initial dosing, at which point Caspase-Glo 3/7 and CellTiter-Glo readouts were performed, respectively.

RNA sequencing library preparation and analysis
cDNA library construction for RNA sequencing (RNA-seq) was performed at Novogene using an NEBNext Ultra mRNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer’s protocol. RNA-seq gene expression quantification was conducted using Sailfish against hg38 Ensembl transcripts (version 79), and differential expression analysis was done using Voom and Limma R package. A detailed description of experimental procedures can be found in the Supplementary Methods. The data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE129012.

Mass-spectrometry proteomics
Trypsin digestion, Tandem Mass Tag labeling, and LC-MS/MS analysis were conducted as previously described (20). A detailed description of experimental procedures can be found in the Supplementary Methods. The data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013796.

Real-time PCR
MV411 cells (1 × 10⁶) treated with vehicle or 0.1 μmol/L AZD4573 were collected at the indicated timepoints. MV-4-11 tumors were also harvested, placed in a Lysing Matrix D tube (Millipore) with Buffer RLT (Qiagen) supplemented with 1% β-mercaptoethanol, and mechanically disrupted using the Millipore FastPrep. RNA isolation and cDNA conversion were conducted using RNAeasy Mini (Qiagen) and High Capacity cDNA RT (Applied Biosystems) kits, respectively, following the manufacturers’ protocols. Relative MCL1 mRNA levels were quantified on an ABI Prism 7900HT Real-time PCR system (ThermoFisher) using Taqman Assays (Applied Biosystems, MCL1: Hs01050896_m1, 18s rRNA: 4319413E). Relative MCL1 expression at each timepoint was calculated using the –ΔΔCₚ method by normalizing cycle threshold values to the 18s rRNA reference.

In vivo studies
Subcutaneous xenografts
Studies were conducted at AstraZeneca in accordance with animal research guidelines from the NIH and the AstraZeneca Institutional

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Animal Care and Use Committee and as previously described (19). Injected cell numbers for cell line xenografts are as follows: 5 × 10^6 for MV-4–11, MOLT-8, and OCI-LY10, and 1 × 10^7 for all others. AZD4573 was dosed i.p.) at either 5 or 15 mg/kg, twice daily with a 2-hour split dose (BID q12h), on a 2-day on/5-day off or biweekly schedule. Venetoclax was dosed daily at 100 mg/kg by oral gavage. AZD5991 was dosed i.v. at 60 mg/kg. For combination studies, venetoclax was administered first, and AZD4573 was administered within 30 min.

AML disseminated patient-derived xenografts

Study was conducted at Champions Oncology using nine models (21). Following 4 weeks of AZD4573 therapy, mice (n = 3 per model) were euthanized for end of study analysis on the spleen, bone marrow, and peripheral blood by FACS analysis to detect human CD45+/CD33+/CD3+ acute myeloid leukemia (AML) blasts.

T-cell lymphoma patient-derived xenograft

Study was conducted under Dana-Farber Cancer Institute Animal Care and Use Committee protocol #13-034 and Public Health Service animal assurance #A3023-01. A full characterization of the model, DFTL-78024, is available online at www.PRoXe.org. Animal work was performed in NSG mice purchased from Jackson Laboratories as published previously (22). When sufficiently engrafted, mice were treated for four cycles of AZD4573 and monitored daily for clinical signs of disease and humanely euthanized when they reached a clinical endpoint. Annexin V was assessed in human CD45+ tumors isolated from peripheral blood 6 hours after the second day 2 dose as previously described (23).

PK/PD/efficacy model development

Model parameters are listed in Supplementary Table S2, and full methodology and derivation are available in the Supplementary Methods.

Data sharing statement

For original data, please contact justin.cidado@astrazeneca.com.

