Synergistic Inhibition of ErbB Signaling by Combined Treatment with Seliciclib and ErbB-Targeting Agents

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Abstract

Purpose: The aims of this study were to investigate whether the cyclin-dependent kinase inhibitor seliciclib could synergize with agents that target ErbB receptors and to elucidate the molecular mechanism of the observed synergy.

Experimental Design: Synergy between seliciclib and ErbB receptor targeted agents was investigated in various cell lines using the CalcuSyn median effect model. The molecular mechanism of the observed synergy was studied in cultured cells, and the combination of seliciclib and the epidermal growth factor receptor (EGFR) inhibitor erlotinib was evaluated in an H358 xenograft model.

Results: Seliciclib synergized with the anti-HER2 antibody trastuzumab in a breast cancer cell line, which overexpresses the HER2 receptor, and with the erlotinib analogue AG1478 in non–small cell lung cancer cell lines. In the H358 non–small cell lung cancer cell line, synergy involved decreased signaling from the EGFR, with AG1478 directly inhibiting kinase activity while seliciclib decreased the levels of key components of the receptor signaling pathway, resulting in enhanced loss of phosphorylated extracellular signal-regulated kinase and cyclin D1. The combination of seliciclib and erlotinib was evaluated further in an H358 xenograft and shown to be significantly more active than either agent alone. An enhanced loss of cyclin D1 was also seen in vivo.

Conclusions: This is the first report that investigates combining seliciclib with an EGFR inhibitor. The combination decreased signaling from the EGFR in vitro and in vivo and was effective in cell lines containing either wild-type or mutant EGFR, suggesting that it may expand the range of tumors that respond to erlotinib, and therefore, such combinations are worth exploring in the clinic.

Protein kinases are key regulators of essential cellular processes, many of which are involved in the pathogenesis of cancer. This important role in tumorigenesis, together with the fact that kinases contain a distinct active site, which is amenable for targeting with small molecules, has made kinases an attractive area for drug discovery (1). However, it was not until the successful clinical development of imatinib, a small molecule inhibitor of BCR-ABL, that the first targeted protein kinase inhibitor became an established cancer treatment (2).

Members of the ErbB family, like many other growth factor receptors, dimerize upon ligand stimulation and undergo autophosphorylation through their cytoplasmic tyrosine kinase domains. Once activated, the ErbB kinases transduce their signals via a number of cellular protein kinases, including extracellular signal-regulated kinase (ERK) and protein kinase B, to ultimately result in up-regulation of cyclin D1 levels leading to activation of cyclin-dependent kinases (CDK) and the initiation of cellular proliferation (3). Overstimulation of ErbB receptor tyrosine kinase signaling has been documented in a number of human cancers, including overexpression of HER2 (ErbB2) in up to 30% of breast cancer patients (4), whereas epidermal growth factor receptor (EGFR, ErbB1) is overexpressed in ovarian carcinomas (35-60%), head and neck tumors (70-100%), and non–small cell lung cancer (NSCLC; 50-90%; refs. 5, 6). Two main approaches have been pursued for the development of drugs that target the ErbB family. Humanized monoclonal antibodies, such as trastuzumab and cetuximab, bind to the HER2 and EGFRs, respectively, thereby blocking receptor dimerization/activation and facilitating removal of these proteins from the cell surface (3). In contrast, erlotinib and gefitinib are small molecules that bind directly to the EGFR ATP-binding active site, blocking tyrosine kinase activity (7). For each of these agents, the subsequent loss of mitogenic signaling results in the cessation of cellular proliferation.

The CDKs are a second kinase family that have attracted a considerable amount of interest from a drug discovery perspective; with the three most advanced compounds, seliciclib (CYC202, R-roscovitine), alvocidib (flavopiridol), and SNS-032 (formerly BMS-387032), all currently in phase II clinical development (8, 9). Through their key role of phosphorylating proteins involved in the regulation of cell cycle checkpoints, CDKs control the orderly progression of the cell division cycle (8). In cancer cells, activation of CDKs,
mainly by overexpression of their cognate partners, the cyclins, or loss of the endogenous inhibitors, such as p16, leads to inappropriate proliferation of cells containing genetic alterations, cells which would normally be arrested and either repaired or induced to undergo apoptosis at specific cell cycle checkpoints (10). In addition to controlling the cell cycle, some CDKs, such as CDK7 and CDK9, regulate transcription by phosphorylating the carboxy-terminal domain of RNA polymerase II. Seliciclib, alvocidib, and SNS-032 all inhibit CDK7 and/or CDK9, thereby leading to inhibition of transcription and down-regulation of proteins, such as Mcl-1 and cyclin D1 (11, 12), which have short half-lives (13, 14).

