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Targeting AXL and mTOR Pathway Overcomes Primary and Acquired Resistance to WEE1 Inhibition in Small-Cell Lung Cancer

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Abstract

Purpose—Drugs targeting DNA repair and cell-cycle checkpoints have emerged as promising therapies for small-cell lung cancer (SCLC). Among these, the WEE1 inhibitor AZD1775 has shown clinical activity in a subset of SCLC patients, but resistance is common. Understanding primary and acquired resistance mechanisms will be critical for developing effective WEE1 inhibitor combinations.

Experimental Design—AZD1775 sensitivity in SCLC cell lines was correlated with baseline expression level of 200 total or phosphorylated proteins measured by reverse-phase protein array (RPPA) to identify predictive markers of primary resistance. We further established AZD1775 acquired resistance models to identify mechanism of acquired resistance. Combination regimens

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Disclosure of Potential Conflicts of Interest

J.V. Heymach is a consultant/advisory board member for AstraZeneca. L.A. Byers reports receiving other commercial research support from AstraZeneca, and is a consultant/advisory board member for AbbVie, AstraZeneca, and Medivation. No potential conflicts of interest were disclosed by the other authors.

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were tested to overcome primary and acquired resistance to AZD1775 in *in vitro* and *in vivo* SCLC models.

Results—High-throughput proteomic profiling demonstrate that SCLC models with primary resistance to AZD1775 express high levels of AXL and phosphorylated S6 and that WEE1/AXL or WEE1/mTOR inhibitor combinations overcome resistance *in vitro* and *in vivo*. Furthermore, AXL, independently and via mTOR, activates the ERK pathway, leading to recruitment and activation of another G2-checkpoint protein, CHK1. AZD1775 acquired resistance models demonstrated upregulation of AXL, pS6, and MET, and resistance was overcome with the addition of AXL (TP0903), dual-AXL/MET (cabozantinib), or mTOR (RAD001) inhibitors.

Conclusions—AXL promotes resistance to WEE1 inhibition via downstream mTOR signaling and resulting activation of a parallel DNA damage repair pathway, CHK1. These findings suggest rational combinations to enhance the clinical efficacy of AZD1775, which is currently in clinical trials for SCLC and other malignancies.

Introduction

Small-cell lung cancer (SCLC), accounting for 15% of lung cancer cases, is the most aggressive form of lung cancer and is characterized by early metastasis and rapid development of drug resistance (1, 2). Unlike in non-small cell lung cancer (NSCLC), for which available therapies have substantially advanced over the past decade, treatment options for SCLC are exceedingly limited, and currently there are no approved targeted agents for this disease (3). Most patients experience relapse within months of completing first-line treatment and do not respond to second-line treatment (3).

Standard treatment regimens for SCLC mainly consist of DNA-damaging agents, such as platinum-based chemotherapy with or without radiation (4–6). In a previous study, we demonstrated that SCLC is characterized by increased expression of several key mediators of DNA damage repair, including PARP1, which regulates homologous recombination and other forms of DNA repair, and the checkpoint kinase 1 (CHK1) protein, a G2-cell-cycle checkpoint regulator (7). Inhibitors of both PARP1 and CHK1 have demonstrated activity in preclinical models of SCLC and are now being tested in clinical trials (3, 8–10). The initial high response rate of SCLC to DNA-damaging agents is likely due to the inherent genetic alterations in SCLC, including the near-ubiquitous inactivation of *TP53* and *RB1* (11–14), which make SCLC deficient in G₁–S cell-cycle checkpoint regulators and completely dependent on G₂–M checkpoint regulators upon DNA damage.

WEE1 G2 checkpoint kinase (WEE1) is another vital component of the G₂–M cell-cycle checkpoint that prevents entry into mitosis upon cellular DNA damage (15, 16). The G₂–M checkpoint is mainly regulated by the inhibitory phosphorylation of cyclin-dependent kinase 1 (CDK1 or CDC2) at Tyr15, which is primarily done by WEE1 (17). Inhibition of WEE1 has shown promise as a therapeutic strategy in multiple cancer types, especially those with inactivated *TP53* (18). Initial studies with WEE1 inhibitors demonstrated synergistic activity with chemotherapeutic agents such as gemcitabine, cisplatin, and temozolomide in other cancer types (19–22), and the selective WEE1 inhibitor AZD1775 (formerly MK1775) has

shown activity as a single agent in multiple cancers, including sarcoma, glioblastoma, and head and neck cancer (23, 24).

AZD1775 is currently being tested in a phase IB monotherapy clinical trial for the treatment of advanced solid tumors, including SCLC (NCT02482311). Earlier response data from the trial showed that 2 of 4 patients with SCLC had a response to single-agent AZD1775 (25). Furthermore, as with any targeted therapy, we predict acquired resistance will eventually develop in patients who initially respond. As such, the clinical impact of WEE1 targeting will be enhanced by a greater understanding of molecular mechanism of primary and acquired resistance and the development of effective combination strategies.

In the current study, we identified SCLC models sensitive and resistant to AZD1775 *in vitro* (primary resistance) and then developed models with acquired (secondary) resistance to AZD1775. Using reverse-phase protein array (RPPA)-based proteomic profiling, we then identified druggable targets and/or pathways that were highly expressed in resistant models. Our ultimate goals were to determine the top pathways associated with primary and acquired AZD1775 resistance and investigate rational therapeutic combinations (*in vitro* and *in vivo*) that could potentially be applied in the clinic to overcome resistance to WEE1 targeting in SCLC.

Methods

Cell lines and characterization

Cell lines (10 human-derived SCLC and three NSCLC cell lines) were provided by Dr. John Minna (The University of Texas Southwestern Medical Center, Dallas, TX) or obtained from ATCC. Complete cell line information is provided in the Supplementary Materials and Methods and in Supplementary Table S1.

All cell lines were tested and authenticated by short tandem repeat profiling (DNA Fingerprinting) (26) within 6 months of the study and routinely tested for Mycoplasma species before any experiments were performed. Also see Supplementary Materials and Methods.

Chemical compounds

AZD1775 and LY2606368 were manufactured by MD Anderson's Institute for Applied Chemical Science. TP0903 was provided by Tolero Pharmaceuticals. Temozolomide, cabozantinib, and RAD001 were obtained from Selleck Chemicals. All compounds were dissolved in dimethyl sulfoxide for *in vitro* treatments.

Generation of AZD1775-resistant cells

To increase the resistance of three AZD1775-sensitive SCLC cells (H1836, H524, and H1048) to AZD1775, the cells were treated with constant and increasing drug doses. The parental cells were first treated with 10 nmol/L, and the concentration was increased every 15 days in accordance with seven sequential treatments (25, 50, 100, 200, 400, 800 nmol/L) with a 1,000 nmol/L final concentration of AZD1775. Resistant cells were subjected to AZD1775 exposure for 3 days and then allowed 4 days to recover. The cells were compared

with parental cells at the end of each cycle to assess resistance. At the end of the last cycle, the cells were treated with the highest drug concentration (1,000 nmol/L) for 15–21 days and then kept without the drug for 3 weeks before assessing cell viability by CellTiter Glo assay. A cell viability assay confirmed acquired resistance to AZD1775 in all three cell lines.

Mice

For the syngeneic mouse model, 6-week-old female athymic nude mice (Envigo) were used. These animals were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) of The University of Texas MD Anderson Cancer Center and the NIH Guide for the Care and Use of Laboratory Animals.

Establishment of flank xenografts and studies in nude mice

The primary AZD1775-resistant H865 cell line was used for this study. For subcutaneous injections, 0.5×10^6 human SCLC (H865) cells were injected into one flank of each mouse with Matrigel (1:1, BD Biosciences).

Treatment schedule of SCLC in vivo models

Mice with mouse SCLC tumors received one of the following treatments: (i) vehicle; (ii) AZD1775 (60 mg/kg 5/7, every day, orally); (iii) TP0903 (50 mg/kg, 5/7, every day, orally); (iv) RAD001 (5 mg/kg 2/7, every day, orally); or (v) combination of AZD1775+TP0903 or AZD1775+RAD001. Once tumor volume reached 1,500 mm³, mice were euthanized according to institutional regulations.

Calculation of drug parameters

For single-agent analysis, we estimated IC₅₀ values by using the software program drexplorer (27), which fitted multiple dose–response models and selected the best model using the residual standard error.

In drug combination analysis, the Bliss independence model was used to estimate additive effect (<http://onlinelibrary.wiley.com/doi/10.1111/j.1744-7348.1939.tb06990.x/abstract;jsessionid=E87F2639E0C805EA671F6922AFA74C8D.f01t03>). Area under the curve (AUC) was calculated for the curve from theoretical additive effect (AUC₀) and from treatment with the drug combination (AUC₁). AUC was defined as the difference between AUC₁ and AUC₀ (AUC = AUC₁ – AUC₀). Therefore, a negative value of AUC suggests a more than additive effect (or synergy effect) and a positive value of AUC suggests a less than additive effect (or antagonistic effect).