Results

Identification of a highly selective and potent CDK9 inhibitor, AZD4573

Understanding the binding of a high-quality probe compound to CDK9 helped guide optimization of a selective and potent CDK9 inhibitor with suitable physicochemical properties that would result in a short compound half-life and enable intravenous administration in humans. This structure-based drug discovery effort ultimately led to the identification of AZD4573 (Fig. 1A; ref. 24), which potently inhibits the CDK9 enzyme (IC50 at Ks and high ATP concentrations are <0.003 μM/L and <0.004 μM/L, respectively). Kinase selectivity profiling measured the affinity of AZD4573 across a set of 468 kinases and relevant variants using the KINOMEscan platform. At a screening concentration of 0.1 μM/L, which is more than 100-fold above the measured IC50 in a CDK9 biochemical assay, AZD4573 resulted in 90% reduction of binding to the cognate ligand capture matrix for 16 kinases (Fig. 1B). For 14 of the top hits, the IC50 for AZD4573 was determined at a physiologically relevant concentration of ATP, revealing >10-fold selectivity for CDK9 compared with 13 of 14 kinases and >100-fold selectivity compared with 8 of 14 kinases (Fig. 1C). Similarly, AZD4573 exhibited >25-fold cellular selectivity for CDK9 over other CDKs upon short-term treatment of MCF7 cells (Fig. 1D; Supplementary Fig. S1A). MCF7 is a favorable model for assessing AZD4573 selectivity and mechanism of action because a frameshift mutation in the CASP3 gene results in the absence of functional caspase-3 protein, preventing potentially confounding apoptosis induction and cell death.

Kinetic analysis of CASPASE-3 cleavage (“caspase activation”), a hallmark of committed apoptosis, and loss of viability in cell lines cultured with AZD4573 over 24 hours showed that potent and selective CDK9 inhibition can kill cancer cells. Though results varied slightly, they highlighted a rapid response to AZD4573 in sensitive models and a direct correlation of caspase activation with loss of cell viability (Fig. 1E; Supplementary Fig. S1B and S1C). Conversely, the insensitive cell lines exhibited no caspase activation nor loss of viability until prolonged transcription inhibition caused nonspecific cytotoxicity (Supplementary Fig. S1D). This observation underscored the need to determine the requisite duration of target engagement to achieve maximal effect in tumor cells while mitigating unwanted toxicity in normal tissue. Therefore, the AML cell line MV-4–11 was pulsed with AZD4573 for various lengths of time, washed out, and then assessed for caspase activation and loss of cell viability at 12 and 24 hours, respectively. This study showed that approximately 6 to 8 hours of CDK9 inhibition was sufficient to achieve maximal cell death equivalent to continuous exposure of AZD4573 (Fig. 1F).

CDK9 inhibition by AZD4573 results in rapid turnover of MCL-1

In previous studies, MCL-1 overexpression rescued CDK9 inhibitor–mediated apoptosis, seemingly validating MCL-1 depletion as the mechanism driving the antitumor effects (25, 26). However, overexpression studies relying on supraphysiological protein levels come with known caveats, especially in systems requiring a delicate balance between multiple proteins (i.e., apoptosis regulation by BCL-2 family proteins; ref. 27). Therefore, an agnostic approach utilizing integrated transcriptomic and proteomic time-course experiments was employed to elucidate this mechanism. For reasons listed above, the AZD4573-insensitive cell line, MCF7, was used for these experiments to ensure cell death did not introduce artifact into the assay. Cells were treated with vehicle or AZD4573 for short periods of time (0, 4, and 8 hours) based upon target engagement requirements (Supplementary Fig. 1F). Biological triplicates were subjected to RNA-seq or LC-MS/MS, which demonstrated high reproducibility based upon principal component analysis (Supplementary Fig. S2A).

