Phase I Study of Single-Agent AZD1775 (MK-1775), a Wee1 Kinase Inhibitor, in Patients With Refractory Solid Tumors

Khanh Do, Deborah Wilsker, Jiuping Ji, Jennifer Zlott, Tomoko Freshwater, Robert J. Kinders, Jerry Collins, Alice P. Chen, James H. Doroshow, and Shivaani Kummar

See accompanying article on page 3485

Khanh Do, Jennifer Zlott, Jerry Collins, Alice P. Chen, James H. Doroshow, and Shivaani Kummar, National Cancer Institute, Bethesda, MD; Deborah Wilsker, Jiuping Ji, and Robert J. Kinders, Leidos Biomedical Research, Frederick National Laboratory for Cancer Research, Frederick, MD; and Tomoko Freshwater, Merck Research Laboratories-Oncology, Boston, MA.

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Corresponding author: Shivaani Kummar, MD, Division of Cancer Treatment and Diagnosis, National Cancer Institute, 31 Center Dr, Bldg 31, Rm 3A44, Bethesda, MD 20892; e-mail: kummar@mail.nih.gov.

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ABSTRACT

Purpose

Wee1 tyrosine kinase phosphorylates and inactivates cyclin-dependent kinase (Cdk) 1/2 in response to DNA damage. AZD1775 is a first-in-class inhibitor of Wee1 kinase with single-agent antitumor activity in preclinical models. We conducted a phase I study of single-agent AZD1775 in adult patients with refractory solid tumors to determine its maximum-tolerated dose (MTD), pharmacokinetics, and modulation of phosphorylated Tyr15-Cdk (pY15-Cdk) and phosphorylated histone H2AX (γ H2AX) levels in paired tumor biopsies.

Patients and Methods

AZD1775 was administered orally twice per day over 2.5 days per week for up to 2 weeks per 21-day cycle (3 + 3 design). At the MTD, paired tumor biopsies were obtained at baseline and after the fifth dose to determine pY15-Cdk and γ H2AX levels. Six patients with *BRCA*-mutant solid tumors were also enrolled at the MTD.

Results

Twenty-five patients were enrolled. The MTD was established as 225 mg twice per day orally over 2.5 days per week for 2 weeks per 21-day cycle. Confirmed partial responses were observed in two patients carrying *BRCA* mutations: one with head and neck cancer and one with ovarian cancer. Common toxicities were myelosuppression and diarrhea. Dose-limiting toxicities were supraventricular tachyarrhythmia and myelosuppression. Accumulation of drug ($t_{1/2}$ approximately 11 hours) was observed. Reduction in pY15-Cdk levels (two of five paired biopsies) and increases in γ H2AX levels (three of five paired biopsies) were demonstrated.

Conclusion

This is the first report of AZD1775 single-agent activity in patients carrying *BRCA* mutations. Proof-of-mechanism was demonstrated by target modulation and DNA damage response in paired tumor biopsies.

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INTRODUCTION

Cells depend on cell cycle checkpoints to allow time for DNA repair and maintenance of genomic integrity before they begin cell division. DNA damage response signaling cascades culminate in the phosphorylation of Wee1 tyrosine kinase, which is involved in the inhibitory phosphorylation of cyclin-dependent kinase 1 and 2 (Cdk1 and Cdk2).^{1,2} Wee1-dependent phosphorylation of Cdk1 results in activation of the G₂ checkpoint and cell cycle arrest, whereas phosphorylation of Cdk2 allows for regulation of DNA replication and maintenance of stalled replication forks during S phase. Wee1 inhibition has been shown to result in the dysregulation of Cdk1 and Cdk2 activity, unfettered

activation of DNA origins of replication, stalling of replication forks, and double-stranded DNA breaks in preclinical models.³

AZD1775 (previously MK-1775) is a first-in-class, pyrazolo-pyrimidine derivative and potent small-molecule inhibitor of Wee1 kinase (half-maximal inhibitory concentration, 5.18 nmol/L). Early preclinical studies focused on chemopotentiality, based on the rationale that abrogation of cell cycle checkpoints with AZD1775 would allow for premature entry into cell division with unrepaired DNA lesions resulting in cell death.⁴⁻⁶ Increased cytotoxicity was observed in p53-deleted compared with p53 wild-type cell lines, which may be the result of inhibition of the G₂ checkpoint in the setting of cells with a defective G₁ checkpoint, which would favor

mitotic lethality.⁵ More recent observations suggest that the mechanism of AZD1775 cytotoxicity is based on defective DNA damage response rather than premature entry into mitosis and is independent of p53 status.^{7,8} In vitro studies of AZD1775 in various cell lines, including LoVo (*TP53* wild-type colorectal adenocarcinoma), showed strong induction of pS345-Chk1 and the DNA damage response. An increase in phosphorylated histone H2AX (γ H2AX) –positive cells was observed, with no increase in phosphorylated histone H3 (pHH3), a marker of mitosis, suggesting that the mechanism of cytotoxicity was primarily DNA damage rather than premature mitosis.⁷ Single-agent preclinical activity was observed, independent of p53 status, suggesting that other mechanisms may play a role in determining sensitivity to Wee1 inhibition.^{7,8} On the basis of these promising preclinical data, we conducted a phase I study of single-agent AZD1775 in adult patients with refractory solid tumors to determine safety, maximum-tolerated dose (MTD), pharmacokinetics (PK), and antitumor activity. Levels of phosphorylated Tyr15-Cdk (pY15-Cdk) and γ H2AX were evaluated in paired tumor biopsies obtained at the MTD to check for evidence of target modulation and DNA damage response, respectively.