Inhibitors of both kinase families have shown some clinical activity, erlotinib in NSCLC (15), trastuzumab in breast cancer (16, 17), and alvocidib in B-cell chronic lymphocytic leukemia (18). However, most agents will be used in the clinic ultimately in combination with other anticancer drugs. It was recently shown that the combination of trastuzumab and alvocidib was synergistically cytotoxic in HER2-overexpressing breast cancer cell lines (19–21). To initially ascertain whether seliciclib could also be combined synergistically with trastuzumab, the combination was evaluated in the SKBr3 breast cancer cell line that overexpresses the HER2 receptor. These studies were then extended to determine whether seliciclib could act synergistically in combination with agents, such as AG1478 and erlotinib, which target the EGFR, a second member of the ErbB family. The results from these studies showed that seliciclib synergized with trastuzumab in SKBr3 cells and with AG1478 in the NSCLC cell lines H358, H292, and H1650, which express wild-type or mutant EGFR. In each case, synergy involved inhibition of the ErbB signaling pathway, which could be shown both in vitro and in vivo in an NSCLC xenograft model.

After drug treatment, the number of viable cells in each well was estimated by incubating in media containing 10% alamar blue (Roche) and measuring the absorbance at 488 to 595 nm. Drug interactions were analyzed using the CalcuSyn software package (BioSoft), which is based on the median effect model of Chou and Talalay (22). A combination index (CI) of 1 indicated an additive drug interaction, whereas a CI of >1 was antagonistic and a score lower than 1 was synergistic.

**Western blot analysis.** Cells were seeded at $\approx 8 \times 10^3$ per plate on 10 cm plates and left to settle overnight. Compounds were added to the plates, and cells were incubated for the indicated times. Media were removed from each of the wells and centrifuged at 1,000 $\times$ g for 5 min to pellet any cells that had become detached from the plate surface. Cells that remained attached to the plate were washed once with ice-cold buffer A [50 mmol/L HEPES (pH 7.0), containing 20 mmol/L NaCl, 1 mmol/L DTT, and protease inhibitor cocktail (Merck)] and then scraped into 0.35 mL buffer B (buffer A containing 10 mmol/L sodium PPI, 10 mmol/L sodium fluoride, and 1 mmol/L sodium orthovanadate). The resuspended cells were then pooled with the appropriate media cell pellet and lysed by sonication (2 × 3 bursts using Sanyo Soniprep 150 at 5 A). The protein concentration of each lysate was determined using the bicinchoninic acid assay (Perbio Science). Lysates (20–30 $\mu$g protein/well) were resolved on 3% to 8% acrylamide Tris-acetate gels or 10% acrylamide Bis-Tris gels (Invitrogen), and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) using the Invitrogen wet transfer system. Membranes were blocked for 1 h at room temperature in PBS containing 0.02% (v/v) Tween 20 (PBST) and 5% (w/v) fat-free dried milk. Primary antibody incubations were carried out overnight at 2°C in PBST containing 3% (v/v) dried milk, using the following primary antibodies: HER2 and EGFR (Calbiochem), phosphorylated Tyr1068 EGFR (New England Biolabs), cyclin D1 (Lab Vision), ERK2, and phosphorylated ERK1/ERK2 (Abcam), phosphorylated Ser2473 protein kinase B (Upstate), Asp-214 cleaved poly(ADP)-ribose polymerase (BD Pharmingen), Mcl-1 (Santa Cruz), CTD RNA polymerase II phosphorylated Ser7 (Covance), and $\beta$-actin. Membranes were washed thrice in PBST and then incubated for 1 h with the appropriate horseradish peroxidase–conjugated secondary antibody (Perbio) at 1:5,000 dilution in PBST containing 3% (w/v) milk. Membranes were washed thrice in PBST before development using an enhanced chemiluminescence kit (Amersham Corporation). Bands corresponding to each protein were scanned, and the band intensities were measured using NIH image.1

**Flow cytometry.** H358, H292, or H1650 cells were seeded in 10 cm plates at $\approx 5 \times 10^5$ per plate and left to settle overnight. Cells were treated with 1 × IC50 seliciclib, AG1478, or both drugs together. After 72 h treatment, cells were harvested by trypsinization, washed twice in PBS, and then fixed overnight in 70% (v/v) ethanol at -20°C. Cells were washed twice in PBS containing 1% (w/v) bovine serum albumin and then incubated with 50 $\mu$g/mL propidium iodoide and 50 $\mu$g/mL RNase A for 20 min at room temperature. Cells were analyzed for DNA content by flow cytometry using the CellQuest program on a Becton Dickinson LSR flow cytometer.

**In vivo studies.** All in vivo experiments were done at Institute for Drug Development. Female (nu/nu) mice were injected s.c. with $\approx 1 \times 10^7$ H358 cells per mouse at a single site on their flanks. Tumors were allowed to grow to $\approx 110 \text{mm}^3$ before being pair-matched by tumor size into treatment groups (nine mice per group). One group was treated with seliciclib (50 mg/kg) as a twice daily i.p. injection for 5 consecutive days followed by a 2 day break; the treatment was then repeated for a total of four cycles. Erlotinib (100 mg/kg) was given daily by oral gavage for 28 consecutive days. The group treated with the combination was dosed in the same manner as both single-agent groups. Mice were weighed at least twice a week to assess toxicity of the

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1 http://rsb.info.nih.gov/ij/
treatments, and the tumors were measured with calipers at least twice a week to determine tumor growth. During the first week of treatment, there was some weight loss in the animals. However, this seemed to be associated with the initial vehicle used for seliciclib (50 mmol/L HCl), as the weight loss also occurred in the vehicle control group (max weight loss, 11% between days 8 and 11). On day 10, the vehicle was changed to 10% cremophor EL, 10% ethanol, and 80% saline for the control and the two groups receiving seliciclib; all three groups started gaining weight immediately.