A cancer-associated gene list emphasizing driver oncopgenes, including the antiapoptotic BCL-2 and IAP family proteins, was curated from multiple sources to identify candidate genes whose rapid mRNA and protein depletion could drive AZD4573-mediated cell death (Supplementary Methods and Supplementary Table S3). Through this agnostic approach, the only oncogene and antiapoptotic protein with significantly diminished mRNA and protein levels after 4 and 8 hours of treatment with AZD4573 compared with vehicle treatment was MCL-1 (Fig. 2A and B; Supplementary Tables S4–S6). Conversely, the other antiapoptotic BCL-2 and IAP family proteins were quite stable (BFL-1 and BCL-B were not detected) despite reports that XIAP was rapidly modulated upon CDK9 inhibition (28). Many other oncopgenes, despite rapid mRNA turnover, either had prolonged protein half-lives or were not detected (Supplementary Table S5). This supports MCL-1 modulation as a major driver of AZD4573-mediated tumor cell apoptosis.
Figure 1. Identification of a highly selective and potent CDK9 inhibitor, AZD4573. A, Chemical structure of the CDK9 inhibitor, AZD4573. B, TREEspot image of 468 kinases screened with AZD4573 using the KINOMEscan platform. Percent control corresponds to the amount of kinase bound to its cognate ligand following AZD4573 treatment and relative to vehicle control. C, Biochemical selectivity of 0.1 μmol/L AZD4573 at 5 mmol/L ATP for CDK9 compared with 14 top kinase hits from KINOMEscan. AZD4573 IC_{50} values for each kinase were determined and plotted as fold change over the CDK9 IC_{50}. Selectivity thresholds are depicted by dashed lines and shaded regions. D, Cellular selectivity for multiple CDKs was determined in MCF-7 cells using putative phosphorylation endpoints for the respective kinase. Lysates of MCF-7 cells treated with a dose response of AZD4573 for 6 hours were immunoblotted and quantified by densitometry to derive IC_{50} values. E, MV-4-11 cells treated with 0.1 μmol/L AZD4573 were assessed for caspase activation and loss of viability at multiple time points relative to controls. F, MV-4-11 cells treated with 0.1 μmol/L AZD4573 were washed out at the indicated times and assessed for caspase activation at 12 hours (red) or viability at 24 hours (blue).
Figure 2. Acute inhibition of CDK9 with AZD4573 induces apoptosis through rapid depletion of MCL-1. **A and B**, Differential transcriptomic (**A**) and proteomic (**B**) analysis of MCF-7 cells treated with 0.1 µmol/L AZD4573 for 4 hours (left) or 8 hours (right). Changes in transcript and protein levels are defined as not significant (gray, FDR > 0.05), significant (yellow, FDR < 0.05), and significant with a strong effect (orange, FDR < 0.05 and two-fold change). Other key, measurable antiapoptotic proteins are highlighted in the volcano plots. **C**, Lysates from MV-4-11 cells treated with a range AZD4573 concentrations for 6 hours were immunoblotted for the indicated proteins. **D**, Protein and RNA lysates from MV-4-11 cells treated with 0.1 µmol/L AZD4573 for 24 hours were harvested at the indicated times. MCL1 transcript level was measured by RT-qPCR, whereas pSer2-RNAP2, MCL-1, BCL-2, BCL-XL, and cleaved caspase-3 protein levels were evaluated by immunoblot. Transcript and protein levels are normalized to the pretreatment time point.
Transcript for AZD4573: Inductive of apoptosis upon depletion of MCL-1

Effect of acute AZD4573 treatment on MCL-1 was measured in the sensitive MV-4-11 cell line. AZD4573 treatment for 6 hours increased pSer2-RNAP2 and MCL-1 in a dose-dependent manner (IC50 = 14 μmol/L), and upon sufficient MCL-1 depletion, cleaved caspase-3 levels increased. Although caspase activation only occurred in sensitive cell lines, suppression of pSer2-RNAP2 and MCL-1 occurred in all assayed cell lines, which shows that some cancers rely on survival factors other than MCL-1 (assayed cell lines, which shows that some cancers rely on survival factors other than MCL-1). AZD4573 treatment for 6 hours reduced caspase activation and loss of cell viability. When MV-4-11 cells were treated with a combination of AZD4573 and AZD5991, there was good concordance between caspase and viability data. AZD4573 treatment for 6 hours was also associated with apoptosis induction for those cell lines (Supplementary Fig. S1B–S1D).

Temporal effects of AZD4573 on relevant biomarkers were examined to understand the relationship between rapid biomarker modulation, caspase activation, and loss of cell viability. Within 1 hour and 4 hours, respective (Fig. 2D), Supplementary Fig. S2E). Cleaved caspase-3 levels began to increase between 4 and 6 hours. Importantly, levels of other antiapoptotic proteins, BCL-2 and BCL-XL, remained unchanged over the same dose range (Fig. 2C) and time frame (Fig. 2D), consistent with the transcriptomic and proteomic findings.

Because caspase-3 cleavage can occur through nonapoptotic mechanisms (29), experiments were performed to ensure AZD4573-mediated caspase activation and subsequent cytotoxicity resulted from engagement of the intrinsic apoptosis pathway. Individual knockdown of apoptosis effector molecules, BAK and BAX, and the diffuse large B-cell lymphoma (DLBCL) cell line OCI-LY10 only partially reduced caspase activation following 6 hours of AZD4573 treatment, but simultaneous knockdown completely suppressed caspase activation (Supplementary Fig. S2F). In MV-4-11 cells, AZD4573 rapidly decreased mitochondrial outer membrane potential and increased caspase activation and phosphatidylinerine exposure as measured by tetramethylrhodamine ethyl ester staining, Caspase-3/7 Glo, and Annexin V staining, respectively, consistent with MCL-1 inhibition kinetics and triggering of intrinsic apoptosis (19). These events were closely followed by loss of cellular ATP, signaling the loss cell viability (Supplementary Fig. S2G).