PATIENTS AND METHODS

Patient Population

Patients age 18 years or older with histologically confirmed solid tumors that had progressed through at least one line of standard therapy for metastatic disease were eligible. Patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 2, be able to swallow whole capsules, and have adequate organ function, defined as absolute neutrophil count $\geq 1,500/\mu\text{L}$, platelet count $\geq 100,000/\mu\text{L}$, total bilirubin $\leq 1.5 \times$ institutional upper limit of normal (ULN), AST and ALT $\leq 3 \times$ ULN, and creatinine $\leq 1.5 \times$ ULN. Patients enrolled in the expansion cohort at the MTD were required to have disease amenable to biopsy and be willing to undergo paired tumor biopsies. Previous treatment must have been completed at least 4 weeks before enrollment. Exclusion criteria included active brain metastases or GI processes that could interfere with adequate absorption of study agents. Concurrent agents known to be strong inhibitors or inducers of CYP3A4 were not permitted because of the potential for PK interactions. This trial was conducted under a National Cancer Institute–sponsored investigational new drug application with institutional review board approval. Protocol design and conduct followed all applicable regulations, guidance, and local policies.

Study Design

This was an open-label, phase I trial of single-agent AZD1775 in patients with refractory solid tumors. AZD1775 was supplied by the National Cancer Institute's Division of Cancer Treatment and Diagnosis under a collaborative research and development agreement with Merck and AstraZeneca. The starting dose (dose level 1 [DL1]) of AZD1775 was 225 mg administered orally twice per day over 2.5 days for 1 week of a 21-day cycle. With subsequent dose escalations, AZD1775 was given orally at 225 mg (DL2) or 300 mg (DL3) twice per day for 2.5 days per week for 2 consecutive weeks of a 21-day cycle. Dose escalation and schedule were chosen on the basis of data from initial phase I studies of AZD1775 in combination with chemotherapeutic agents in which plasma exposure after the fifth dose was above the threshold level established in preclinical studies for antitumor activity. Hence, the study was designed to evaluate the tolerability of an increased number of days of exposure above the threshold required to optimize antitumor activity. This trial followed a standard 3 + 3 design.

Toxicities were graded according to Common Terminology Criteria for Adverse Events version 4.0. Dose-limiting toxicity (DLT) was defined as neutropenia (neutrophil count $< 0.5 \times 10^9/\text{L}$) for more than 5 days; neutropenia

(neutrophil counts $< 1 \times 10^9/\text{L}$) with fever; thrombocytopenia (platelet count $< 25 \times 10^9/\text{L}$) or thrombocytopenia (platelet count $< 50 \times 10^9/\text{L}$) accompanied by bleeding; any grade 3 or 4 nonhematologic toxicity not a consequence of tumor progression; and grade 3 nausea, vomiting, or diarrhea refractory to appropriate supportive care. Treatment was held for grade 3 hematologic toxicities and restarted at the same dose with improvement to grade 2. Patients were dose reduced for grade 3 or higher nonhematologic toxicities if toxicities could not be corrected to grade 1 or better within 24 hours with supportive care (or grade 2 or better for diarrhea). At the MTD, six additional patients were enrolled in the expansion cohort with mandatory tumor biopsies before drug administration and again 2 to 5 hours after the fifth dose of drug during the first week of treatment. Radiologic response assessments by computed tomography scans were performed at baseline and every two cycles. Tumor response was evaluated according to RECIST version 1.1.⁹

After a partial response (PR) was confirmed in a patient with *BRCA*-mutant head and neck cancer, we amended the study to enroll an additional six patients at the MTD with advanced solid tumors and documented *BRCA1/2* mutations to evaluate the antitumor activity of AZD1775 in this patient population.

Safety Assessments

History and physical examination and laboratory evaluations (CBC and serum chemistries) were performed before each treatment week for the first cycle and at the beginning of each subsequent 21-day cycle. An electrocardiogram was obtained at baseline, after the fifth dose during the first week of treatment, and as clinically indicated thereafter.

PK Analyses

Plasma levels of AZD1775 were determined by using a validated liquid chromatography–tandem mass spectrometry assay with a lower limit of quantitation of 2 ng/mL. Blood samples were collected predose and at multiple time points during cycle 1. Standard PK parameters were calculated by using a noncompartmental method (Phoenix WinNonlin 6.3; Certera, Princeton, NJ).

Fig 1. Best response in patients with *BRCA1/2* mutations. Two patients had partial responses.