Tumormeasurementswereconvertedintovolumesusingtheformula: tumor volume (mm3) = width2 (mm) / length (mm)0.5. The percentage of tumor growth inhibition was determined with the formula: 1 - (mean change in treated tumor volume / mean change in control tumor volume) / 100. For each group, statistical significance was determined by comparing to the control group using a one-way ANOVA followed by a Dunnett’s test. Significance between different treatment groups was determined using a two-sided unpaired Student’s t test.

Immunohistochemical studies. Female (nu/nu) mice were injected s.c. with −1 × 10^7 H358 cells per mouse at a single site on their flanks. Tumors were allowed to grow to −110 mm^3 before being pair-matched by tumor size into treatment groups (two mice per group). Treatment was for 5 consecutive days; erlotinib (100 mg/kg) was given daily by oral gavage, whereas seliciclib (100 mg/kg) was given as a twice daily i.p. injection. On day 5, the mice were sacrificed and the tumors removed, fixed overnight in formalin, and then preserved in paraffin blocks.

Slices (4 μm) were cut from the paraffin blocks; wax was removed from the tissue sections by washing twice for 10 min in Histoclear (National Diagnostics). The sections were then rehydrated in 95% (v/v) alcohol, 70% (v/v) alcohol, and PBS twice for 5 min in each solution. Endogenous peroxidase activity was blocked by treating the sections with 0.1% (v/v) hydrogen peroxide in PBS for 20 min, followed by two 5 min washes in PBS. The sections were boiled for 10 min in 10 mmol/L citrate buffer (pH 6.0) containing 0.05% (v/v) Tween 20. The slides were allowed to cool for 10 min and then washed twice in PBS. Immunohistochemistry staining of the sections was done using the Vectastain avidin-biotin complex method kit (Vector Laboratories) following the manufacturer’s instructions. The primary antibody was raised against cyclin D1 (SP4, Abcam) and was used at a 1:50 dilution. On completion of the immunostaining process, sections were washed for 5 min in water, counterstained for 30 s with Mayer’s hematoxylin, and washed for 10 min in water. Sections were then dehydrated through 70% (v/v) alcohol and 95% (v/v) alcohol, twice for 5 min in each solution and Histoclear twice for 10 min. Finally, sections were mounted with a drop of DPX resin (BDH) and a coverslip. Slides were viewed using a Nikon eclipse E800 light microscope, and images were taken with a DS-Fi1 Nikon color digital camera. Axiovision viewer was used to prepare images of the sections.

Results and Discussion

The aim of this series of experiments was to determine whether agents that target either CDKs or ErbB receptors can be combined synergistically. Recent experiments have shown that the anti-HER2 antibody trastuzumab synergizes with the CDK9 inhibitor alvocidib (19–21). The data presented herein extend these findings by exploring the synergy between seliciclib, an inhibitor of CDK2, CDK7, and CDK9, and either trastuzumab or the EGFR kinase inhibitors, AG1478 and erlotinib. The

Fig. 1. Seliciclib plus trastuzumab down-regulates HER2 levels in SkBr3 cells. A, SkBr3 cells were treated for 72 h using increasing concentrations of seliciclib and trastuzumab, either alone or in a fixed ratio of 1,400:1, as described in Materials and Methods. The data were analyzed using the CalcuSyn program, and a graph from a representative experiment is shown. CI values are defined in the legend for Table 1. SkBr3 cells were incubated with the indicated concentration of trastuzumab (B), seliciclib (C), or seliciclib + trastuzumab (D) for 24 h before harvesting. Protein lysates (30 μg) were resolved on 3% to 8% acrylamide Tris-acetate gels or 10% acrylamide Bis-Tris gels, transferred to nitrocellulose membranes, and probed with the antibodies shown. Results are representative of two independent experiments.
reported for alvocidib (20, 24). Down-regulation of the HER2 does not down-regulate HER2 levels in SkBr3 cells (23). In consistent with previous findings demonstrating that this drug Trastuzumab treatment had no effect on HER2 levels at levels of HER2 and cyclin D1 proteins were determined. Both agents for 24 hours, cell lysates were prepared, and the HER2 signaling pathway was done. SkBr3 cells expressing HER2 (19–21).

consistent with earlier observations, which showed that additive in MCF7 cells (CI = 0.96). These findings are 50%). In contrast, the inhibition of cell growth was only (Fig. 1A and Table 1), generating a CalcuSyn CI of 0.66 at ED 50 values of 16.3 and 10.0 mol/L in SkBr3 and MCF7 cells, respectively. Co-incubation of SkBr3 cells with seliciclib and trastuzumab resulted in a synergistic inhibition of cell growth (19). Seliciclib inhibited the growth of both cell lines with IC 50 values for ED 50 (the point on the curve where the cell number is 50% of that in the untreated controls). Results are the average of at least three independent experiments. The CI value definitions are as follows: 1.45 to 1.2 is moderately antagonistic, 1.2 to 1.1 is slightly antagonist, 1.1 to 0.9 is additive, 0.9 to 0.85 is slightly synergic, 0.85 to 0.7 is moderately synergic, and 0.7 to 0.3 is synergistic.