AZD4573 induces rapid cell death across a diverse panel of hematologic cancer cell lines

With MCL-1 reportedly essential for the survival of various tumor types, particularly leukemias and lymphomas (30, 31), AZD4573 was screened in a diverse panel of cancer cell lines to assess the breadth of anticancer activity. Caspase activation and loss of viability were measured following 6 and 24 hours of treatment with AZD4573, respectively. Greater activity was observed with AZD4573 across hematologic cancer cell lines (geometric mean EC50 = 0.166 μmol/L, GI50 = 0.023 μmol/L) compared with solid tumor cell lines (geometric mean EC50 = 6.871 μmol/L and GI50 = 0.774 μmol/L), although a subset of solid tumor cell lines was sensitive (Fig. 3A and B, Supplementary Table S7). Importantly, there was good concordance between caspase and viability data. Nonselective CDK9 inhibitors also exhibited robust activity across both hematologic and solid tumor lineages, although longer-term readouts were employed (32). Likewise, when viability was assessed after 72-hour continuous AZD4573 exposure, equipotency was observed across all cell lines suggesting that prolonged transcriptional repression results in general cytotoxicity or cytostasis (Supplementary Fig. S3A), consistent with data from the time-course assays (Fig. 1E, Supplementary Fig. S1B–S1D).

The same panel of cancer cell lines was also assessed for 6-hour caspase activation when treated with the selective MCL-1 inhibitor, AZD5991, one of multiple MCL-1 inhibitors in clinical development (19, 33). There was a strong correlation between the activity observed with AZD4573 and AZD5991 (r² = 0.76; Fig. 3C), providing additional evidence that depletion of MCL-1 is the primary mechanism driving AZD4573-mediated cell killing. Therefore, when MV-4-11 cells were treated with a combination of AZD4573 and AZD5991 in vitro and assayed for caspase activation, no combination benefit was observed across the dose matrix (Supplementary Fig. S3B). Furthermore, measurement of MCL-1 and BCL-XL protein levels for a subset of AZD4573-sensitive and -insensitive cell lines revealed that higher levels of BCL-XL corresponded to decreased sensitivity to AZD4573 (Supplementary Fig. S3C). This is consistent with a lack of BCL-XL depletion upon acute AZD4573 treatment (Fig. 2D) and previous reports of the MCL-1:BCL-XL expression ratio predicting response to CDK9 inhibitors and BH3 mimetics (13, 34).

Intermittent dosing of AZD4573 drives regression of MV-4-11 tumor xenografts

To determine if observed in vitro activity translated to in vivo efficacy, AZD4573 was evaluated in a hematologic subcutaneous xenograft model using an intermittent dosing schedule that was selected to provide the requisite acute target inhibition identified in vitro studies (~6 hours). Mice bearing MV-4-11 tumors were treated weekly with 5 or 15 mg/kg AZD4573, dosed IP BID q2h on consecutive days (5/5 and 15/15 mg/kg), resulting in a tolerated dose-dependent response. The 15/15 mg/kg dose led to regressions for all mice that were sustained out to more than 125 days (Fig. 4A, Supplementary Fig. S4A). Notably, when this study was repeated to explore other biweekly schedules, similar robust and durable single-agent activity was observed (data not shown).

AZD4573 treatment was also compared head-to-head with AZD5991 in the sensitive MV-4-11 model and relatively insensitive OCI-AML3 model. Both the 15/15 mg/kg AZD4573 regimen and a single i.v. dose of 60 mg/kg AZD5991 resulted in regressions of MV-4-11 tumors and minimal tumor growth inhibition (TGI) of OCI-AML3 tumors (Supplementary Fig. S4B and S4C). These strikingly similar effects in vivo for the two inhibitors provide further evidence of the primarily MCL-1–dependent mechanism of action for acute AZD4573 treatment.