Pharmacodynamic End Point Analyses

Paired tumor biopsies (18-gauge core needle biopsies, two cores) were obtained and analyzed for the levels of pY15-Cdk, γ H2AX, and pHH3. pY15-Cdk levels were measured by using a quantitative immunofluorescence assay validated for use on formalin-fixed paraffin-embedded human tissue. The assay uses purified monoclonal antibody pY15-Cdk2 (clone EPR2233Y; Abcam, Cambridge, MA), which recognizes both pY15-Cdk1 and pY15-Cdk2, and pHH3-biotinylated rabbit monoclonal antibody (clone D2C8; Cell Signaling Technology, Danvers, MA) for the identification of mitotic activity in cells. Goat-anti-rabbit Alexa Fluor 488 conjugate was used as the reporter (Invitrogen, Grand Island, NY). Assay validation criteria and results are included in the Appendix (online only). The γ H2AX levels were measured by using a validated immunofluorescence assay as described previously.¹⁰

RESULTS

Patient Population and Disposition

A total of 25 patients were enrolled onto this study and treated between December 2012 and September 2014, including a six-patient expansion cohort for evaluation of pharmacodynamic end points and a six-patient expansion cohort of patients with *BRCA1/2* mutations. Median age was 52 years (range, 22 to 78 years), male-to-female ratio was 8:17, and the mean number of prior treatments was five (range, one to nine). Twenty-one patients were evaluable for response; two patients withdrew after the first week of treatment, opting for surgery in one case and standard of care in the second case because of hardship from travel to the National Institutes of Health Clinical Center. Two patients experienced DLTs after the first week of drug treatment on DL3. Four patients had clinical progression before the planned restaging after two cycles, and seven patients were found to have progression

of their disease at the first restaging after two cycles. The mean time on study was three cycles (range, one to seven cycles).

Nine patients with *BRCA1/2* mutations were enrolled onto this study, two in the escalation phase and seven in the *BRCA* expansion cohort (Figure 1). The patient with *BRCA2*-mutant fallopian tube carcinoma on DL1 of the escalation phase had stable disease (by scans and tumor markers) at three cycles but opted to withdraw from the study to receive local radiation therapy for palliation of symptoms. In the escalation phase, one patient with *BRCA1*-mutant squamous cell carcinoma (SCC) of the base of the tongue had a confirmed PR (Figure 2) and remained on study for six cycles at DL2. On the basis of this response, the study was amended to enroll six additional patients with *BRCA1/2* mutations at the MTD to further evaluate response in this population. Of the seven patients enrolled in the expansion cohort, six were evaluable for response. One patient with pancreatic cancer was replaced because she withdrew after the first week of treatment, opting for standard of care with her local physician. A patient with *BRCA1*-mutant papillary serous ovarian cancer had a confirmed PR. Two patients with *BRCA2*-mutant papillary serous ovarian carcinoma had progressive disease (PD) after two cycles, and another with *BRCA1*-mutant papillary serous ovarian carcinoma and a *TP53* mutation had initial 14% decrease in index lesions with decrease in cancer antigen 125 after two cycles, but had PD after four cycles.

Adverse Events

As shown in Table 1, the most frequent adverse events were hematologic and GI. Toxicities were manageable at the MTD (DL2). Grade 4 myelosuppression occurred in two patients, one patient with

Fig 2. Computed tomography scan results of a patient with squamous cell carcinoma of the head and neck who had a *BRCA1* germline mutation. This patient had a confirmed partial response with more than 30% reduction in target lesions noted after two cycles, persisting through four cycles of treatment with AZD1775. (A) Baseline, (B) after two cycles, and (C) after four cycles.

Table 1. Summary of Adverse Events Attributed to Study Treatment for AZD1775 Dose Cohort