Statistical analysis

The Student *t* test was used to compare gene expression between SCLC and normal lung samples. Spearman rank correlation was used to identify proteins associated with IC₅₀ values. Proteins showing *P* < 0.05 in the analysis were used to construct the heatmap with samples ordered by AZD1775 IC₅₀ value. Bimodality index, a metric to investigate bimodal expression, was calculated using the SIBER software (<http://bioinformatics.oxfordjournals.org/content/29/5/605.short>). Spearman rank correlation was

also used to correlate gene expression data. We used the R software program to perform all statistical analyses (28).

Other methods

The details of other methods, including targeted *WEE1* and *AXL* knockdown, cell viability assay, RNA isolation, reverse transcription, PCR, Western blot analysis, apoptosis, and cell-cycle analysis, *in vivo* models and dosing, and RPPA, are given in the Supplementary Materials and Methods.

Results

WEE1 is overexpressed in SCLC

We initially sought to determine the expression of *WEE1* in SCLC patient tumors. We analyzed *WEE1* mRNA expression levels in 68 SCLC and 26 normal lung tissue samples. Compared with normal lung tissue samples, SCLC samples had >4-fold higher *WEE1* expression ($P < 0.0001$; Fig. 1A, left). Comparison of protein levels between 63 SCLC and 114 NSCLC cell line samples confirmed higher *WEE1* protein expression in SCLC cell line samples ($P < 0.0001$; FC = 1.66; Fig. 1A, right). We then further analyzed *WEE1* mRNA expression in nine human SCLC cell lines, which we compared with three NSCLC cell lines (H1299, H2822, and H1666) using real-time reverse transcriptase PCR. We found that *WEE1* mRNA expression in all SCLC cell lines examined was higher than in NSCLC cell lines (Supplementary Fig. S1A), further indicating that *WEE1* expression is significantly increased in SCLC. Furthermore, expression levels of *WEE1* mRNA and *WEE1* protein were highly correlated in the SCLC cell lines ($n = 54$; $r = 0.734$; $P < 0.0001$; Supplementary Fig. S1B).

Genetic knockdown of WEE1 causes DNA damage and apoptosis in most SCLC cell lines

To determine the effect of *WEE1* targeting on DNA damage and cell survival, we performed siRNA-mediated knockdown of *WEE1* in four human SCLC cell lines with a range of *WEE1* expression levels. The knockdown efficiency was confirmed by real-time reverse transcriptase PCR (Fig. 1B) and immunoblot analysis (Fig. 1C) 24 hours after transfection. *WEE1* is required for the temporal regulation of CDK2 in the S-phase and CDK1 in the G₂ phase of the cell cycle; therefore, *WEE1* targeting is expected to lead to S-phase defects (DNA double-strand breaks) and G₂-M defects (premature mitosis; ref. 17). As expected, we observed appreciable DNA damage, as evident from increased levels of phosphorylated (p-) γ H2AX upon *WEE1* knockdown (Fig. 1C) in three human SCLC cell lines (H82, H1836, and H526). However, there was no significant induction of γ H2AX levels in two resistant cell lines H865 and H1930 (Fig. 1C; Supplementary Fig. S1C and S1D). Furthermore, H82, H1836, and H526 knockdown cells still progressed through mitosis, as was evident from increased levels of mitotic marker phosphorylated histone H3 (PH3; Fig. 1C); in contrast to H865 and H1930 where PH3 levels did not significantly change (Fig. 1C; Supplementary Fig. S1C and S1D). Similarly, appreciable apoptosis was observed in the same three cell lines (Fig. 1D), but not in H865 (Fig. 1D) 48 hours after transfection. Together, these results demonstrate that inhibiting *WEE1* expression caused DNA damage and decreased survival

in most, but not all, SCLC cell lines and suggest that a subset of SCLC has primary resistance.

Pharmacologic inhibition of WEE1 by AZD1775 shows a range of sensitivity in SCLC in vitro

The WEE1 inhibitor AZD1775 is currently being analyzed in a phase IB clinical trial for advanced solid tumors, including SCLC (NCT02482311; ref. 25). Preliminary results from the clinical trial suggest that a subset of patients with SCLC exhibits *de novo* resistance to treatment with single-agent AZD1775. To explore the sensitivity of SCLC to AZD1775, we measured the median half-maximal inhibitory concentration (IC₅₀) in a panel of nine SCLC cell lines after 5 days of treatment. SCLC cell lines displayed a range of sensitivity to AZD1775 (IC₅₀ ranging from 30 nmol/L to >1 μmol/L), with about 30% of SCLC cell lines showing relative resistance to WEE1 targeting alone (IC₅₀ > 100 nmol/L; Fig. 1E). The concentration of 100 nmol/L was chosen to define resistance because concentrations above this are unlikely to be clinically achievable (29, 30).

To investigate potential mechanisms of *de novo* pharmacologic resistance, we first compared differences in apoptosis upon treatment with AZD1775 between sensitive (H82 and H526) and putatively resistant (H865 and H1417) cell lines. As expected, single-agent AZD1775 (100 nmol/L for 24 hours) induced apoptosis in sensitive, but not resistant, cell lines (Fig. 1F). This result confirmed the initial observation that H865 and H1417 exhibit resistance to WEE1 targeting.

Because WEE1 is a vital regulator of the cell cycle, we next tested whether cell-cycle effects of AZD1775 were different between sensitive and resistant cell lines. Treatment with AZD1775 (100 nmol/L for 48 hours) resulted in appreciable accumulation of cells in the sub-G₁ phase (36%) and an increase in the G₂-M cell population (increase of 6%) in the AZD1775-sensitive cell lines (H82, H1836, H526; Fig. 1G; Supplementary Fig. S1D). However, AZD1775 did not have any effect on the cell-cycle profile of AZD1775-resistant cells (H865, H1930, and H1417; Fig. 1G; Supplementary Fig. S1E). Western blot analysis confirmed the downregulation of WEE1 downstream target, pCDC2_Y15, in SCLC cell lines (H82, H526, H865, and H1417; Supplementary Fig. S1F). These data suggest that SCLC cells exhibit a range of sensitivity to the WEE1 inhibitor AZD1775, with resistant cells showing no appreciable change in viability, apoptosis, or cell-cycle profile in response to treatment.

WEE1 inhibition is synergistic with temozolomide independent of MGMT expression or initial sensitivity to WEE1 targeting in SCLC cell lines

The oral DNA-alkylating agent temozolomide is an established treatment for several cancer types (31, 32). Clinical studies have shown activity of single-agent temozolomide in only a minority of SCLC patients (33, 34); however, preclinical data from SCLC models and other cancers support the ability of DNA damage repair inhibitors to potentiate the effect of temozolomide, and clinical trials are investigating combinations of temozolomide with PARP inhibitors (3, 34). We investigated the effects of temozolomide in combination with

WEE1 targeting by genetic knockdown (*WEE1* siRNA) or pharmacologic inhibition (AZD1775) on cell viability, apoptosis, DNA damage, and DNA repair in SCLC *in vitro*.

We analyzed cell viability in a panel of SCLC cell lines ($n = 10$) treated with AZD1775 or temozolomide alone or in a fixed ratio ranging from 30 nmol/L temozolomide:3 nmol/L AZD1775 to 3 μ mol/L temozolomide:0.3 μ mol/L AZD1775. All 10 cell lines, including those with primary AZD1775 resistance, exhibited synergy (more than additive effect) across different dose ranges to AZD1775 and temozolomide (Fig. 2A and B; Supplementary Fig. S2A). Response to the combination of AZD1775 and temozolomide was independent of O⁶-methylguanine-DNA-transferase (*MGMT*) expression, which has previously been associated with clinical response to single-agent temozolomide (ref. 35; Fig. 2B).

Single-agent WEE1 targeting, by genetic knockdown (*WEE1* siRNA) or AZD1775 (100 nmol/L), caused apoptosis in AZD1775-sensitive (H82 and H1836) cell lines but not in AZD1775-resistant (H865 and H1930) cell lines (Fig. 2C). However, treatment with the combination of WEE1 targeting by *WEE1* siRNA or AZD1775 with temozolomide led to increased apoptosis in all cell lines irrespective of their *de novo* response to WEE1 inhibition (Fig. 2C).