In vitro analysis of the combination of seliciclib and trastuzumab. To evaluate the interaction between seliciclib and trastuzumab, combination experiments were done in two breast cancer cell lines: SkBr3, which overexpresses HER2, and MCF7, which expresses low levels of HER2 (19). Incubation with trastuzumab (1-100 nmol/L) reduced the proliferation of SkBr3 cells by up to 40%, but had no significant effect on MCF7 cells (data not shown), in agreement with previous data (19). Seliciclib inhibited the growth of both cell lines with IC 50 values of 16.3 and 10.0 μmol/L in SkBr3 and MCF7 cells, respectively. Co-incubation of SkBr3 cells with seliciclib and trastuzumab resulted in a synergistic inhibition of cell growth (Fig. 1A and Table 1), generating a CalcuSyn CI of 0.66 at ED 50 (the point at which the combination inhibits cell growth by 50%). In contrast, the inhibition of cell growth was only additive in MCF7 cells (CI = 0.96). These findings are consistent with earlier observations, which showed that trastuzumab is synergistic with alvocidib in cells that over-express HER2 (19–21).

To understand the mechanism behind the synergistic seliciclib/trastuzumab combination, analysis of the effects on the HER2 signaling pathway was done. SkBr3 cells were incubated with seliciclib, trastuzumab, or the combination of both agents for 24 hours, cell lysates were prepared, and the levels of HER2 and cyclin D1 proteins were determined. Trastuzumab treatment had no effect on HER2 levels at concentrations of up to 28 nmol/L (Fig. 1B), which was consistent with previous findings demonstrating that this drug does not down-regulate HER2 levels in SkBr3 cells (23). In contrast, treatment with seliciclib decreased HER2 levels in a dose-dependent manner (Fig. 1C) as has previously been reported for alvocidib (20, 24). Down-regulation of the HER2 receptor levels by seliciclib was most likely due to its inhibitory effects on CDK7 and CDK9 (25, 26), resulting in decreased phosphorylation of the carboxy-terminal domain of RNA polymerase II with a subsequent reduction in transcription of the receptor mRNA and loss of the HER2 protein through degradation (27). The effect of seliciclib on transcription was confirmed by examining the extent of phosphorylation of the carboxy-terminal domain of RNA polymerase II (Fig. 1C). At concentrations above the IC 50 value, the level of phosphorylation was dramatically reduced, as were the levels of the McI-1 protein, an established marker for seliciclib-induced transcriptional inhibition (Fig. 1C; ref. 11). When given together, trastuzumab and seliciclib produced greater down-regulation of the HER2 receptor than either single agent, suggesting that the drug combination enhanced the down-regulation of receptor levels (Fig. 1D).

Mitogenic stimuli transduced by the HER2 signaling pathway result in the expression of cyclin D1 and the consequent activation of CDKs leading to cellular proliferation (3). Indeed it has been shown that cyclin D1 is required for transformation by the HER2 receptor (28) and that cyclin D1–deficient mice are resistant to HER2-mediated tumorigenesis (29, 30). At the concentrations tested in these experiments, trastuzumab had no effect on cyclin D1 levels (Fig. 1B), whereas seliciclib significantly decreased cyclin D1 levels (~60% reduction at 18 μmol/L) in a dose-dependent manner (Fig. 1C); like McI-1, cyclin D1 also has a very short half-life (14) and is subject to transcriptional control by either alvocidib or seliciclib (12, 14). The loss of cyclin D1 was slightly enhanced by combining seliciclib with trastuzumab (~75% reduction at 18 μmol/L; Fig. 1D), presumably as a consequence of decreased HER2 signaling and seliciclib-mediated inhibition of cyclin D1 transcription. These data indicate that in SkBr3 cells, seliciclib enhanced the efficacy of trastuzumab by promoting the down-regulation of the HER2 receptor and loss of the cell cycle regulator cyclin D1, which has been shown to be critical for propagating the HER2 mitogenic signal and transformation (28–30).

In vitro analysis of the combination of seliciclib and AG1478. A second ErbB receptor family member, EGFR (ErbB1), has also been the focus of significant anticancer drug development. Erlotinib, an EGFR tyrosine kinase inhibitor, has been approved for the treatment of advanced NSCLC, having shown survival benefit in the treatment of NSCLC (15), whereas cetuximab, a monoclonal antibody that targets the external domain of EGFR, has shown survival benefit in combination with irinotecan for the treatment of colorectal cancer (31). To date, there have been no published studies evaluating combinations between CDK inhibitors and EGFR inhibitors. To extend the analysis of synergistic interactions between seliciclib and inhibitors of the ErbB family, seliciclib was evaluated in combination with the EGFR tyrosine kinase inhibitor AG1478 (32, 33), an analogue of erlotinib.