A mechanistic PK/PD/efficacy model relates CDK9 inhibition, MCL-1 depletion, and induction of apoptosis in response to AZD4573 treatment

Using a series of studies with MV-4-11 tumor xenografts, a PK/PD/ efficacy model was developed to quantify the dynamic relationship between AZD4573 PKs and tumor pSer2-RNAP2 and MCL-1 PDs (Fig. 4B). Model parameter values were estimated by fitting the model to compiled in vivo PK/PD study individual animal data (Supplementary Table S2), and an example model fit is shown in Fig. 4B. The free AZD4573 concentration that results in half-maximal inhibition of pSer2-RNAP2 production rate was estimated to be 0.011 to 0.021 μmol/L, which was consistent with the IC50 derived from in vitro studies (Fig. 1D). The kinetics of MCL-1 mRNA and protein modulation were also consistent with in vitro data, demonstrating an estimated mRNA and protein half-life of 5 and 17 minutes, respectively.
The model was extended to quantify the relationship between extent of MCL-1 protein suppression and rate of cell death induction in vivo as measured by reduction in MV-4-11 tumor xenograft volume or increase in cleaved caspase-3 in PK/PD study tumor samples. The parameter values describing this relationship were estimated by fitting the model to sets of mean tumor volumes from multidose level efficacy studies (Supplementary Table S2). The rate of tumor cell death induction was modeled as a saturable first-order process that exhibited a steep apoptotic response to the suppression of tumor MCL-1. The estimated tumor MCL-1 protein level associated with half-maximal rate of cell death induction (Mcl50) was consistent across studies and estimated to be approximately 25% of baseline. The estimated maximum first-order rate constant for cell death induction (Kmax) was also consistent across studies and estimated to be 0.16 to 0.19 hr⁻¹.

Satisfactory model fits to observed tumor cleaved caspase-3 kinetic data were also obtained using the same Mcl50 and Kmax values to describe the relationship between MCL-1 protein suppression and the rate of caspase activation from the pool of tumor proCASPASE-3. Modeling suggested that each daily split dose results in the death of a consistent fraction of xenograft tumor cells, underscored by the similar overall efficacy for 2-day on/5-day off and biweekly dosing schedules (Fig. 4B). Each 15 mg/kg split dose was modeled to reduce pSer2-RNAP2 below 20% of baseline levels for approximately 4 hours. This extent and duration of pSer2-RNAP2 reduction caused suppression of MCL-1 below the determined Mcl50 for approximately 4 hours, which subsequently resulted in approximately 55% MV-4-11 tumor volume reduction that was sufficient to sustain progressive reduction in tumor volume on a biweekly dosing schedule (Fig. 4B). Application of this derived exposure–effect relationship to other preclinical models, including OCI-LY10, estimated equivalent parameters and predicted the in vivo response (Supplementary Fig. S4D; Supplementary Table S2).

In vitro sensitivity to AZD4573 accurately predicts in vivo efficacy in hematologic tumor xenograft models

Having established a detailed understanding of the relationship between AZD4573 exposure, MCL-1 suppression, and apoptosis induction in the MV-4–11 xenograft model, in vivo efficacy analysis was expanded to include ten more hematologic models. Based on in vitro 6-hour caspase activity parameters used to define sensitivity (EC50 ≤ 45 nmol/L based on modeled MV-4–11 EC99 maximum caspase activation ≥ 60%), certain models were predicted to be
Figure 4.
A mechanistic PK/PD/efficacy model relates CDK9 inhibition, MCL-1 depletion, and caspase activation to in vivo response with AZD4573 treatment. A. An MV-4-11 subcutaneous xenograft model was treated with three cycles of vehicle or AZD4573 (5 mg/kg or 15 mg/kg, BID q2h, 2 days on/5 days off). Tumor volumes are presented as geometric mean ± SEM (n = 5). Shaded area indicates treatment period. B. The top portion depicts a schematic of the established PK/PD/efficacy model. A three-compartment PK model was used to describe the observed plasma and tumor AZD4573 concentration data. Tumor pSer2-RNAP2 and MCL-1 PD was modeled using a series of linked indirect response models. Unperturbed tumor growth was modeled as a first-order process, and MCL-1 was modeled as inhibiting the induction of apoptosis in the MV-4-11 cells. A series of transit compartments were used to bridge the time delay between cell death (hours) and observed tumor shrinkage (days). On the bottom, graphs show the model fit (lines) to observed PK and PD data (individual data points) where darker lines indicate population mean with lighter lines indicating 10% to 90% percentiles expected based on estimated interindividual variability. Efficacy graphs show the model fit to observed tumor volume data. All modeling data are based upon MV-4-11 subcutaneous tumor xenograft studies.
AZD4573 demonstrates antitumor activity in disseminated leukemia and lymphoma patient-derived xenograft models