WEE1 siRNA and single-agent AZD1775 efficiently abrogated WEE1 expression, activated the downstream substrate pCDC2 (Y15) in all three tested cell lines (H82, H1836, and H865; Fig. 2D). However, we observed significant induction of γ H2AX, PH3, and cleaved caspase-3 in H82 and H1836 as compared with the resistant cell line, H865 (Fig. 2D; Supplementary Fig. S2B). These effects were further enhanced by treatment with temozolomide in all three tested cell lines (H82, H865, H1836) irrespective of initial sensitivity to WEE1 targeting (Fig. 2D). To further confirm our finding we analyzed the expression of γ H2AX pre- and post-AZD1775 treatment by RPPA in three sensitive (H82, H1836, and H1048) and three resistant (H865, H1417, and H1930) cell lines (Supplementary Fig. S2C). As shown in Supplementary Fig. S2C, AZD1775 treatment (100 nmol/L, 24 hours) significantly enhanced γ H2AX levels in sensitive models ($P < 0.001$), whereas there was no significant change in the resistant models ($P > 0.6$; Supplementary Fig. S2C).

AZD1775-sensitive cell lines (H82 and H1836) showed decreased expression of DNA repair proteins (pATM_S1981, MRE11, RAD51, and E2F1) after WEE1 inhibition with or without temozolomide (Fig. 2E), unlike *de novo* WEE1 inhibitor-resistant cells. The DNA repair proteins were, however, abrogated in all cell lines after combination treatment with temozolomide, independent of sensitivity to WEE1 targeting (Fig. 2E).

Taken together, these results show that WEE1 targeting by AZD1775 combined with temozolomide interacts synergistically to decrease viability, increasing DNA damage and enhancing apoptosis in SCLC cell lines irrespective of *MGMT* status or initial response to AZD1775 alone. However, the SCLC cell lines showing primary resistance to AZD1775 have an intact DNA repair mechanism even after DNA damage, which may contribute to the resistance mechanism.

AXL expression is increased in AZD1775-resistant SCLC

Next, using RPPA, we compared the expression of 195 proteins between the most sensitive ($IC_{50} < 30$ nmol/L) and resistant SCLC cell lines ($IC_{50} > 100$ nmol/L) by *t* test to identify potential predictive biomarkers. Among all proteins measured, the receptor tyrosine kinase AXL was the top marker of *in vitro* resistance to AZD1775 ($P = 0.002$; Fig. 3A and B), and S6_S240/244, a downstream target of AXL and the PI3K/mTOR pathways, was the next strongest marker of resistance ($P = 0.001$; Fig. 3A–C). Basal expression levels of other members of the mTOR pathway, such as S6_S235/236 and TSC2_pT1462, were also significantly higher in cells showing *de novo* resistance to AZD1775 ($P = 0.003$; Supplementary Fig. S3A). Western blot analysis of the three most sensitive (H1836, H82, H1048) and most resistant (H1417, H865, H1930) cell lines confirmed that AZD1775-resistant cell lines had significantly higher expression of AXL than AZD1775-sensitive cell lines, in which expression of AXL remained unchanged after treatment with AZD1775 (100 nmol/L for 24 hours; Fig. 3D).

AXL is a receptor tyrosine kinase frequently overexpressed in cancers that have undergone epithelial-to-mesenchymal transition (EMT) and EMT has been implicated as an important mediator of resistance to certain targeted therapies (e.g., EGFR and HER2 inhibitors) in several other cancers, including head and neck squamous cell cancers, NSCLC, and melanoma (36). EMT has been previously described as being associated with resistance in several cancer types and recent data from our group shows an association between EMT and resistance to several drugs including cisplatin and PARP inhibitors (37). AXL is highly expressed in EMT and based on the strong correlation of AXL with AZD1775 resistance *in vitro*, we sought to determine the frequency of AXL expression in SCLC patient tumors.

Analysis of 110 SCLC patient samples showed that AXL was bimodally expressed (bimodality index = 1.27; Fig. 3E), with 30% of tumors having high AXL levels. Expression of AXL was also inversely correlated with WEE1 expression (Fig. 3F); however, AXL expression levels were superior to WEE1 expression levels ($P > 0.05$) in predicting response to AZD1775. Taken together, these results demonstrate that AXL is overexpressed in a subset of treatment-naïve SCLC clinical samples and that high basal (pretreatment) AXL expression is associated with primary resistance to AZD1775 *in vitro* which persists following treatment with AZD1775.

AXL-activated mTOR pathway limits sensitivity to AZD1775

Given the role of AXL in activation of the downstream AKT/ mTOR pathway, we investigated the differences in this pathway between three AZD1775-sensitive (H82, H1836, and H1048) and three AZD1775-resistant (H865, H1930, and H1417) SCLC cell lines. We found that pAKT_S473 was suppressed by AZD1775 in both resistant and sensitive cell lines (Fig. 3G; Supplementary Fig. S3B). However, mTOR activity was not abrogated in resistant cell lines, which had higher basal levels and sustained phosphorylation of S6_S240/244 (Fig. 3G) following treatment with AZD1775. To further confirm this observation we performed RPPA analysis pre- and post-AZD1775 treatment in 3 sensitive (H82, H1836, and H1048) and three resistant (H1417, H865, and H1930) cell lines (Supplementary Fig. S3C). As demonstrated by Supplementary Fig. S3C, AZD1775

resistant cells had higher basal level of S6_S240/244 as compared with sensitive cells. AZD1775 treatment successfully abrogated S6_S240/244 level in sensitive cells ($P < 0.0001$), whereas there was no significant change in resistant models ($P > 0.05$; Supplementary Fig. S3C). These results demonstrate that activation of the mTOR pathway by sustained phosphorylation of S6_S240/244 was consistent in all AZD1775-resistant cell lines, and this was not abrogated by treatment with AZD1775.

Cotargeting AXL overcomes primary AZD1775 resistance in SCLC cell lines

To determine whether AXL overexpression directly contributes to primary WEE1 inhibitor resistance, we tested the effect of AXL inhibition (by TP0903) or knockdown (*AXL* siRNA) on WEE1 inhibitor response in AZD1775-resistant SCLC cell lines (H865, H1930, and H1417). TP0903 sensitized the cells to AZD1775, decreasing viability, relative to AZD1775 alone (Fig. 4A) at almost all doses (20 nmol/L, 40 nmol/L, and 80 nmol/L). The combination of AZD1775 (100 nmol/L) and TP0903 (20 nmol/L) also significantly increased apoptosis at 24 hours in all three cell lines (Fig. 4B). Similar effects were observed with genetic knockdown of *AXL* by siRNA (Supplementary Fig. S4A and S4C); treatment with AZD1775 decreased the viability of cells with *AXL* knockdown significantly more than in the scramble and parental cells. We also observed that pAKT and pS6_S240/244 (downstream of AXL) were suppressed by TP0903, an effect that was further enhanced with the combination of TP0903 and AZD1775 (Fig. 4D).

Thus, these results confirm that cotargeting AXL by genetic knockdown and pharmacologic inhibition decreased viability, enhanced apoptosis, and suppressed signaling through the mTOR pathway in cell lines with *de novo* resistance to AZD1775.

AXL-mediated persistent mTOR activation promotes primary resistance to WEE1 targeting in SCLC

To further confirm the role of mTOR activation in AZD1775 resistance, we tested the effect of targeting mTOR in combination with AZD1775 using the allosteric mTORC1 inhibitor everolimus (henceforth referred to as RAD001) which is approved by the FDA for the treatment of other cancer types, including neuroendocrine tumors. We observed that the combination of AZD1775 with RAD001 significantly enhanced the cytotoxicity of AZD1775 and decreased cell viability compared with either drug alone in AZD1775-resistant cell lines (H865, H1417, and H1930; Fig. 4E). Similar to AXL inhibition, the combination of RAD001 (100 nmol/L) and AZD1775 (100 nmol/L) also significantly enhanced apoptosis in AZD1775-resistant cell lines (Fig. 4F). As expected, the combination increased the expression of markers of DNA damage (γ H2AX) and apoptosis (cleaved caspase-3) in the resistant cells (Supplementary Fig. S4B).

To confirm that these findings were not due to any potential off-target effects of AZD1775, we next treated WEE1 knockdown resistant cells with RAD001. *WEE1* KD alone does not cause any appreciable difference in viability in any of the resistant cell lines (Supplementary Fig. S4C). However, treatment with RAD001 significantly decreased the viability of *WEE1* knockdown cells (compared with parental or scramble siRNA-treated cells; Fig. 4G). As expected, treatment with RAD001 alone was sufficient to decrease the phosphorylation of p-

mTOR_S2448 and pS6_S240/244, which was further abrogated with the addition of AZD1775 (Fig. 4H). Taken together, these results show that SCLC cell lines with primary resistance to WEE1 targeting maintain sustained activation of mTOR and that pharmacologic inhibition of mTOR resensitizes cells to WEE1 targeting.