Adverse Event*	Grade												Total [%]
	Dose Level 1 (n = 3)				Dose Level 2 (n = 19)				Dose Level 3 (n = 3)				
	1	2	3	4	1	2	3	4	1	2	3	4	
Hematologic†													
Leucopenia	1 (1)	1 (1)	—	1	4 (5)‡	4 (3)	1 (1)	—	2 (2)	—	—	1 (1)	15 [60]
Lymphopenia	1 (1)	—	1 (1)	1	4 (2)	5 (3)	2 (1)	—	1 (1)	—	—	1 (1)	16 [64]
Anemia	1 (1)	—	2	—	4 (4)	2 (2)	1 (1)	—	1 (1)	1 (1)	1 (1)	—	13 [52]
Thrombocytopenia	0 (1)‡	—	1	—	4 (4)	4 (4)	1 (1)	—	1 (1)	—	—	1 (1)	12 [48]
Neutropenia	—	—	—	1	—	5 (4)	2 (2)	—	—	1 (1)	—	1 (1)	10 [40]
Febrile neutropenia	—	—	—	—	—	—	—	—	—	—	1 (1)	—	1 [4]
Gastrointestinal													
Nausea	1 (2)‡	1	—	—	10 (10)	4 (2)	1 (1)	—	1 (1)	—	—	—	18 [72]
Vomiting	1 (1)	1	—	—	10 (10)	5 (2)	—	—	1 (1)	—	—	—	18 [72]
Diarrhea	—	—	—	—	11 (12)‡	3 (1)	1 (1)	—	2 (2)	—	—	—	17 [68]
Abdominal pain	—	—	—	—	4 (2)	1 (1)	—	—	—	—	—	—	5 [20]
Bloating	1	—	—	—	1 (1)	2 (1)	—	—	—	—	—	—	4 [16]
Flatulence	—	—	—	—	3 (1)	—	—	—	—	—	—	—	3 [12]
Oral mucositis	—	—	—	—	1	—	—	—	1 (1)	—	—	—	2 [8]
Gastritis	—	—	—	—	—	1 (1)	—	—	—	—	—	—	1 [4]
Cardiac and vascular													
Hot flashes	2 (2)	—	—	—	2 (2)	—	—	—	—	—	—	—	4 [16]
Flushing	1 (1)	—	—	—	—	—	—	—	1 (1)	—	—	—	2 [8]
QTc prolongation	—	—	—	—	2 (2)	—	—	—	1 (1)	—	—	—	3 [12]
Supraventricular tachycardia	—	—	—	—	—	—	—	—	—	—	1 (1)	—	1 [4]
Sinus tachycardia	—	—	—	—	—	—	—	—	—	1 (1)	—	—	1 [4]
Palpitations	—	—	—	—	—	—	—	—	—	1 (1)	—	—	1 [4]
Metabolism and nutritional													
Anorexia	—	1	—	—	5 (2)	1	—	—	—	—	—	—	7 [28]
Hypophosphatemia	—	—	—	—	—	2 (1)	1	—	—	—	—	—	3 [12]
Investigations													
Increase in ALT	—	—	—	—	5 (3)	—	—	—	—	—	—	—	5 [20]
Increase in AST	—	—	—	—	3 (3)	—	—	—	1	—	—	—	4 [16]
Increase in alkaline phosphatase	—	—	—	—	4 (3)	—	—	—	—	—	—	—	4 [16]
Increase in GGT	—	—	—	—	1	1	—	—	—	—	—	—	2 [8]
Increase in bilirubin	—	—	—	—	1	1 (1)	—	—	—	—	—	—	2 [8]
Other													
Chills	—	—	—	—	2 (2)	—	—	—	—	—	—	—	2 [8]
Myalgia	—	—	—	—	3 (1)	1	—	—	—	—	—	—	4 [16]
Fatigue	—	—	—	—	1 (1)	1 (1)	—	—	1 (1)	—	—	—	3 [12]
Increase in creatinine	—	—	—	—	2 (2)	—	—	—	1 (1)	—	—	—	3 [12]
Dysgeusia	—	—	—	—	1 (1)	—	—	—	—	—	—	—	1 [4]
Weight loss	—	—	—	—	1	—	—	—	1 (1)	—	—	—	2 [8]
Dehydration	—	—	—	—	—	—	—	—	—	1 (1)	—	—	1 [4]
Skin pain	—	—	—	—	1 (1)	—	—	—	—	—	—	—	1 [4]

Abbreviation: GGT, gamma-glutamyltransferase.

*The total number of patients experiencing the drug-related adverse event during treatment on study (numbers in parentheses represent adverse events occurring during the first cycle of treatment only). In each case, the worst grade event for each patient is shown.

†One patient had grade 5 aspiration pneumonia, febrile neutropenia, and sepsis in the setting of grade 4 hematologic toxicities at dose level 3.

‡Adverse event grade worsened after the first cycle of treatment.

Ewing sarcoma enrolled in DL1 who was heavily pretreated with five prior systemic therapies including two immunotherapy-based treatments, and four courses of radiation to the spine, and a patient with SCC of the base of the tongue at DL3 who had a DLT. DLTs occurred in two patients during the first week of treatment at DL3, and these patients were taken off study. One patient developed a supraventricular tachyarrhythmia 3 days after the fifth dose of the first week of treatment. The patient developed hemodynamic compromise with demand ischemia but soon reverted to sinus rhythm with vagal maneuvers and did not warrant cardioversion. Subsequent cardiac eval-

uation detected no underlying cardiac disease and no permanent cardiac sequelae from study treatment. The patient with SCC of the base of the tongue developed grade 4 myelosuppression 5 days after the first week of treatment. His course was complicated by aspiration pneumonia resulting in death as a consequence of sepsis.

PK Analysis

Plasma-concentration profiles of the first 18 patients showed that for the 15 patients who received AZD1775 at the 225 mg twice per day dosing schedule for either 1 or 2 weeks (DL1 and DL2, respectively),

Fig 3. Mean plasma concentration curves for AZD1775 after multiple dosing. Blood samples were collected predose and during cycle 1 at 1, 2, 4, 6, and 8 hours after the first dose; before the third dose; and before and at 1, 2, 4, 6, and 8 hours after the fifth dose. Plasma concentrations increased after each dose to an average maximum serum concentration of 1,650 nmol/L after the fifth dose. Area under the serum concentration-time curve on day 3 was two- to three-fold higher than on day 1.

plasma concentrations for AZD1775 increased after each dose to an average maximum serum concentration of 1,650 nmol/L after the fifth dose (Figure 3). Total exposure (area under the serum concentration-time curve) on day 3 was two- to three-fold higher than on day 1. Average plasma exposure was higher for the three patients at 300 mg twice per day dosing (DL3), but there was overlap with results for 225 mg twice per day; too few patients were enrolled in DL3 to determine if exposure was proportional or more than proportional to the dose. The accumulation of drug over 2.5 days on the twice-per-day regimen was consistent with a half-life of approximately 11 hours and showed linear kinetics (Appendix Table A1, online only).