In addition to subcutaneous cell line xenograft models, in vivo studies were expanded to include i.v. disseminated patient-derived xenograft (PDX) models of AML and T-cell lymphoma. Nine AML PDX models were evaluated for the ability of AZD4573 to decrease tumor burden in the bone marrow. By the end of study, five of the nine models exhibited greater than 50% reduction of leukemic blasts in the bone marrow (Fig. 5B). The angioimmunoblastic T-cell lymphoma PDX model, DFTL-78024, which exhibited strong MCL-1 dependence based upon BH3 profiling (Supplementary Fig. S5J; ref. 35), was also evaluated. Treatment with AZD4573 led to significantly more CD45+ tumor cells undergoing apoptosis, as measured by increases in Annexin V staining following the second dose on day 2, which ultimately led to a significant survival benefit compared with vehicle (P value = 0.0018; Fig. 5C). Together, these data establish that AZD4573 is efficacious in models of disseminated disease.

AZD4573 combinations with venetoclax are tolerated and efficacious

Despite broad monotherapy activity for AZD4573 across hematologic tumor models, several models exhibited little to no response (Supplementary Fig. S5A–S5C). Furthermore, in some sensitive models, AZD4573 resulted in extensive TGI, yet tumors regrew immediately following cessation of dosing, suggesting either the presence of an underlying refractory cell population or insufficient tumor cell death during the dosing period. To produce more durable responses, combinations with the selective BCL-2 inhibitor, venetoclax, were explored given the observed benefit for AZD5991 combinations with venetoclax (19).

AZD4573 monotherapy treatment in the SU-DHL-4 (GCB-DLBCL) tumor xenograft model exhibited robust antitumor activity, although overall regressions were not achieved (Supplementary Fig. SSF). In vitro, SU-DHL-4 cells were sensitive to single-agent AZD4573 (EC50 = 16 nmol/L; max caspase activation = 79%) and relatively insensitive to venetoclax (EC50 = 94 nmol/L; max caspase activation = 24%). Combination treatment, however, increased the extent of caspase activation (EC50 = 10 nmol/L; max caspase activation = 118%), which was also observed in vivo (Fig. 6A and B, left). Monotherapy treatment with venetoclax was minimally efficacious, consistent with the in vitro response, and single-agent AZD4573 again exhibited extensive TGI but not regressions. The combination of AZD4573 with venetoclax, however, produced highly durable regressions in 100% of treated animals, with all 8 mice remaining tumor-free out to day 63. Similar effects were observed in the OCI-AML3 model, which was intrinsically resistant to both monotherapies (Fig. 6A and B, right). Notably, minimal body weight loss was observed, highlighting that this could be a tolerated combination regimen (Supplementary Fig. SSK and SSL).

The combination benefit in cancer cell line models insensitive to inhibition of BCL-2 or both BCL-2 and MCL-1 was striking. Previous studies have shown that venetoclax displaces proapoptotic molecules like BIM from BCL-2, which can then become sequestered by MCL-1 (36). Similar BCL-2 family protein dynamics were observed here. In vitro treatment with venetoclax revealed a rapid (~3 hours) increase in MCL-1 for both models (Fig. 6C). Similarly, SU-DHL-4 tumor xenograft PD samples treated with a single dose of venetoclax exhibited an increase in MCL-1 protein by 6 hours after dose with maximal levels achieved by 24 hours (Fig. 6D). In OCI-AML3 cells treated with venetoclax and coimmunoprecipitated with either anti-MCL-1 or anti-BIM antibodies, Bim was displaced from BCL-2 and then sequestered by MCL-1 (Fig. 6E), likely stabilizing the protein and creating an MCL-1 dependency, lending further credence to the reported combination mechanism.