Coinhibition of the AXL and/or mTOR pathway enhances the antitumor efficacy of WEE1 inhibition in SCLC

Encouraged by the *in vitro* activity, we next investigated the *in vivo* effect of WEE1+AXL or WEE1+mTOR inhibition. For this, we selected the cell line H865, which exhibited primary resistance to AZD1775 and high basal expression of AXL and pS6_S240/244. Tumor-bearing mice ($n = 10$ per group) were treated with vehicle, AZD1775, TP0903, RAD001, AZD1775+TP0903, or AZD1775+RAD001. Primary resistance of the model was confirmed *in vivo* in the H865 flank xenograft model, in which, as expected, tumors did not respond to single-agent AZD1775 (60 mg/kg, 5/7, every day, orally) (Fig. 4I; Supplementary Fig. S5A) and the tumor growth was similar to that observed with vehicle (Fig. 4I; Supplementary Fig. S5A). We observed a modest tumor delay with single-agent treatment with the AXL inhibitor TP0903 (Fig. 4I; Supplementary Fig. S5A).

In contrast, treatment with the combination of the AXL inhibitor TP0903 (50 mg/kg, 5/7, every day, orally) with AZD1775 (60 mg/kg, 5/7, every day, orally) led to significant delay in tumor growth in all animals, which was superior to either single-agent alone (Fig. 4I and J; Supplementary Fig. S5A), with a tumor/control (T/C) ratio = 0.1 ($P < 0.001$) at day 36. The median overall survival was 36 days for mice treated with vehicle, 39 days for mice treated with AZD1775, and 49 days for mice treated with TP0903, whereas all mice treated with AZD1775+TP0903 survived (100% survival benefit) until the endpoint of data collection at day 80 (Fig. 4J). There was no weight loss in any of the treatment groups throughout the course of this experiment (data not shown).

A similar antitumor efficacy for the combination of AZD1775 and RAD001 (5 mg/kg, 2/7, every day, orally) was observed in these models; this combination caused a significant delay in tumor growth compared with either single-agent alone (Fig. 4K; Supplementary Fig. S5B); T/C = 0.2 ($P < 0.01$) at day 36. The median survival was 37 days for mice treated with vehicle, 39 days for mice treated with AZD1775, and 42 days for mice treated with RAD001; 9 of 10 mice in the AZD1775+RAD001 treatment group survived (90% survival benefit) until the endpoint of data collection at day 80 (Fig. 4L). There was no weight loss in any of the treatment groups throughout the course of this experiment (data not shown).

Taken together, these results further support that simultaneous inhibition of WEE1 and AXL or WEE1 and mTOR had a significantly higher antitumor efficacy (and no significant toxicity) than inhibition of WEE1 alone in a primary resistant flank xenograft SCLC model.

Treatment with AZD1775 upregulates the ERK/p90RSK signaling cascade and enhances DNA repair proteins in primary resistant cell lines

Previous studies have shown that AXL plays a role in the regulation of the ERK/p90RSK pathway (38, 39) and that this pathway plays a cooperative role in mTOR activation (40, 41). Hence, we aimed to elucidate the involvement of the ERK/p90RSK pathway in adaptive

response to treatment with AZD1775 in the three primary AZD1775-resistant SCLC cell lines. To this end, we performed RPPA analysis in primary AZD1775-resistant cell lines before and after treatment with AZD1775 (100 nmol/L), TP0903 (20 nmol/L), or the combination for 24 hours. Interestingly, RPPA analysis showed that treatment with AZD1775 led to persistent upregulation of members of the ERK/p90RSK pathway (pERK1/2, p-p90RSK) and S6_S240/244 and S6_S235/236 (Fig. 5A; Supplementary Fig. S6A and S6B). In addition, we found that several DNA repair proteins, pATR_S428, pCHK1_S345, and pCHK1_S296, were also elevated after treatment with AZD1775 (Fig. 5A; Supplementary Fig. S6A and S6B). To demonstrate the role of AXL, we investigated the level of these markers in the cells treated with the combination of AZD1775 and TP0903, and we observed a consistent decrease in these proteins (Fig. 5A; Supplementary Fig. S6A and S6B) with the combination treatment.

These results suggest that ERK/p90RSK and DNA repair proteins could also be involved in primary resistance to WEE1 targeting. To investigate crosstalk between the two pathways, we treated the AZD1775-resistant cell lines with AZD1775, RAD001, or the combination. As expected, members of the ERK/p90RSK and mTOR pathways either remained unchanged or increased upon treatment with AZD1775 alone (Fig. 5B), whereas expression of S6_S235/236, pERK1/2, and p-p90RSK decreased upon treatment with RAD001 and the combination of AZD1775 and RAD001 (Fig. 5B), supporting the role of mTOR pathway activation in activation of the ERK/p90RSK pathway in AZD1775-resistant cells.

Overall, these data show that upregulation of AXL and its interaction with ERK/p90RSK and mTOR contributes to persistent activation of downstream pathways. Moreover, the mTOR pathway, upon activation, cooperates with ERK/p90RSK to sustain this activation. The upregulation of DNA repair proteins other than WEE1 in these primary AZD1775-resistant cells suggests that activation of a parallel pathway may serve as a substitute for the DNA repair function of WEE1 in these resistant models.

AXL/mTOR-mediated ERK/p90RSK activation leads to nuclear arrangement and activation of CHK1 that substitutes for the role of WEE1 in AZD1775-resistant SCLC models

Earlier reports identified the role of the mTOR and ERK/p90RSK pathways in DNA repair and the cell cycle (40, 41), including activation of CHK1. On the basis of these findings and our finding that both ERK/p90RSK and DNA repair proteins, including CHK1, are upregulated upon treatment with AZD1775 in resistant cells, we aimed to further elucidate the role of AXL in DNA damage response in AZD1775-resistant SCLC cells. An earlier study showed that p90RSK facilitates the nuclear accumulation of CHK1 through S280 phosphorylation, leading to activation of the ATR/CHK1 pathway (42). Accordingly, we observed higher level of pCHK1_S280 in the nuclear extract of AZD1775-resistant cells (H865 and H1930) than in the cytoplasm (Fig. 5C). Furthermore, when the cells were treated with AZD1775 alone, we found either no change or a slight increase in the level of pCHK1_S280 in the nucleus but no change in the cytoplasmic fragment.

Interestingly, when the cells were treated with the AXL inhibitor TP0903 (20 nmol/L for 24 hours), there was a decrease in the level of p-p90RSK and nuclear pCHK1_S280, but no appreciable change was observed in the cytoplasmic fraction (Fig. 5C). The coinhibition of

WEE1 and AXL led to a significant decrease in the nuclear localization of pCHK1_S280. Together, these data strongly suggest that AXL-mediated p90RSK activation induces CHK1 accumulation in the nucleus, which may lead to activation of the DNA damage repair response of CHK1 in these cells.

On the basis of the observation that CHK1 was activated in cells showing primary resistance to AZD1775, as well as the availability of the potent CHK1 inhibitor LY2606368, which is currently being investigated in a clinical trial for recurrent SCLC (NCT02735980), we focused on additional experiments to confirm the involvement of CHK1. Indeed, cotargeting CHK1 increased the apoptotic potential of AZD1775 in the cell lines showing primary resistance to the WEE1 inhibitor (Fig. 5D). Together, these findings confirm that CHK1 is activated in these primary AZD1775-resistant cells as an escape mechanism to overcome accumulation of DNA damage and eventual cell death upon WEE1 targeting; cotargeting CHK1 could overcome the *de novo* AZD1775 resistance in these models.

High AXL/MET/mTOR expression defines acquired resistance to AZD1775 in SCLC in vitro

To study the molecular mechanism by which acquired resistance to AZD1775 emerges, we selected three AZD1775-sensitive cell lines (H1836, H524, and H1048) and exposed them to increasing concentrations of AZD1775 over time until resistance emerged (Supplementary Fig. S7A). At the end of the last cycle, cell viability assays confirmed acquired resistance to AZD1775 in all three cell lines, as shown in Supplementary Fig. S7B. The cells were named H524 AZD-Res, H1836 AZD-Res, and H1048 AZD-Res. Next, we analyzed changes in protein expression and pathway activation between paired parental and resistant cell counterparts using RPPA. Among the 195 proteins that were measured, AXL (fold change = 1.91, $P < 0.05$) and S6_S240/244 (fold change = 2.64, $P < 0.05$) were the top markers upregulated in cells with acquired resistance (Fig. 6A and B) compared with the paired parental cells. Interestingly, higher expression of the prometastatic receptor tyrosine kinase MET was also observed as a top marker of acquired resistance (fold change = 1.68; $P < 0.05$; Fig. 6A and B). Increased total MET has previously been associated with poor outcome in other cancer types, such as metastatic renal cell carcinoma, and with drug resistance in NSCLC. We also observed decreased expression of WEE1 (fold change = 3.12; $P < 0.05$) as a marker of acquired resistance in these models (Fig. 6A and B).