The combination between seliciclib and AG1478 was evaluated initially in three NSCLC cell lines: H358, A549, and H460. These cell lines express different levels of wild-type EGFR with A549 cells expressing the highest levels and H358 cells expressing the lowest levels (Fig. 2A). The IC 50 values for AG1478 were 4, 6.6, and 10.4 μmol/L in H358, A549, and H460 cells, respectively, demonstrating that there was no correlation between EGFR protein levels and sensitivity to

### Table 1. Summary of combination studies involving seliciclib and either trastuzumab or the EGFR inhibitor AG1478

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Schedule</th>
<th>CI at ED 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkBr3</td>
<td>Seliciclib + trastuzumab</td>
<td>0.66</td>
</tr>
<tr>
<td>MCF7</td>
<td>Seliciclib + trastuzumab</td>
<td>0.96</td>
</tr>
<tr>
<td>H358</td>
<td>Seliciclib/AG1478</td>
<td>0.96</td>
</tr>
<tr>
<td>A549</td>
<td>Seliciclib/AG1478</td>
<td>0.74</td>
</tr>
<tr>
<td>A549</td>
<td>Seliciclib + AG1478</td>
<td>1.13</td>
</tr>
<tr>
<td>H460</td>
<td>Seliciclib/AG1478</td>
<td>1.33</td>
</tr>
<tr>
<td>H460</td>
<td>AG1478/seliciclib</td>
<td>0.74</td>
</tr>
<tr>
<td>H460</td>
<td>Seliciclib + AG1478</td>
<td>1.36</td>
</tr>
<tr>
<td>H460</td>
<td>Seliciclib + AG1478</td>
<td>1.34</td>
</tr>
</tbody>
</table>

NOTE: Seliciclib was tested in combination with either trastuzumab or AG1478 in various cell lines, using the protocol described in Materials and Methods. Concomitant (+) and sequential (/) treatment schedules were tested, and the resulting CI values were shown for ED 50 (the point on the curve where the cell number is 50% of that in the untreated controls). Results are the average of at least three independent experiments. The CI value definitions are as follows: 1.45 to 1.2 is moderately antagonistic, 1.2 to 1.1 is slightly antagonistic, 1.1 to 0.9 is additive, 0.9 to 0.85 is slightly synergistic, 0.85 to 0.7 is moderately synergic, and 0.7 to 0.3 is synergistic.

results show synergistic suppression of tumor growth both in vitro and in vivo.

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AG1478. A similar pattern of sensitivity has been reported for erlotinib, leading to the description of these three cell lines as sensitive, intermediate, and resistant, respectively (34). It has been reported that A549 and H460 cells contain high levels of constitutively active ERK or protein kinase B, respectively (35), which are two downstream effectors of the EGFR signaling pathway. This was confirmed by Western blot analysis; A549 cells expressed relatively high levels of phosphorylated ERK, whereas H460 cells contained high levels of phosphorylated protein kinase B (Fig. 2A). Neither pathway seemed to be activated to such an extent in H358 cells (Fig. 2A) in line with its greater sensitivity to EGFR inhibitors. Seliciclib IC50 values

![Figure 2](https://example.com/figure2.png)

Fig. 2. Analysis of the combination of seliciclib and AG1478 in NSCLC cells. A, lysates (30 μg) from untreated A549, H460, and H358 cells were resolved on 3% to 8% acrylamide Tris-acetate gels or 10% acrylamide Bis-Tris gels, transferred to nitrocellulose membranes, and probed with the antibodies shown. Results are representative of two independent experiments. B, H358 cells were treated for 72 h using increasing concentrations of seliciclib and AG1478, either alone or in a fixed ratio of 2.125:1, as described in Materials and Methods. The resultant data were analyzed using the CalcuSyn program, and graphs from a representative experiment for each treatment schedule are shown. CI values are defined in the legend for Table 1. C, H358 cells were incubated in the presence (+) or absence (−) of either seliciclib or AG1478 for 72 h before harvesting. In H358 cells, the IC50 values for seliciclib and AG1478 were 8.5 and 4.0 μmol/L, respectively. Western blots were done as in A.
were determined for H358, A549, and H460 cells and corresponded to 8.5, 10, and 14 μmol/L, respectively. The effect of the combination on cell growth was examined by addition of the compounds, either concomitantly or sequentially. In H358 cells, moderate synergy was seen in the combination treatments, whereas the combination had, at best, an additive effect on cell growth in either A549 or H460 cells (summarized in Table 1). Thus, the combination of seliciclib and AG1478 generated the best synergistic interaction in H358 cells, the EGFR inhibitor-sensitive cell line, and was less effective in cells with constitutively active components downstream of the EGFR. A general sequence dependence for combinations involving either CDK inhibitors or EGFR inhibitors has been reported previously, for example, alvocidib and paclitaxel (36, 37) or erlotinib and pemetrexed (38, 39). In each case, the sequence dependence has been hypothesized to be due to one treatment blocking the cells in a phase of the cell cycle where the second treatment is less effective. A degree of sequence dependence was observed with the combination between seliciclib and AG1478. When the different combination schedules were examined in H358 cells, concomitant treatment and pretreatment with AG1478 followed by seliciclib caused modestly synergistic inhibition of cell growth (average ED₅₀ CI values of 0.74 and 0.81, respectively), whereas treatment with seliciclib followed by AG1478 produced only an additive drug interaction (ED₅₀ CI = 0.96; summarized in Table 1 and a representative experiment shown in Fig. 2B). The fact that concomitant treatment resulted in the best synergy was also confirmed when the data was analyzed using a nonlinear regression fit of the data to the equation of Greco (data not shown; ref. 40).