Discussion

Seminal work with nonselective CDK inhibitors showed that induction of tumor cell death required activity against CDK9 (37), which resulted in the rapid depletion of prosurvival factors, like MCL-1 (7, 38). Despite signs of clinical activity, development of these molecules as monotherapies was suspended due to narrow therapeutic windows (2). However, trials investigating their combination potential are currently ongoing. Recently, more selective CDK9 inhibitors have been developed to overcome the noted limitations of nonselective CDK inhibitors (15, 39, 40). This work describes the novel CDK9 inhibitor, AZD4573, that was optimized for high selectivity and potency against CDK9 as well as physicochemical properties enabling intravenous infusion and a short PK half-life. These attributes allow for intermittent dosing of AZD4573, resulting in periods of acute yet sufficient CDK9 inhibition that drives rapid tumor cell apoptosis without causing prolonged transcription inhibition that could erode the therapeutic index. AZD4573 is currently in phase I clinical trials for evaluation in patients with hematologic malignancies (NCT03263637).

There are various studies that have successfully associated CDK9 inhibition with MCL-1 depletion and subsequent induction of tumor cell death (41). However, many come with study design caveats such as prolonged CDK9 inhibition (15, 42), a narrow focus on one indication and/or a limited number of preclinical models (8, 43), and use of nonselective compounds (14, 44) that make it difficult to draw firm conclusions. This study overcame those limitations by utilizing the selective CDK9 inhibitor, AZD4573, and applying multiple approaches to interrogate the mechanism of action of CDK9 inhibition. First, with the agnostic and integrated transcriptomic and proteomic analysis, MCL1 was identified as the most robustly and significantly modulated cancer-associated gene in cells treated acutely with AZD4573. Interestingly, several transcripts and proteins were upregulated after transient AZD4573 exposure, which could result from CDK9 inhibition driving stress responses or repressing another repressor (45). Although inclusion of more than one cell line in these multivariate experiments would have been preferred for hit validation, focused evaluation of MCL-1 depletion in multiple other models revealed a high degree of consistency across tumor types and ranges of AZD4573 sensitivity. This work also highlighted the requirement of an intact intrinsic apoptotic pathway to achieve AZD4573-induced cell death, a high concordance between the activity observed in vitro and in vivo for AZD4573 and the novel MCL-1 inhibitor AZD5991 (19), and a lack of benefit for the combination of AZD4573 and AZD5991. Collectively, these data support MCL-1 depletion as the primary mechanism driving tumor cell death upon acute CDK9 inhibition.
Figure 5.
AZD4573 demonstrates antitumor activity in disseminated leukemia and lymphoma PDX models. A, Other subcutaneous tumor xenograft models from AML, multiple myeloma (MM), and DLBCL cancer cell lines were treated with AZD4573 at 15 mg/kg, BID q2h, 2 days on/5 days off for three cycles (* represents models receiving only two cycles). The percent change from tumor volume at time of first dose is depicted. Red bars denote models predicted from in vitro caspase sensitivity parameters to show signs of efficacy in vivo. B, Human disseminated AML PDX models grown in NOG-EXL mice were treated with four cycles of vehicle or AZD4573 (15 mg/kg, BID q2h, 2-day on/5-day off; n = 3). FACS analysis was used to detect leukemic blasts in the bone marrow (human CD3-/CD33+/CD45+) and presented as mean percent change ± SEM from pretreatment counts. C, Kaplan–Meier survival curves for the DFTL-78024 model treated with four cycles of AZD4573 (n = 5; left). PD response to vehicle or AZD4573 treatment assessed 6 hours after a single 15 mg/kg dose. Tumors from 3 mice per group were assessed for Annexin V staining in human CD45+ tumor cells via flow cytometry (right).
AZD4573 combinations with venetoclax are tolerated and efficacious. A, SU-DHL-4 (left) and OCI-AML3 (right) cells treated in vitro with a dose-response of AZD4573, venetoclax, or the combination were assessed for caspase activation. B, SU-DHL-4 (left) and OCI-AML3 (right) subcutaneous xenograft models were treated with three cycles of vehicle, venetoclax (100 mg/kg QD), AZD4573 (15 mg/kg, BID q2h, 2-day on/5-day off), or the combination. Shaded area indicates treatment period. Tumor volumes are presented as geometric mean ± SEM (n = 5). C, Lysates from SU-DHL-4 (left) and OCI-AML3 (right) cells treated with 0.1 μmol/L venetoclax, 0.1 μmol/L AZD4573, or the combination for various times were immunoblotted for the depicted proteins. D, SU-DHL-4 tumor lysates from mice treated with a single dose of vehicle or venetoclax (100 mg/kg) for the indicated times (n = 3) were immunoblotted for the indicated proteins. E, OCI-AML3 cells were treated with 0.1 μmol/L venetoclax in vitro for the indicated times. MCL-1 and Bim were immunoprecipitated from whole-cell lysates (input) and immunoblotted for the indicated protein.