Dual treatment with AXL and MET inhibitors demonstrates enhanced activity in overcoming acquired resistance to AZD1775 in SCLC in vitro

To further characterize the effects of AXL, MET, and mTOR blockade in overcoming resistance, we conducted viability assays with single-agent and combination regimens [AZD1775±TP0903 (Fig. 6C), AZD1775±RAD001 (Fig. 6D), and AZD1775±cabozantinib (Fig. 6E)] in the parental and AZD1775-resistant counterparts of three cell lines (H524, H1836, and H1048). All combinations were superior to single-agent doses. Treatment with TP0903 and RAD001 showed appreciable response in the resistant cells by decreasing viability at almost all combination doses (Fig. 6C and D). However, simultaneous suppression of WEE1 (AZD1775) and AXL/MET (cabozantinib) resulted in the most remarkable response in these models; combinatorial synergy was observed at all treated doses (Fig. 6E). These results further support that AXL, mTOR, and MET play a significant

role in driving acquired AZD1775 resistance in SCLC and that coinhibition of WEE1 along with a dual AXL/MET inhibitor (cabozantinib) results in resistance reversal in these models. A summary of the pathways driving primary and acquired AZD1775 resistance in SCLC is shown in Fig. 6F.

Discussion

WEE1 inhibition is a novel and promising therapeutic strategy for SCLC, an aggressive disease for which therapeutic advances have been very limited in the past three decades. However, to date, no predictive biomarkers for WEE1 inhibitors have been identified. The current study puts forward four main findings. First, the WEE1 inhibitor AZD1775 is active in a subset of SCLC models and synergizes with temozolomide irrespective of *MGMT* expression. Second, increased basal expression of AXL and activated mTOR pathway are associated with primary AZD1775 resistance, and cotargeting AXL or mTOR overcomes WEE1 inhibitor resistance in SCLC models. Third, AXL/mTOR axis-mediated activation of the ERK/p90RSK pathway leads to accumulation and, thus, activation of parallel DNA repair pathway such as ATR/CHK1, which leads to a bypass of WEE1 inhibition. Finally, upregulation of AXL, p-S6, and a second tyrosine kinase receptor, MET, occurs following the development of acquired AZD1775 resistance. However, this acquired resistance can be reversed through the simultaneous inhibition of WEE1 and AXL/MET or mTOR.

In this study, we demonstrated sensitivity to WEE1 targeting in a subset of SCLC models, which was enhanced by the oral chemotherapy temozolomide irrespective of *MGMT* expression. The finding that temozolomide—AZD1775 is an active combination is not entirely unexpected given prior data supporting temozolomide combinations with other DNA-damaging agents (e.g., PARP inhibitors) in SCLC (3, 5). However, this finding has the potential for immediate translation into the clinic, especially if the combination is well tolerated in a current trial of temozolomide with AZD1775 for glioblastoma (NCT01849146).

By further investigating the protein signature of AZD1775-resistant models, we then demonstrated for the first time a critical role for the receptor tyrosine kinase AXL and downstream pathways in primary and secondary resistance. Moreover, we showed that WEE1 inhibitor resistance can be overcome through AXL or mTOR inhibition. Furthermore, we reported a subset of SCLC patient tumors with high AXL expression, suggesting a population likely to have primary resistance to AZD1775. These findings have important implications for the clinical development of WEE1 inhibitors, both for patient selection with AXL as a predictive biomarker and for the development of novel drug combinations to overcome resistance.

A growing number of studies implicate AXL in resistance to chemotherapy, radiation, and specific targeted agents such as EGFR/HER2 inhibitors in other cancer types (36). Recently, a study from our group demonstrated a potential new functional role of AXL in regulating DNA damage response in NSCLC, head and neck cancer, and triple-negative breast cancer models. In that study, the combination of AXL and PARP inhibitors was highly synergistic

in cell lines (43). The current study builds upon these findings, highlighting a possible mechanism through which AXL may directly contribute to DDR inhibitor resistance.

In our models, AXL drove activation of the mTOR pathway and pharmacologic inhibition of mTOR by RAD001 was sufficient to overcome WEE1 inhibitor resistance *in vitro* and *in vivo*. This finding is consistent with a previous study in leukemia in which dual WEE1/mTOR targeting was active in multiple RAS-expressing malignancies (44). Given that RAD001 was recently FDA-approved for the treatment of other high-grade neuroendocrine cancers of the lung, the synergy observed with combining AZD1775 with RAD001 *in vitro* and *in vivo* has the potential for rapid translation into the clinical setting.

Another interesting finding in our models was upregulation of the ERK/p90RSK pathway as an adaptive response to AZD1775. This is particularly exciting because previous reports have shown cross-talk between the ERK/p90RSK and AKT/mTOR pathways when driving resistance to targeted therapies (40, 41), and AXL is an upstream activator of both pathways (38, 39). In our models, mTOR inhibition was sufficient to decrease the expression of p-p90RSK, indicating that mTOR may activate the RSK pathway. Previous reports have indicated that the involvement of ERK/p90RSK pathway in cell cycle and DNA damage checkpoint regulation (45). In our primary resistant models, we observed retention of pCHK1_S280 in the nucleus, which was abrogated upon AXL inhibition, and coinhibition of WEE1/CHK1-sensitized cells with primary resistance to AZD1775. This finding is supported by previous studies that have also shown that the P90RSK pathway is involved in acceleration of the ATR-CHK1-mediated DNA damage response and in retention of CHK1 in the nucleus (42). Thus, our study shows that AXL overexpression activates the ERK/p90RSK and mTOR pathways which, in turn, may promote cell survival (despite WEE1 targeting) via recruitment and activation of CHK1. Further investigation is required to assess the exact mechanism of CHK1 recruitment in these models.

Finally, in our models of acquired AZD1775 resistance, we observed upregulation of MET in addition to AXL and S6_S240/244. MET is an important therapeutic target for multiple cancers (46–48) and increased expression has been associated with poor prognosis (48). Previous studies have implicated MET in therapeutic resistance to antiangiogenic therapies and radiation (46–48), and earlier reports suggested a role of MET in SCLC tumorigenesis and progression (49). We find that the combination of AZD1775 and a dual AXL/MET inhibitor, cabozantinib (FDA-approved for thyroid and renal cancers) overcomes acquired resistance to WEE1 inhibition even more than combinations with TP0903 or RAD001. The appreciable effect of the AZD1775 and cabozantinib combination in cell lines with acquired AZD1775 resistance may be attributed to the dual inhibition of AXL and MET in these models. A previous study had shown that MET inhibition synergizes with DNA-damaging agents potentially by overriding DNA damage-induced checkpoint arrest via ATR/CHK1 (50), but the exact mechanism of MET-induced acquired resistance to WEE1 requires further investigation in SCLC models.

AXL expression in the patients with SCLC undergoing treatment with AZD1775 in the clinical trial has not yet been reported and is currently not used for patient selection. MET inhibitors are currently in several clinical trials but the data remain largely inconclusive. On

the basis of our findings that MET expression increases in acquired AZD1775 resistance and cotargeting MET and AXL overcomes WEE1 inhibitor resistance, we predict that other cancer types with G₂-M dependency and for which WEE1 inhibitors are in clinical trials (e.g., triple-negative breast cancer, head and neck cancer, and glioblastoma) may implement a similar mechanism to escape, and these cancers could also be sensitive to dual AXL/WEE1, mTOR/WEE1, or dual AXL/MET+WEE1 targeting.

In summary, our study indicates that in SCLC, AXL expression could serve as a candidate biomarker to select patients for WEE1 inhibitor trials, and AXL-mTOR axis mediates primary resistance to WEE1 targeting by mediating the response through the mTOR and p90RSK pathways, ultimately leading to the recruitment of a parallel G₂-M cell-cycle checkpoint kinase, CHK1. On the basis of these findings, we propose the combination regimens, WEE1-temozolomide, WEE1-AXL, WEE1-mTOR, and WEE1-CHK1 targeting, as potential therapeutic strategies to overcome *de novo* resistance to WEE1 inhibitors in patients with SCLC. MET may also contribute to acquired WEE1 inhibitor resistance and combination regimens with WEE1/MET inhibitors warrant further study. The results from this study support further investigation of these combinations in SCLC, a disease with very limited therapeutic options.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Govindan R, Page N, Morgensztern D, Read W, Tierney R, Vlahiotis A, et al. Changing epidemiology of small-cell lung cancer in the United States over the last 30 years: analysis of the surveillance, epidemiologic, and end results database. *J Clin Oncol*. 2006; 24:4539–44. [PubMed: 17008692]
2. William WN Jr, Glisson BS. Novel strategies for the treatment of small-cell lung carcinoma. *Nat Rev Clin Oncol*. 2011; 8:611–9. [PubMed: 21691321]
3. Byers LA, Rudin CM. Small cell lung cancer: where do we go from here? *Cancer*. 2015; 121:664–72. [PubMed: 25336398]
4. O'Brien ME, Ciuleanu TE, Tsekov H, Shparyk Y, Cucevia B, Juhasz G, et al. Phase III trial comparing supportive care alone with supportive care with oral topotecan in patients with relapsed small-cell lung cancer. *J Clin Oncol*. 2006; 24:5441–7. [PubMed: 17135646]