Pharmacodynamic Studies

Six patients were enrolled in the expansion cohort with paired tumor biopsies obtained at baseline and 2 to 5 hours after the fifth dose of the first week of administration of drug. Five of six paired tumor

biopsies showed adequate tumor content and were considered analyzable for pharmacodynamic end points. Dramatic reductions in pY15-Cdk levels (84% and 90% compared with baseline) were found in two of five paired tumor biopsies with three of the five patients also demonstrating concurrent evidence of DNA damage response based on increased levels of γ H2AX (Figure 4). We were unable to detect a decrease in pY15-Cdk for one patient specimen because of low expression of pY15-Cdk at baseline. One patient with *KRAS*-mutant nonsquamous non-small-cell lung cancer with *TP53* mutation on archival tissue had a concurrent increase in pHH3 level as well as γ H2AX level on post-treatment tumor biopsy (Figure 5).

To summarize, we report the results of this first-in-class single-agent study of the Wee1 kinase inhibitor AZD1775 in patients with refractory solid tumors. The MTD was established at 225 mg orally twice per day for 2.5 days each week for 2 consecutive weeks of a 21-day cycle, with manageable hematologic and GI toxicities. Pharmacokinetic profiling demonstrates a dose-dependent increase in exposure with a plasma half-life of approximately 11 hours. To the best of our knowledge, this is the first trial demonstrating single-agent activity of AZD1775 in patients with advanced solid tumors who have *BRCA* mutations. This is also the first clinical trial to demonstrate proof-of-mechanism for this novel inhibitor of the Wee1 kinase, with direct evidence of decrease in pY15-Cdk (target of Wee1 kinase) levels in paired tumor biopsies. In addition, we demonstrate single-agent activity with concurrent evidence of DNA damage response, as shown by an increase in γ H2AX levels in paired tumor biopsies, which supports the notion that inhibition of Wee1 gives rise to DNA damage. Interestingly, only one of five patient specimens demonstrated an increase in pHH3 post-treatment, suggesting that interference with the $G_2 \rightarrow M$ progression and mitotic catastrophe may not be the major downstream consequence of Wee1 inhibition.

Confirmed PRs were observed in two patients with germline *BRCA1* mutations, one with a deletion in exon 15 and another with a mutation in the coding region of exon 11 (C3508G). A deletion in exon 15 results in truncation of the C-terminal domain required for binding of phosphoproteins involved in DNA damage-induced checkpoint control of the $G_2 \rightarrow M$ transition.¹¹ The functional and clinical significance of the C3508G mutation in the coding region of

Fig 4. Quantitation of pY15-Cdk and γ H2AX signaling based on the percentage of the nuclear area positive for the marker. Three of five patients had a demonstrable decrease in pY15-Cdk levels post-treatment. All five patient biopsies had some increase in γ H2AX level post-treatment, with four patients having substantial increases. Postdose (*) or predose (†) biopsy levels less than the lower limit of quantitation did not allow quantitation of the percentage change in marker level after treatment.

Fig 5. Immunofluorescence assay for pY15-Cdk (phosphorylated Tyr15-Cdk), γ H2AX (phosphorylated histone H2AX), and pHH3 (phosphorylated histone H3) in paired tumor biopsies from a patient with nonsquamous non-small-cell lung carcinoma. Post-treatment biopsies were obtained 2 to 5 hours after the fifth dose on day 3 of cycle 1. Hematoxylin and eosin staining demonstrates adequate tumor content. A decrease in pY15-Cdk signal in the post-treatment biopsy is consistent with the downstream effect of Wee1 inhibition. Increases in γ H2AX and pHH3 levels were observed in the post-treatment biopsy. (A) Hematoxylin and eosin, (B) pY15-Cdk, (C) γ H2AX, and (D) pHH3.

exon 11 has not previously been identified and is not known to result in a deleterious mutation; however, the binding region for DNA repair proteins, in particular the retinoblastoma protein, c-Myc, Rad50, and Rad51, is known to be localized to this area.¹² BRCA1-dependent activation of Chk1 is based on complex formation with claspin and ATR, followed by ATR-dependent phosphorylation of Chk1.¹³ Activated Chk1 in turn activates homologous recombination repair through modulation of the BRCA2-Rad51 interaction as well as activation of cell cycle checkpoints through phosphorylation and activation of Wee1 kinase. A point mutation in the region of exon 11 could conceivably affect efficient binding to DNA repair proteins. When coupled with inhibition of Wee1, aberrant activation of Cdk2, and uncoordinated firing of DNA origins of replication, cells may be unable to compensate for the resultant DNA damage.