Despite the strong correlation between AZD4573 and AZD5991 activity in vitro, there were some hematologic cancer cell lines sensitive to AZD4573 but not AZD5991. Although prolonged AZD4573 exposure would expectedly cause turnover of numerous proteins that could cause general cytotoxicity, these were short, 6-hour assays. Therefore, AZD4573 is likely rapidly depleting another factor necessary for cancer cell survival where MCL-1 inhibition alone is insufficient to induce apoptosis.

A quantitative AZD4573 exposure/effect relationship was also established that further substantiates MCL-1 as the downstream target...
mediating the induction of apoptosis. Reduction in pSer2-RNAP2 occurs in a concentration- and time-dependent manner that precedes a proportionate but delayed depletion of MCL-1 protein, and caspase activation occurs once MCL-1 levels reach a critical value in AZD4573-sensitive cancer cells. The relationship between PD response and rate of cell death induction, as assessed by reduction in tumor volume, in vivo was similar to that observed in in vitro studies. At tolerated AZD4573 doses in mice, the extent and duration of MCL-1 suppression was sufficient to drive MV-4-11 subcutaneous tumor xenografts into complete regression when using twice-weekly dose schedules. A mechanistic PK/PD/efficacy model was developed that relates AZD4573 exposure to the extent and duration of pSer2-RNAP2 and MCL-1 suppression necessary to induce caspase activation and reduce tumor size. The derived parameter values for the in vivo model were consistent with the observed in vitro target engagement requirement (~6 hours) in MCL-1–dependent cell lines. Given the applicability in multiple tumor models, this PK/PD/efficacy model was used to inform design of the current phase I trial. With this greater mechanistic understanding and quantitative modeling of AZD4573, the hope is to optimize the dose and schedule to maximize the therapeutic window.

Venetoclax is now approved for treatment of adult patients with chronic lymphocytic leukemia based upon high response rates with monotherapy. In other hematologic cancers, the response rates were much lower, but combinations with standards of care yielded better results and led to the approval of venetoclax in combination with hypomethylating agents or low-dose cytarabine for the treatment of select patients with AML who cannot receive intensive induction chemotherapy (46, 47). Despite these promising responses and exciting approvals, acquired resistance to venetoclax is beginning to emerge, and one mechanism identified predilectionally is compensation by increased levels of MCL-1 (48). Although this could result from selection of subclones with preexisting MCL-1 overexpression, our data suggest that venetoclax can directly induce stabilization of MCL-1 by favoring its binding of BIM. Using models intrinsically resistant to venetoclax, this study showed that adding AZD4573 to the venetoclax regimen resulted in significant and tolerated combination benefit, which is in line with venetoclax combination studies using other CDK9 and MCL-1 inhibitors (14, 49, 50). This suggests that the combination could be used to increase depth and duration of response as well as prevent or overcome the emergence of resistance.

In conclusion, this study has identified AZD4573, a potent and selective CDK9 inhibitor, that induces cancer cell death across preclinical hematologic cancer models at tolerated, intermittent doses primarily through transient depletion of MCL-1. This mechanistic understanding enabled the development of a quantitative PK/PD/efficacy model that can aid in the selection of an optimized clinical dose and schedule to maximize the therapeutic index. These findings collectively supported progression of AZD4573 as a clinical candidate for the treatment of patients with hematologic malignancies.

Disclosure of Potential Conflicts of Interest

J. Cidado, S. Boiko, T. Proia, D. Ferguson, S.W. Criscione, P. Pop-Damklov, W. Shao, J.C. Saeh, S.E. Fawell, and L. Drew are employees/paid consultants for AstraZeneca. M.M. Weinstock is an employee/paid consultant for Travera, Axon, and Bantam Pharmaceuticals, reports receiving commercial research grants from AstraZeneca, Abbvie, Aileron, Daiichi Sankyo, and Verastem, holds ownership interest (including patents) in Ajax and Travera, and reports receiving other remuneration from Genentech/Roche. M. Zinda is an employee/paid consultant for, reports receiving commercial research grants from, and holds ownership interest (including patents) in AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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