5. Pietanza MC, Byers LA, Minna JD, Rudin CM. Small cell lung cancer: will recent progress lead to improved outcomes? *Clin Cancer Res.* 2015; 21:2244–55. [PubMed: 25979931]
6. Turrisi AT III, Glover DJ, Mason BA. A preliminary report: concurrent twice-daily radiotherapy plus platinum-etoposide chemotherapy for limited small cell lung cancer. *Int J Radiat Oncol Biol Phys.* 1988; 15:183–7. [PubMed: 2839439]
7. Byers LA, Wang J, Nilsson MB, Fujimoto J, Saintigny P, Yordy J, et al. Proteomic profiling identifies dysregulated pathways in small cell lung cancer and novel therapeutic targets including PARP1. *Cancer Discov.* 2012; 2:798–811. [PubMed: 22961666]
8. Cardnell RJ, Feng Y, Diao L, Fan YH, Masrourpour F, Wang J, et al. Proteomic markers of DNA repair and PI3K pathway activation predict response to the PARP inhibitor BMN 673 in small cell lung cancer. *Clin Cancer Res.* 2013; 19:6322–8. [PubMed: 24077350]
9. Hong D, Infante J, Janku F, Jones S, Nguyen LM, Burris H, et al. Phase I study of LY2606368, a checkpoint kinase 1 inhibitor, in patients with advanced cancer. *J Clin Oncol.* 2016; 34:1764–71. [PubMed: 27044938]
10. Lok BH, Gardner EE, Schneeberger VE, Ni A, Desmeules P, Rekhtman N, et al. PARP inhibitor activity correlates with SLFN11 expression and demonstrates synergy with temozolomide in small cell lung cancer. *Clin Cancer Res.* 2017; 23:523–35. [PubMed: 27440269]
11. George J, Lim JS, Jang SJ, Cun Y, Ozretic L, Kong G, et al. Comprehensive genomic profiles of small cell lung cancer. *Nature.* 2015; 524:47–53. [PubMed: 26168399]
12. Peifer M, Fernandez-Cuesta L, Sos ML, George J, Seidel D, Kasper LH, et al. Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. *Nat Genet.* 2012; 44:1104–10. [PubMed: 22941188]
13. Rudin CM, Durinck S, Stawiski EW, Poirier JT, Modrusan Z, Shames DS, et al. Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. *Nat Genet.* 2012; 44:1111–6. [PubMed: 22941189]
14. Sato M, Shames DS, Gazdar AF, Minna JD. A translational view of the molecular pathogenesis of lung cancer. *J Thorac Oncol.* 2007; 2:327–43. [PubMed: 17409807]
15. McGowan CH, Russell P. Cell cycle regulation of human WEE1. *EMBO J.* 1995; 14:2166–75. [PubMed: 7774574]
16. Watanabe N, Broome M, Hunter T. Regulation of the human WEE1Hu CDK tyrosine 15-kinase during the cell cycle. *EMBO J.* 1995; 14:1878–91. [PubMed: 7743995]
17. Lindqvist A, Rodriguez-Bravo V, Medema RH. The decision to enter mitosis: feedback and redundancy in the mitotic entry network. *J Cell Biol.* 2009; 185:193–202. [PubMed: 19364923]
18. Matheson CJ, Backos DS, Reigan P. Targeting WEE1 kinase in cancer. *Trends Pharmacol Sci.* 2016; 37:872–81. [PubMed: 27427153]
19. De Witt Hamer PC, Mir SE, Noske D, Van Noorden CJ, Wurdinger T. WEE1 kinase targeting combined with DNA-damaging cancer therapy catalyzes mitotic catastrophe. *Clin Cancer Res.* 2011; 17:4200–7. [PubMed: 21562035]
20. Hirai H, Arai T, Okada M, Nishibata T, Kobayashi M, Sakai N, et al. MK-1775, a small molecule Wee1 inhibitor, enhances anti-tumor efficacy of various DNA-damaging agents, including 5-fluorouracil. *Cancer Biol Ther.* 2010; 9:514–22. [PubMed: 20107315]
21. Indovina P, Marcelli E, Di Marzo D, Casini N, Forte IM, Giorgi F, et al. Abrogating G(2)/M checkpoint through WEE1 inhibition in combination with chemotherapy as a promising therapeutic approach for mesothelioma. *Cancer Biol Ther.* 2014; 15:380–8. [PubMed: 24365782]
22. Matheson CJ, Venkataraman S, Amani V, Harris PS, Backos DS, Donson AM, et al. A WEE1 inhibitor analog of AZD1775 maintains synergy with cisplatin and demonstrates reduced single-agent cytotoxicity in medulloblastoma cells. *ACS Chem Biol.* 2016; 11:921–30. [PubMed: 26745241]
23. Guertin AD, Li J, Liu Y, Hurd MS, Schuller AG, Long B, et al. Preclinical evaluation of the WEE1 inhibitor MK-1775 as single-agent anticancer therapy. *Mol Cancer Ther.* 2013; 12:1442–52. [PubMed: 23699655]
24. Krehling JM, Gemmer JY, Reed D, Letson D, Bui M, Altiock S. MK1775, a selective Wee1 inhibitor, shows single-agent antitumor activity against sarcoma cells. *Mol Cancer Ther.* 2012; 11:174–82. [PubMed: 22084170]

25. Bauer TM, Jones SF, Greenlees C, Cook C, Mugundu GM, Jewsbury PJ, et al. Abstract CT013: A phase Ib, open-label, multicenter study to assess the safety, tolerability, pharmacokinetics, and antitumor activity of AZD1775 monotherapy in patients with advanced solid tumors: initial findings. *Cancer Res.* 2016; 76(14 Suppl):CT013.
26. Masters JR, Thomson JA, Daly-Burns B, Reid YA, Dirks WG, Packer P, et al. Short tandem repeat profiling provides an international reference standard for human cell lines. *Proc Natl Acad Sci.* 2001; 98:8012–7. [PubMed: 11416159]
27. Tong P, Coombes KR, Johnson FM, Byers LA, Diao L, Liu DD, et al. drexplorer: A tool to explore dose-response relationships and drug-drug interactions. *Bioinformatics.* 2015; 31:1692–4. [PubMed: 25600946]
28. R Development Core Team. R: a language and environment for statistical computing. Vienna, Austria: The R Foundation for Statistical Computing; 2011.
29. Do K, Wilsker D, Ji J, Zlott J, Freshwater T, Kinders RJ, et al. Phase I study of single-agent AZD1775 (MK-1775), a Wee1 kinase inhibitor, in patients with refractory solid tumors. *J Clin Oncol.* 2015; 33:3409–15. [PubMed: 25964244]
30. Leijen S, van Geel RM, Pavlick AC, Tibes R, Rosen L, Razak AR, et al. Phase I study evaluating WEE1 inhibitor AZD1775 as monotherapy and in combination with gemcitabine, cisplatin, or carboplatin in patients with advanced solid tumors. *J Clin Oncol.* 2016; 34:4371–80. [PubMed: 27601554]
31. Bower M, Newlands ES, Bleeher NM, Brada M, Begent RJ, Calvert H, et al. Multicentre CRC phase II trial of temozolomide in recurrent or progressive high-grade glioma. *Cancer Chemother Pharmacol.* 1997; 40:484–8. [PubMed: 9332462]
32. Gajewski TF. Temozolomide for melanoma: new toxicities and new opportunities. *J Clin Oncol.* 2004; 22:580–1. [PubMed: 14726506]
33. Gay CM, de Groot PM, Catherine Pietanza M, Byers LA. Durable, exceptional response to temozolomide in a patient with extensive-stage small cell lung cancer (ES-SCLC) metastatic to brain. *Cancer Treat Res Commun.* 2017; 10:17–20.
34. Pietanza MC, Kadota K, Huberman K, Sima CS, Fiore JJ, Sumner DK, et al. Phase II trial of temozolomide in patients with relapsed sensitive or refractory small cell lung cancer, with assessment of methylguanine-DNA methyltransferase as a potential biomarker. *Clin Cancer Res.* 2012; 18:1138–45. [PubMed: 22228633]
35. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* 2005; 352:997–1003. [PubMed: 15758010]
36. Gay CM, Balaji K, Byers LA. Giving AXL the Axe: targeting AXL in human malignancy. *Br J Cancer.* 2017; 116:415–23. [PubMed: 28072762]
37. Stewart CA, Tong P, Cardnell R, Sen T, Masrourpour FM, Fan Y, et al. Abstract 3870: SLFN11 is a biomarker of sensitivity to PARP inhibition and chemotherapy in small cell lung cancer (SCLC). *Cancer Res.* 2016; 76(14 Suppl):3870.
38. Axelrod H, Pienta KJ. Axl as a mediator of cellular growth and survival. *Oncotarget.* 2014; 5:8818–52. [PubMed: 25344858]
39. Korshunov VA. Axl-dependent signalling: a clinical update. *Clin Sci.* 2012; 122:361–8. [PubMed: 22187964]
40. Dent P. Crosstalk between ERK, AKT, and cell survival. *Cancer Biol Ther.* 2014; 15:245–6. [PubMed: 24424114]
41. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci.* 2011; 36:320–8. [PubMed: 21531565]
42. Li P, Goto H, Kasahara K, Matsuyama M, Wang Z, Yatabe Y, et al. P90 RSK arranges Chk1 in the nucleus for monitoring of genomic integrity during cell proliferation. *Mol Biol Cell.* 2012; 23:1582–92. [PubMed: 22357623]
43. Balaji K, Vijayaraghavan S, Diao L, Tong P, Fan Y, Carey JP, et al. AXL inhibition suppresses the DNA damage response and sensitizes cells to PARP inhibition in multiple cancers. *Mol Cancer Res.* 2017; 15:45–58. [PubMed: 27671334]