To elucidate the molecular mechanism involved in the synergistic effects of seliciclib and AG1478, H358 cells were treated with either agent alone or in combination. These experiments were done with concomitant treatment, as this seemed to be the best regimen. Cell lysates were prepared, and proteins were analyzed by Western blotting (Fig. 2C). Initially the effects of the compounds on the level of the EGFR and its phosphorylation status were studied. Treatment with either AG1478 or seliciclib decreased modestly the level of the EGFR in a dose-dependent manner; at 2.25 times the IC₅₀ value, both single-agent treatments resulted in a ~60% reduction in signal than with either of the two individual drug treatments (Fig. 2C). In addition, AG1478 treatment resulted in a dose-dependent decrease in EGFR phosphorylation, whereas seliciclib treatment had a limited effect on phosphorylated EGFR levels. The reduction in the levels of EGFR phosphorylation was enhanced by the concomitant treatment (Fig. 2C), suggesting that the combination lowered both the quantity and activation status of the EGFR.

To further explore the effects of the combination of seliciclib and AG1478 on the EGFR signaling pathway, the phosphorylation status of ERK, and cyclin D1 protein levels were examined. Neither seliciclib nor AG1478 had any significant effect on ERK phosphorylation at the concentrations tested. However, the combination was very effective at reducing ERK phosphorylation to barely detectable levels, suggesting an enhanced inhibition of phosphorylated EGFR levels. The reduction in the levels of EGFR phosphorylation was enhanced by the concomitant treatment (Fig. 2C), suggesting that the combination lowered both the quantity and activation status of the EGFR.

Fig. 3. Seliciclib plus AG1478 induces increased apoptosis. A, duplicate flasks of cells were treated with 2.25 × IC₅₀ seliciclib, AG1478, or both compounds for 72 h before harvesting. The DNA content of the cells was analyzed by flow cytometry after propidium iodide staining. Sub-G₁ cells contain less DNA than normal diploid cells and are considered to represent apoptotic cells. In H358 cells, the IC₅₀ values for seliciclib and AG1478 were 8.5 and 4.0 μmol/L, respectively; in H292 cells, the IC₅₀ values for seliciclib and AG1478 were 15.0 and 5.0 μmol/L, respectively; in H1650 cells, the IC₅₀ values for seliciclib and AG1478 were 10.4 and 13.0 μmol/L, respectively. Results are representative of two independent experiments. B, lysates (30 μg) from cells treated for 72 h at 2.25 × IC₅₀ value were resolved on 3% to 8% acrylamide Bis-Tris gels or 10% acrylamide Bis-Tris gels, transferred to nitrocellulose membranes, and probed with the antibodies shown. Results are representative of two independent experiments.
downstream of the majority of ErbB receptor signaling pathways. Indeed, a reduction in cyclin D1 levels may be required to achieve a response to EGFR inhibitors both in vitro and in patients (41, 42). Seliciclib concentrations above cellular IC₅₀ values produced a modest decrease in cyclin D1 levels in H358 cells (reductions of 5% at 1.5 × IC₅₀ and 45% at 2.25 × IC₅₀), whereas at the concentrations tested AG1478 had very little effect on the levels of this protein. When applied together, the two drugs dramatically reduced cyclin D1 expression in a dose-dependent manner equivalent to ~90% and ~98% reduction at the two concentrations (Fig. 2C). The loss of cyclin D1, one of the key drivers of the cell cycle, will have contributed to the effect of the combination on cellular proliferation.

The combination of seliciclib and AG1478 can induce apoptosis in cell lines that express either wild-type or mutant EGFR. Seliciclib treatment is known to cause apoptotic cell death (11, 43), but significant induction of apoptosis is not observed in many cell lines treated with AG1478, erlotinib, or trastuzumab (21, 44–46). Using poly(ADP)-ribose polymerase (PARP) cleavage as a marker of apoptosis, Western blot analysis showed that at the concentrations tested in H358 cells, AG1478 caused little apoptosis (Fig. 2C). In contrast, seliciclib induced a dose-dependent increase in cleaved PARP, which was not enhanced in the presence of AG1478 (Fig. 2C). Taken together, these data suggest that in H358 cells, the combination of seliciclib and AG1478 caused a synergistic down-regulation of the EGFR signaling pathway, resulting in a cessation of cell proliferation rather than increased apoptotic cell death.