Development of agents targeting Wee1 has focused on p53-deficient tumors, based on the rationale that cells defective in the G₁ checkpoint as a result of p53 loss-of-function are heavily dependent on the G₂ checkpoint for survival.^{5,14,15} In this study, archival tissue from five patients confirmed *TP53* mutations, although no responses were observed in these patients. Two of the patients with *TP53* mutations had concurrent *BRCA1/2* mutations, leading us to expect to see responses; however, one patient had PD after two cycles, and the other patient had an initial 14% decrease in index lesions and decrease in cancer antigen 125 after two cycles but had PD after four cycles. One patient with *KRAS*-mutant nonsquamous non-small-cell lung cancer and a *TP53* mutation documented from molecular profiling of archival tissue had concurrent pHH3 and γ H2AX level increases in the

post-treatment tumor biopsy compared with baseline levels. It is interesting to note that this particular patient's tumor specimens invoked both mechanisms of response to Wee1 inhibition, which may be attributed to Wee1 inhibition in the context of *KRAS* mutation because Ras has previously been implicated in ATR activation, sustained Cdk2 activation, and mitogenic signaling.¹⁶ The results of this study underscore the role that complementary repair pathways may play in the response to DNA damage and show that the effect of Wee1 inhibition is not limited to *TP53*-mutant cancers but is heavily dependent on a variety of contextually specific cellular mechanisms, presenting opportunities for development of novel combinations with this agent.

Recent efforts have focused on dual inhibition of components of the ATR-Chk1-Wee1 axis based on the hypothesis that simultaneous inhibition of Chk1 and Wee1 would allow for a more complete disruption of cell cycle checkpoint control, resulting in cytotoxic synergism.^{17,18} In addition, recent in vitro studies evaluating the combination of Wee1 inhibition and mTOR inhibition in *RAS*-driven malignancies show enhanced antitumor activity with the combination compared with either agent alone.¹⁹ Cdk1 is known to play a role in phosphorylation of various mTORC1 signaling factors during mitosis.²⁰ The development of AZD1775 in combination with an mTOR inhibitor in *RAS*-driven malignancies presents a promising approach when targeted therapies remain elusive in this population of patients.

Finally, the preliminary responses seen in two patients with *BRCA* mutations in this study present an intriguing opportunity for further investigation of this agent in patients harboring defects in

homologous recombination repair. Recent evidence indicates that poly (ADP-ribose) polymerase (PARP) binding to Chk1 at stalled replication forks is required for S-phase checkpoint activation, independent of its binding to claspin and BRCA1.²¹ This observation and the clinical activity observed in this study in patients with *BRCA* mutations forms the basis for future combination studies of AZD1775 with PARP inhibitors in patients carrying defects in the DNA repair pathways.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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AUTHOR CONTRIBUTIONS

Conception and design: Khanh Do, Robert J. Kinders, Jerry Collins, Alice P. Chen, James H. Doroshow, Shivaani Kummar

Financial support: James H. Doroshow

Administrative support: James H. Doroshow

Provision of study materials or patients: James H. Doroshow

Collection and assembly of data: Khanh Do, Deborah Wilsker, Jiuping Ji, Jennifer Zlott, Tomoko Freshwater, Robert J. Kinders, Jerry Collins, Shivaani Kummar

Data analysis and interpretation: Khanh Do, Deborah Wilsker, Jiuping Ji, Jennifer Zlott, Tomoko Freshwater, Robert J. Kinders, Jerry Collins, Alice P. Chen, James H. Doroshow, Shivaani Kummar

Manuscript writing: All authors

Final approval of manuscript: All authors

GLOSSARY TERMS

BRCA1: a tumor suppressor gene known to play a role in repairing DNA breaks. Mutations in this gene are associated with increased risks of developing breast or ovarian cancer.

BRCA2: a tumor suppressor gene whose protein product is involved in repairing chromosomal damage. Although structurally different from *BRCA1*, *BRCA2* has cellular functions similar to *BRCA1*. *BRCA2* binds to RAD51 to fix DNA breaks caused by irradiation and other environmental agents. Also known as the breast cancer 2 early onset gene.

cell cycle: an ordered progression of events, from a resting state to DNA synthesis to cell division. The major phases of the cell cycle are G₀/G₁ (GAP 1), S (synthetic) phase, G₂ (GAP 2), and M (mitosis).

p53: a transcriptional activator of genes with a p53-binding site and an inhibitor of genes lacking a p53 binding site. Expression of high levels of wild-type p53 is associated with cell cycle arrest and apoptosis. Mutations in p53 are seen in several tumors.

poly (ADP-ribose) polymerase (PARP): a family of nuclear enzymes that facilitate DNA repair via poly (ADP-ribose)ylation of histones and DNA repair enzymes.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**Phase I Study of Single-Agent AZD1775 (MK-1775), a Wee1 Kinase Inhibitor, in Patients With Refractory Solid Tumors**