44. Weisberg E, Nonami A, Chen Z, Liu F, Zhang J, Sattler M, et al. Identification of Wee1 as a novel therapeutic target for mutant RAS-driven acute leukemia and other malignancies. *Leukemia*. 2015; 29:27–37. [PubMed: 24791855]
45. Ray-David H, Romeo Y, Lavoie G, Deleris P, Tcherkezian J, Galan JA, et al. RSK promotes G2 DNA damage checkpoint silencing and participates in melanoma chemoresistance. *Oncogene*. 2013; 32:4480–9. [PubMed: 23108403]
46. De Bacco F, D'Ambrosio A, Casanova E, Orzan F, Neggia R, Albano R, et al. MET inhibition overcomes radiation resistance of glioblastoma stem-like cells. *EMBO Mol Med*. 2016; 8:550–68. [PubMed: 27138567]
47. Kim SM, Kim H, Yun MR, Kang HN, Pyo KH, Park HJ, et al. Activation of the Met kinase confers acquired drug resistance in FGFR-targeted lung cancer therapy. *Oncogenesis*. 2016; 5:e241. [PubMed: 27429073]
48. Zhou L, Liu XD, Sun M, Zhang X, German P, Bai S, et al. Targeting MET and AXL overcomes resistance to sunitinib therapy in renal cell carcinoma. *Oncogene*. 2016; 35:2687–97. [PubMed: 26364599]
49. Gelsomino F, Rossi G, Tiseo M. MET and small-cell lung cancer. *Cancers*. 2014; 6:2100–15. [PubMed: 25314153]
50. Medova M, Aebersold DM, Blank-Liss W, Streit B, Medo M, Aebi S, et al. MET inhibition results in DNA breaks and synergistically sensitizes tumor cells to DNA-damaging agents potentially by breaching a damage-induced checkpoint arrest. *Genes Cancer*. 2010; 1:1053–62. [PubMed: 21779429]

Translational Relevance

WEE1 inhibitor, AZD1775, recently entered phase I clinical trial for advanced solid tumors, including small-cell lung cancer (SCLC). Initial results indicate that AZD1775 has promising activity in a subset of patients, but the development of predictive biomarkers for patient selection and combinations that address resistance mechanisms will be important to maximize its clinical impact. In this study, we investigated for the first time the molecular mechanism of primary and acquired resistance to WEE1 targeting and identify novel combinations that overcome both primary and acquired resistance in SCLC models. This is the first demonstration of AXL playing a role in DNA damage response (DDR) inhibitor resistance. AXL expression in SCLC patients under AZD1775 clinical trial has not been reported and is currently not used for patient selection. Our study indicates that in SCLC, AXL expression could serve as a candidate biomarker to select patients for WEE1 inhibitor trials.

Figure 1.

WEE1 is overexpressed in SCLC, and genetic knockdown of *WEE1* induces DNA damage and causes apoptosis in SCLC cell lines. **A**, RNA sequencing analysis showing the gene expression profile of *WEE1* in 68 SCLC samples and 26 normal lung tissue samples. SCLC samples had significantly higher ($P < 0.0001$; ANOVA) *WEE1* gene expression levels than normal lung tissue, with a fold change of 4.23 (left). RPPA analysis showing protein expression of *WEE1* in 63 SCLC and 114 NSCLC cell lines. SCLC cells had 1.66-fold higher *WEE1* expression than NSCLC cells ($P < 0.0001$; right). See also Supplementary Fig. S1A. **B**, Quantitative reverse transcriptase PCR analysis showing the efficiency of siRNA-mediated knockdown of *WEE1* in four human SCLC cell lines: H82, H1836, H526, and H865. Parental cells (no siRNA; Con) and scramble shRNA cells (SCR) were used as controls in each case. *GAPDH* was used as the reference in this analysis. **C**, Western blot

analysis confirming the knockdown efficiency of *WEE1*, expression of phosphorylated γ H2AX, and phosphorylated histone H3 (PH3) levels in total protein lysates from the parental, scramble, and *WEE1*-knockdown (KD) cell lines H82, H1836, H526, and H865, 24 hours after transfection. Actin was used as the loading control. See also Supplementary Fig. S1B and S1C. **D**, Annexin V–propidium iodide–based flow cytometry 48 hours after transfection showing significantly increased apoptosis in the *WEE1* KD cell lines H82, H1836, and H526. There was no significant induction of apoptosis in *WEE1* KD H865 cells. **E**, Cell viability in response to treatment with AZD1775 in a panel of nine human SCLC cell lines, demonstrating that SCLC *in vitro* models have a range of sensitivity to single-agent AZD1775; about 30% of the cell lines were resistant to the drug (red box; $IC_{50} > 100$ nmol/L). The genetic profile information for each cell line is presented in Supplementary Table S1. **F**, Single-agent treatment with AZD1775 (100 nmol/L) for 24 hours significantly increased apoptosis in H82 and H526 but did not increase apoptosis in H865 and H1417 cells. **G**, Flow cytometry–based cell-cycle analysis demonstrated that treatment with AZD1775 (100 nmol/L) for 48 hours caused sub- G_1 accumulation and increased G_2 –M arrest in cells showing initial sensitivity to single-agent AZD1775 (H82). However, there was no significant change between the control and AZD1775-treated cell-cycle signatures in cells showing *de novo* resistance to *WEE1* targeting (H865). See also Supplementary Fig. S1D. In all panels, data are derived from three independent experiments conducted in triplicate (error bars indicate SEM). The *P* values were calculated using the Student *t* test. **, $P < 0.01$; ***, $P < 0.001$.

Figure 2. WEE1 inhibition is synergistic with temozolomide (TMZ) independent of MGMT expression or initial sensitivity to WEE1 targeting in SCLC cell lines. **A**, Dose–response curve of human SCLC cell lines ($n = 10$) treated with temozolomide (red), AZD1775 (green), and AZD1775+ temozolomide (black), from a 5-day CellTiter Glo assay. Doses started at 3.1 $\mu\text{mol/L}$ and were serially diluted at 3-fold dilutions for five dose ranges. See also Supplementary Fig. S2A. **B**, Difference in area under the curve (AUC) analysis demonstrating the degree of synergy of AZD1775+ temozolomide in human SCLC cell lines ($n = 10$). A negative value of AUC suggests a more than additive effect (synergistic) and a positive value of AUC suggest a less than additive effect (antagonistic). The bars are color-coded according the expression of *MGMT* in these cell lines, demonstrating that the combination is synergistic in all cell lines tested irrespective of *MGMT* expression. **C**, Two *de novo* AZD1775-sensitive cell lines (H82 and H1836) and two *de novo* AZD1775-resistant cell lines (H865 and H1930) were treated with single-agent AZD1775 (100 nmol/L) or siRNA *WEE1* knockdown (KD) alone or combined with temozolomide (1 $\mu\text{mol/L}$) for 24 hours and apoptosis was assessed by Annexin–propidium iodide FACS assay. Data are derived from three independent experiments conducted in triplicate (error bars).

indicate SEM). The *P* values were calculated using the Student *t* test: **, $P < 0.01$; ***, $P < 0.001$. See also Supplementary Fig. S2B and S2C. **D**, Scramble (SCR), H82, H865, and H1836 cells were treated with AZD1775 (100 nmol/L) and temozolomide (1 μ mol/L) for 24 hours; *WEE1* KD cells were treated with just temozolomide (1 μ mol/L) for 24 hours. The cells were lysed at 24 hours and subjected to Western blot analysis for phosphorylated WEE1 (pWEE1_S642), pCDC2_Y15 (WEE1 downstream effector), γ H2AX (DNA damage marker), PH3 (mitotic marker), cleaved caspase-3 (apoptotic marker), and actin (loading control). **E**, SCR and *WEE1* KD H82, H865, and H1836 cells were treated as described in **D** and subjected to Western blot analysis for DNA repair markers pATM_S1981, MRE11, RAD51, and E2F1. Actin was used as a loading control.

Figure 3.