To explore the induction of apoptosis in more detail and to address whether the combination was also synergistic in cell lines expressing mutant EGFR, these studies were expanded to include two additional NSCLC cell lines. H1650 cells express mutant EGFR; they are sensitive to erlotinib and undergo apoptosis after treatment (47). H292 cells express wild-type EGFR and are very sensitive to erlotinib both in vitro and in vivo (34), although erlotinib fails to induce apoptosis in this cell line (47). Initially, cytotoxicity experiments were done to show that the combination was synergistic in these cell lines. In
H1650 cells, the combination showed moderate synergy with an optimum CI of 0.71 at ED$_{50}$, whereas for H292 cells, the combination showed synergy with an ED$_{50}$ CI of 0.37, indicating improved synergism over that in both H358 and H1650 cell lines. To evaluate the effect of the combination on apoptosis, the three cell lines were treated with the compounds at 2.25 times their appropriate IC$_{50}$ value and analyzed by flow cytometry. In all three cell lines, seliciclib treatment caused an increase in the percentage of cells with a sub-G$_1$ DNA content (apoptotic cells) and in the number of cells in the G$_2$-M phase of the cell cycle (Fig. 3A), which is in agreement with previous reports (48). After AG1478 treatment, the predominant effect on the cell cycle was to cause an increase in the number of cells in G$_1$; only in the mutant EGFR expressing H1650 cells was there a large increase in the number of apoptotic cells, consistent with previous findings (47). The observation that in these three cell lines significant apoptosis was only induced by AG1478 in the cell line expressing mutant EGFR was also in line with the clinical finding that patients with EGFR mutations were more likely to show a response to EGFR inhibitors than patients that express wild-type EGFR (49, 50).

In H358 cells, the flow cytometry results agreed well with the cleaved PARP data (Fig. 2C) in that the combination of the two agents did not result in a greater induction of apoptosis than seliciclib treatment alone (Fig. 3A). In H1650 cells, the combination of drugs led to an even greater increase in the apoptotic population compared with the single-agent treatments and represented at least an additive induction of apoptosis (Fig. 3A). Finally, in H292 cells, the combination treatment resulted in a substantial increase in the number of cells with a sub-G$_1$ DNA content above that seen with seliciclib treatment alone (Fig. 3A). This equated to a synergistic induction of cell death, which was particularly significant, given that AG1478 alone failed to induce apoptosis in this cell line. Moreover, the enhanced synergistic induction of apoptosis in the H292 cell line also correlated with the CalcuSyn data, which suggested that the synergy was greatest in this cell line.

As a second method of monitoring the induction of apoptosis, protein lysates extracted from cells treated with compounds were analyzed by Western blot for the appearance of cleaved PARP (Fig. 3B). There was excellent correlation between the two methods of measuring apoptosis. In H1650 cells, both single-agent treatments resulted in the appearance of cleaved PARP whereas the combination treatment generated an enhanced signal. In H292 cells, seliciclib alone caused an induction of PARP cleavage whereas AG1478 had no effect. The combination treatment induced an increase in the amount of PARP cleavage clearly greater than the seliciclib treatment alone, suggesting, once again, that the combination treatment resulted in a synergistic induction of cell death in H292 cells. Although these data suggest that the extent of apoptosis obtained with the combination treatment can vary depending on the cell line, importantly, the combination of the two drugs was beneficial regardless of the EGFR status of the cell line.

In vivo evaluation of the combination of seliciclib and erlotinib. The combination of seliciclib and erlotinib was evaluated in vivo in an H358 NSCLC xenograft model. Mouse bearing H358 tumors of ~110 mm$^3$ were given seliciclib (50 mg/kg) i.p. twice per day for 5 consecutive days, and then after a 2 day treatment break, this schedule was repeated for a total of 28 days. At this dose level, seliciclib can reach a C$_{max}$ of 46 µmol/L (data not shown), a concentration above that needed to see synergy in vitro but significantly below the maximum tolerated dose. Based on published reports, erlotinib (100 mg/kg) was dosed by oral gavage once per day for 28 consecutive days (34); for combination treatment, mice received both compounds on the same schedules as for each single agent. Control mice received seliciclib vehicle on a twice daily schedule. On day 49, 3 weeks after all treatments had ceased, control mice (nine of nine mice alive) had tumors with a mean volume of 700 mm$^3$, which represented a >6-fold increase in tumor size (Fig. 4A). Mice treated with single-agent seliciclib or erlotinib (seven of nine mice alive, respectively) had mean tumor volumes of 717 and 444 mm$^3$, respectively, which indicated that neither agent had significant activity on their own at these doses. The relatively low level of erlotinib activity (44% tumor growth inhibition) correlated with the modest activity reported previously for this tumor model (34). In stark contrast, by day 49, mice treated with the combination of seliciclib and erlotinib (eight of nine mice alive) had a mean tumor volume of 153 mm$^3$, representing 93% tumor growth inhibition. The ratio of median treated tumor volume to median control tumor volume for the combination was 22% on day 49, demonstrating that the combination of the two compounds led to significant tumor growth delay. When the treated groups were compared with the vehicle control using the one-way ANOVA test followed by Dunnett’s multiple comparison test, the only group that was statistically different was the combination group ($P = 0.001$). If the individual groups were compared against each other using an unpaired Student’s $t$ test, then the combination was also significantly different from the single-agent seliciclib and erlotinib treatments ($P < 0.002$).