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Khanh Do

No relationship to disclose

Deborah Wilsker

No relationship to disclose

Jiuping Ji

No relationship to disclose

Jennifer Zlott

No relationship to disclose

Tomoko Freshwater

Employment: Merck

Stock or Other Ownership: Merck

Robert J. Kinders

No relationship to disclose

Jerry Collins

No relationship to disclose

Alice P. Chen

No relationship to disclose

James H. Doroshow

No relationship to disclose

Shivaani Kummar

No relationship to disclose

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Appendix

Pharmacokinetic Methods

Human plasma samples were analyzed for levels of AZD1775 based on protein precipitation of AZD1775 in a 96-well format. The analyte and its stable isotope-labeled internal standard 2[H_8]-AZD1775 were chromatographed on an Atlantis HILIC column (2.1 × 50 mm; 5 μ m) using HILIC high pressure liquid chromatography and detected with a tandem mass spectrometer using a turbo-ion-spray interface in the positive ionization mode. The multiple reaction monitoring transitions monitored are m/z 501.3 to 442.3 for AZD1775 and m/z 509.3 to 450.3 for the internal standard. The lower limit of quantification for the assay was 2 ng/mL based on a volume of 50 μ L of plasma. The dynamic range was 2 to 1,000 ng/mL for AZD1775. EDTA was used as the anticoagulant, and plasma samples were stored at −20°C.

Pharmacodynamic Methods

Specimen collection and handling. Collection and handling conditions were optimized for immunofluorescence assays on human core needle biopsies commonly used at the National Institutes of Health Clinical Center. After collection, all specimens were immediately flash-frozen and then fixed in 10% neutral buffered formalin (NBF) before paraffin-embedding, sectioning, and staining.¹⁰ Biopsy specimens were fixed for 21 hours in 10% NBF and were processed in parallel.

Method Validation

pY15-Cdk (phosphorylated Tyr15-Cdk). Initial testing of antibody specificity of the pY15-Cdk2 rabbit monoclonal antibody (clone EPR2233Y; Abcam, Cambridge, MA) was compared with a panel of pY15-Cdk1 and pY15-Cdk2 antibodies from several manufacturers. Each antibody was qualified by Western blotting to verify recognition of a protein product of the appropriate molecular weight. We determined that commercial antibodies raised against pY15-Cdk2 recognize a product of 36 kDa in *HCT116-CDK2^{-/-}* isogenic knockout cells (Horizon Discovery, Cambridge, United Kingdom) that is equivalent in size to pY15-Cdk1. By using an enzyme-linked immunosorbent assay approach, capture antibodies specific to total Cdk1, Cdk2, and Cdk7 were used to determine that several antibodies raised against either pY15-Cdk1 or pY15-Cdk2 recognize phosphorylated tyrosine 15 on both Cdk1 and Cdk2 but not Cdk7. The rabbit monoclonal antibody to pY15-Cdk2 was selected for further testing. Antibody specificity was confirmed with the use of murine jejunum and brain as positive and negative tissue controls. As expected, pY15-Cdk expression was observed in proliferating cells in the crypts of the jejunum whereas no expression was seen in the brain tissue. Rabbit monoclonal isotype control antibody staining showed no positive signal in the mouse jejunum. A peptide competition assay was performed by using a synthetic phosphopeptide (Abgent, San Diego, CA) of the 15 amino acids surrounding and including phosphorylated tyrosine 15 of Cdk1, which successfully competed away the positive signal of EPR2233Y from the crypts of the jejunum. We chose the pY15-Cdk2 (EPR2233Y) rabbit monoclonal antibody as a robust antibody for recognition of both pY15-Cdk1 and pY15-Cdk2 for the immunofluorescence assay on NBF-fixed paraffin-embedded tissue.

pHH3. Histone H3 phosphorylated at Ser10 (pHH3; D2C8) biotinylated rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) specificity was tested by several approaches. Cell lysates were evaluated by Western blot for the presence of a single band at 17 kDa that increased after cells were treated with 100 nmol/L paclitaxel for 16 hours. The antibody was confirmed to recognize mitotic figures on xenograft tissue and signal was competed using an excess of pHH3 (Ser 10) blocking peptide (Cell Signaling Technology). Mouse jejunum was used as a positive control tissue and showed positive pHH3 in the crypts of the microvillae by immunofluorescence assay on cross-sectioned tissue. pHH3 marker fit-for-purpose was generated by determining increased pHH3 signal in xenograft tissues from mice treated with 10 mg/kg paclitaxel intravenously.