Increased basal expression of AXL and activated mTOR pathway is associated with primary AZD1775 resistance. **A**, Spearman correlation of differential expression of 195 reverse-phase protein array (RPPA) markers and sensitivity [half-maximal inhibitory concentration (IC_{50})] to AZD1775. The top markers of sensitivity are marked with arrows and color-coded according to the P values. **B**, Heatmap of RPPA markers significantly correlated with AZD1775 response in human SCLC cell lines ($P < 0.05$). In the top index, RPPA marker expression is denoted by red (high) and blue (low); cell lines with relatively low AZD1775

IC₅₀ values are marked in green and those with relatively high IC₅₀ values are marked in red. **C**, Box plot of AXL and pS6_S240/244 protein expression correlated with dichotomized IC₅₀ of AZD1775, as determined by *t* test. See also Supplementary Fig. S3A. **D**, Three AZD1775-sensitive (IC₅₀ < 30 nmol/L) cell lines (H1836, H82, and H1048) and three AZD1775-resistant (IC₅₀ > 100 nmol/L) cell lines (H1417, H865, and H1930) were treated with AZD1775 (100 nmol/L) for 24 hours and assessed for AXL expression by Western blot analysis. **E**, Bimodality of *AXL* expression in SCLC patient samples [*n* = 110; bimodality index (BI) = 1.27]. **F**, Scatter plot showing the correlation of *AXL* with WEE1 expression in SCLC cell lines (*n* = 69). The *P* value was calculated by Spearman correlation. **G**, Two AZD1775-sensitive H82 and H1836) and two AZD1775-resistant (H865 and H1930) cell lines treated with AZD1775 (100 nmol/L) for 24 hours were subjected to Western blot analysis for pWEE1_S642, total and phosphorylated pCDC2_Y15, total and phosphorylated pAKT_S473, total and phosphorylated pS6_S240/244, and actin as a loading control. See also Supplementary Fig. S3B and S3C.

Figure 4.

Cotargeting AXL and mTOR overcomes *de novo* AZD1775 resistance in SCLC *in vitro* and *in vivo*. **A**, Viability (5-day assay) of AZD1775-resistant cell lines (H865, H1930, and H1417) treated with different concentrations of AZD1775 with or without the AXL inhibitor TP0903 (10 nmol/L, 20 nmol/L, 40 nmol/L, and 80 nmol/L). Data are derived from three independent experiments conducted in triplicate (error bars indicate SEM). The *P* values were calculated using the Student *t* test: *, *P* < 0.01; ***, *P* < 0.001. **B**, AZD1775-resistant cells (H865, H1930, and H1417) were treated with AZD1775 (100 nmol/L), TP0903 (20

nmol/L), or both for 24 hours and apoptosis was measured by Annexin-Propidium Iodide-based FACS analysis. Data are derived from three independent experiments conducted in triplicate (error bars, SEM). The *P* values were calculated using the Student *t* test:***, *P* < 0.001. **C**, Viability (in a 5-day assay) of control (con), scramble (SCR), and *AXL* knockdown (*AXL* KD) cells after treatment with single-agent AZD1775. The *P* values were calculated using the Student *t* test:***, *P* < 0.001. See also Supplementary Fig. S4A. **D**, H865, H1930, and H1417 cells were treated with AZD1775 (100 nmol/L) and/or TP0903 (20 nmol/L) for 24 hours and then subjected to Western blot analysis for expression of pAXL_Y702, AKT, pAKT_S473, S6, S6_S240/244, and actin (as a loading control). **E**, Viability (5-day assay) of AZD1775-resistant cell lines (H865, H1930, and H1417) treated with different concentrations of AZD1775 with or without the mTOR inhibitor RAD001 (0.03 μmol/L, 0.1 μmol/L, 0.3 μmol/L, and 1 μmol/L). Data are derived from three independent experiments conducted in triplicate (error bars indicate SEM). The *P* values were calculated using the Student *t* test:***, *P* < 0.001. **F**, AZD1775-resistant cells (H865, H1930, and H1417) were treated with AZD1775 (100 nmol/L), RAD001 (100 nmol/L), or the combination for 24 hours and apoptosis was measured by Annexin-Propidium iodide-based FACS analysis. Data are derived from three independent experiments conducted in triplicate (error bars, SEM). The *P* values were calculated using the Student *t* test:***, *P* < 0.001. **G**, Viability (in a 5-day assay) of control, SCR, and WEE1 KD cells after treatment with single-agent RAD001. The *P* values were calculated using the Student *t* test:***, *P* < 0.001. See also Supplementary Fig. S4B. **H**, Cells were treated as described in **B** and then subjected to Western blot analysis for expression of p-mTOR_S2448, pWEE1_S642, AKT, pAKT_S473, S6, S6_S240/244, and actin (loading control). **I**, Individual tumor volume changes in H865 xenograft mice treated with vehicle, AZD1775 (60 mg/kg 5/7, every day, orally), TP0903 (50 mg/kg, 5/7, every day, orally), or combination (*n* = 10 per group). Significant differences between the combination and AZD1775 alone are displayed. The *P* values were calculated using the Student *t* test. **J**, Survival of mice treated with vehicle, AZD1775, TP0903, or the combination (*n* = 10 per group). The *P* value was established by the Mantel-Cox test. **K**, Individual tumor volume changes in H865 xenograft mice treated with vehicle, AZD1775 (60 mg/kg, 5/7, every day, orally), RAD001 (5 mg/kg, 2/7, every day, orally) or combination (*n* = 10 per group). Significant differences between the combination and AZD1775 alone are displayed. The *P* values were calculated using the Student *t* test. **L**, Survival of mice treated with vehicle, AZD1775, RAD001, or combination (*n* = 10 per group). The *P* value was established by the Mantel-Cox test:***, *P* < 0.001. See also Supplementary Fig. S5.

Figure 5.

AXL/mTOR signaling activates other DNA repair proteins via the ERK/p90RSK pathway.

A, RPPA analysis corresponding to the indicated mTOR pathway, ERK/p90RSK, and DNA repair markers in AZD1775-resistant SCLC cells, which were treated with vehicle (DMSO), 100 nmol/L AZD1775, 20 nmol/L TP0903, or the combination for 24 hours in triplicate (false discovery rate < 0.05). See also Supplementary Fig. S6. **B**, AZD1775-resistant cells (H865, H1930, and H1417) were treated with AZD1775 (100 nmol/L), RAD001 (100 nmol/L), or the combination for 24 hours and then subjected to Western blot analysis for expression of total ERK1/2, pERK1/2_T202/204, S6_S235/236 total p90RSK, phospho-p90RSK_T359/S363, and actin (loading control). **C**, H865 and H1930 cells were treated with AZD1775 (100 nmol/L) and TP0903 (20 nmol/L) for 24 hours and then the nuclear and cytoplasmic lysates were subjected to Western blot analysis for expression of pCHK1_S280, actin (cytoplasmic loading control), and PCNA (nuclear loading control). **D**, AZD1775-resistant cells (H865, H1930, and H1417) were treated with AZD1775 (100 nmol/L), LY2606368 (30 nmol/L), or the combination for 24 hours and apoptosis was measured by Annexin-propidium iodide-based FACS analysis. Data are derived from three independent experiments conducted in triplicate (error bars indicate SEM). The P values were calculated using the Student t test: ***, $P < 0.001$.

Figure 6.

Acquired AZD1775 resistance in SCLC models can be reversed. **A**, Heatmap and **B**, boxplots of the comparative RPPA analysis of AZD1775-sensitive (parental) cells and cells with acquired resistance to AZD1775 (AZD-Res) for H524, H1048, and H1836 SCLC cell lines. Overexpression of AXL, MET, and S6_S240/244 and abrogation of WEE1 expression was observed in the resistant models. FC indicates fold change. **C–E**, Cell viability assay of H524 AZD-Res, H1048 AZD-Res, and H1836 AZD-Res treated with single-agent AZD1775 (red lines), single-agent (green lines) AXL inhibitor TP0903 (**C**), single-agent

mTOR inhibitor RAD001 (**D**), single-agent dual AXL/MET inhibitor cabozantinib (**E**) and combinations (black lines). The combinations showed superior effects compared with single agents alone. **F**, Schematic summarizing the proposed mechanism by which AXL drives primary and acquired resistance to AZD1775 in SCLC and possible treatment combinations to overcome this resistance. Increased basal expression of AXL and sustained activation of the mTOR pathway leads to activation of the ERK/p90RSK pathway and finally to recruitment of a parallel DNA repair mechanism by CHK1 activation. The combination of AZD1775 inhibition with AXL, mTOR, or CHK1 targeting would abrogate this resistance pathway and result in enhanced efficacy of AZD1775 in SCLC models. AXL/MET and mTOR is overexpressed in cells with acquired resistance, and targeting AXL and MET with a dual inhibitor shows enhanced response in these models. See also Supplementary Fig. S7.