To explore the proposed mechanism of action in vivo, a second H358 xenograft was done; two mice per treatment group were dosed for 5 days with erlotinib orally daily (100 mg/kg), seliciclib i.p. twice a day (100 mg/kg), or the combination of the two agents. After 5 days, the mice were sacrificed and the tumors were removed for immunohistochemical staining with an antibody raised against cyclin D1. Representative fields of view are shown in Fig. 4B and graphed in Fig. 4C. It was clear that the combination treatment resulted in a dramatic reduction in the amount of cyclin D1 staining compared with either single-agent treatment, in terms of both the number of cells stained and the intensity of staining in the cells. An unpaired Student’s $t$ test suggested that the number of cells stained in the combination samples was significantly lower than the vehicle control or either of the single-agent treatments ($P < 0.0001$). Thus, the mechanistic in vivo results mirror the results seen in vitro; targeting one of the key drivers of cell proliferation, cyclin D1, by blocking growth factor signaling and preventing its transcription has synergistic antitumor activity both in vitro and in vivo.

With the exception of the recent data on alvocidib in B-CLL (18), to date CDK inhibitors have had relatively modest single-agent activity in the clinic. This is also true of several ErbB inhibitors, which have been clinically approved despite their relatively low response rate. For example, the EGFR inhibitor erlotinib had a response rate of only 9% in the pivotal single-agent NSCLC trial (15). Likewise, trastuzumab, which was given only to patients that overexpress the HER2 receptor, achieved just an 11% response rate in the original phase II
single agent trial in this preselected population (16). Evaluation of the potential for combining either of these classes of agents is therefore of great value to try and increase the clinical options for patients. This report discusses the potential synergistic effects of using two agents to cooperatively repress cyclin D1 expression and thereby limit cellular proliferation. A proof-of-principle clinical trial has been done recently based on a similar hypothesis using erlotinib and the rexinoid bexaratone; the latter agent contributes by increasing the proteosomal degradation of cyclin D1 (51). The combination of erlotinib and bexaratone in aerodigestive tract cancers was well tolerated, and in a 24-patient population, four achieved partial responses and nine had stable disease (51). In addition, reductions in cyclin D1 protein levels were detected in buccal scrapes taken posttreatment. Significantly, the patients that achieved clinical benefit were atypical erlotinib responders; they were predominately male, current or former smokers, and infrequently had bronchioalveolar carcinoma histology. This provides proof-of-concept clinical support for the idea that combinations of agents that target cyclin D1 can expand the clinical activity of EGFR inhibitors beyond the typically responsive population.

A second finding of this study was the median overall survival time, which was 14.1 months for the combination compared with 6.7 months for single-agent erlotinib (51). This is of particular significance because frequently patients that respond to EGFR inhibitors have relatively transient responses and eventually relapse with disease progression (52). Resistance to the agent is often due to the appearance of “gatekeeper” mutations (e.g., T790M) at the active site of the kinase that block inhibitor binding (53). Recently, a second mechanism of resistance that involves the amplification of an alternative proto-oncogene, such as the c-MET receptor, which interacts directly with the ErbB3 receptor activating the phosphatidylinositol 3-kinase pathway and stimulating cellular proliferation, has been identified (54). However, because of the emergence of a new oncogenic pathway, such as c-MET, still depends on the production of cyclin D1 as one of the main drivers of cell proliferation, the enhanced reduction in the levels of cyclin D1 by either the erlotinib/bexaratone or potentially the erlotinib/seliclib combination could result in extended response times by limiting the ability to switch receptor dependency.

In summary, preclinically, both alvocidib and selicilib have shown in vitro synergy with a wide range of agents, including both standard chemotherapies and targeted agents; as with other novel targeted therapies, combination therapy may turn out to be the optimum way to exploit these agents in the clinic. In our study, we present the first report of synergy between CDK inhibitors and EGFR inhibitors. The combination of selicilib and AG1478 was synergistic in vitro in cells that expressed either mutant EGFR or wild-type EGFR and resulted in dramatic synergy in vivo in a NSCLC xenograft model. At the molecular level, synergy seemed to involve augmented down-regulation of ErbB receptor levels and inhibition of downstream signaling, resulting in an enhanced loss of cyclin D1, one of the downstream components of ErbB signaling pathways, a key regulator of cellular proliferation, and a protein that has been highlighted already as a potential biomarker of clinical response to erlotinib (42). These data suggest that the combination of these agents could potentially improve the efficacy of ErbB inhibitors and is worthy of further investigation in the clinic.

Disclosure of Potential Conflicts of Interest

I. Fleming, S. McClue, S. Frame, S. Green, and M. Hogben are employed by Cyclacel Limited.

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