BOND-MAX Autostainer Staining Protocols

The following antibody working solutions were prepared and loaded into the BOND-MAX Processing Module: 2.8 μ g/mL pY15-Cdk2 (clone EPR2233Y) antibody prepared in Bond Primary Antibody Diluent (Leica Biosystems, Buffalo Grove, IL), and goat-anti-rabbit Alexa Fluor 488 (Invitrogen, Life Technologies, Grand Island, NY) antibody prepared 1:100 in 1× Bond Wash Solution. For pHH3 immunofluorescence assay, the biotinylated pHH3 antibody (clone D2C8) was prepared in Bond Primary Antibody Diluent at 1.8 μ g/mL, and Streptavidin-Alexa Fluor 660 (Invitrogen) was prepared 1:100 in 1× Bond Wash Solution. For γ H2AX (phosphorylated histone H2AX) immunofluorescence assay, the JBW301 monoclonal antibody (EMD Millipore, Merck KGaA, Darmstadt, Germany) was used.¹⁰

Calibrators and Controls

Drug response calibrators were developed from U87-MG xenograft tissue obtained at 4 hours after the tenth dose of drug from mice treated with AZD1775 administered twice per day at 62.5 mg/kg or 12.5 mg/kg or treated with vehicle control. Calibrator panel slides were

prepared with one tumor quadrant from each treatment group. A positive control (murine jejunum) and a negative control (murine brain) sections were also included on the same calibrator panel slides. Specifications for calibrator performance and acceptable variability were determined by statistical analysis of calibrator percent nuclear area positive (a surrogate for nuclear enumeration in the microscopic field) levels from three slides with at least five fields imaged per slide. Data were reported on a per-slide basis for statistical analysis. Paraffin blocks were prepared by using the predose needle biopsy from each patient and a flash-frozen positive control tissue (murine jejunum). The postdose biopsy was processed in parallel. Both specimens were run on the same slide with the positive control so all specimen and control tissues were subjected to the same incubation conditions and staining protocol. Four slides were stained for each biopsy. Two calibrator slides were run in each cassette of the BOND-MAX Autostainer in the first and last positions flanking the clinical specimen slides. Positive control tissue (murine jejunum) and negative control tissue (murine brain) were qualitatively assessed.

Image Capture and Quantitation

Four stained slides were scanned using the Aperio ScanScope FL image capture system (Leica Biosystems) for each biopsy with at least three 20× fields from three slides analyzed. The scanned slides were separated by at least three sections (15 μm) to minimize vertical field overlap and isolate individual cells. Two calibrator slides were scanned per clinical specimen, with at least five 20× fields from each xenograft tissue calibrator per slide analyzed. The acceptable levels for the calibrator were determined for each lot of calibrator blocks and primary and reporter antibodies. Fixed size, 20× images were extracted from full tissue scans using Aperio ImageScope software. Images that were selected for extraction were derived from tumor tissue determined to be of sufficient quality as assessed during the evaluation of an adjacent hematoxylin and eosin–stained section of each specimen by a board-certified pathologist (reviewed in Kinders, et al: Clin Cancer Res 20:2578-2586, 2014). Nontumor tissue such as stroma or collagen was excluded from analysis. Definiens Tissue Studio software (Definiens AG, Munich, Germany) was used for quantitative analysis of all calibrator and clinical biopsy material. Thresholds for pY15-Cdk marker and 4,6 diamidino-2-phenylindole expression were determined in preclinical studies using xenograft material. Proof of clinical readiness of the staining, image capture, and analysis was established in representative human samples and calibrator material. The number of blue channel–positive pixels (4,6 diamidino-2-phenylindole) that were also green channel–positive (pY15-Cdk) were counted and scored as the percent nuclear area positive. For γH2AX, the validated assay protocol was followed, and image capture and analysis were performed by using a Nikon 80i fluorescent microscope (Nikon Instruments, Melville, NY) equipped with a Retiga 2000R CCD camera (QImaging, Surrey, BC, Canada) and a custom macro capture script.¹⁰

Table A1. Summary of Pharmacokinetic Parameters by Dose Cohort

AZD1775 Dose Cohort	Parameter	Day 1				Day 2	Day 3	Day 3			
		C _{max} (nM)	T _{max} (hours)	AUC _{0-8 hours} (nM · hour)	C _{8-hour} (nM)	C _{trough} (nM)	C _{trough} (nM)	C _{max} (nM)	T _{max} (hours)	AUC _{0-8 hours} (nM · hour)	C _{8-hour} (nM)
Dose level 2 (225 mg twice per day for 2.5 days; n = 17)	Mean	740	3.53	3,990	422	494	776	1,490	3.65	9,630	1,030
	SD	266	1.12	1,400	180	225	341	688	1.77	4,610	509
	Median	667	4.00	3,980	402	475	657	1,390	4.00	8,460	783
Dose level 3 (300 mg twice per day for 2.5 days; n = 3)	Mean	1,040	3.00	5,840	586	985	1,560	2,570	2.67	17,800	1,980
	SD	296	1.73	1,450	209	154	414	554	1.15	3,790	455
	Median	911	4.00	5,310	555	1,020	1,630	2,660	2.00	18,100	2,020

Abbreviations: AUC, area under the serum concentration-time curve; AUC_{0-8 hours}, area under the serum concentration-time curve from dosing to 8 hours after dosing; C_{8-hour}, serum concentration 8 hours after dosing; C_{max}, maximum serum concentration; C_{trough}, minimum serum concentration between doses; SD, standard deviation; T_{max}, time to reach peak serum concentration.