AKT inhibition synergistically enhances growth-inhibitory effects of gefitinib and increases apoptosis in non-small cell lung cancer cell lines

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Objectives: EGFR inhibitors are ineffective against most EGFR wild-type non-small cell lung cancer, for which novel treatment strategies are needed. AKT signalling is essential for mediating EGFR survival signals in NSCLC. We evaluated the combination of gefitinib and two different AKT inhibitors, the allosteric inhibitor AKTI-1/2 and the ATP-competitive pan-AKT inhibitor AZD5363, in EGFR-mutant (HCC-827 and PC-9) and -wild-type (NCI-H522, NCI-H1651), non-small cell lung cancer cell lines.

Materials and methods: Drug interaction was studied in two EGFR mutant and two EGFR wild-type non-small cell lung cancer cell lines by calculating combination index (CI) using median effect analysis. The effects on p-EGFR, p-ERK, p-AKT, p-S6 and apoptosis were studied by Western blot analysis.

Results: The combination of gefitinib and AKTI-1/2 or AZD5363 showed synergistic growth inhibition in all cell lines. CI values for the combination of gefitinib and AKTI-1/2 were 0.35 (p = 0.0048), 0.56 (p = 0.036), 0.75 (p = 0.13) and 0.64 (p = 0.0003) in NCI-H522, NCI-H1651, HCC-827 and PC-9 cell lines, respectively; CI values of 0.45 (p = 0.0087) and 0.22 (p = 0.0001) were observed in NCI-H522 and PC-9 cells, respectively, when gefitinib was combined with AZD5363. Additive inhibition of signalling output through AKT and key downstream proteins (S6) and increased apoptosis were demonstrated.

Conclusion: Dual inhibition of EGFR and AKT may be a useful up-front strategy for patients with EGFR-mutant and -wild-type non-small cell lung cancer.

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1. Introduction

Lung cancer affects approximately 1.6 million people each year and is the leading cause of cancer-related deaths worldwide, with 342,000 estimated deaths in Europe in 2008 [1]; non-small cell lung cancer (NSCLC) represents approximately 85% of all cases. In the advanced disease setting conventional chemotherapy has reached a plateau in efficacy with a median survival of 8–11 months [2]. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as gefitinib, are successful treatments for the approximately 10% of NSCLC with an activating EGFR mutation [3,4], but have limited efficacy in an EGFR wild-type unselected population. EGFR signals through intermediates including the PI3K–Akt–mTOR–S6 and RAF–MEK–ERK networks [5]. De-novo resistance to EGFR TKIs may be linked to genetic alterations downstream of EGFR, including KRAS (15–20%), BRAF (2–3%), PI3K (2%), AKT (1%) and PTEN (30%) [6]. However, alternative mechanisms other than driver gene alterations may also be involved in conferring resistance to EGFR TKIs, through activation of alternative signalling [7]. There is growing evidence that AKT signalling is essential for mediating EGFR survival signals in NSCLC [8,9]. Persistent activation of AKT has been linked to resistance to EGFR TKI in NSCLC in vitro models [10–13]. In addition, concurrent AKT and EGFR inhibition has shown synergistic activity in a panel of NSCLC cell lines [14]. We explored this further by studying the effect of gefitinib, a selective EGFR TKI, in combination with two different AKT inhibitors on cell growth inhibition and modulation of signalling pathways in EGFR mutant and EGFR wild-type NSCLC cell lines. The AKT inhibitors used include a generic allosteric inhibitor of AKT 1 and 2 (AKTI-1/2), and the ATP-competitive
pan-AKT inhibitor AZD5363; the latter compound is currently in clinical development.

2. Materials and methods

2.1. Cell lines and cell culture

NSCLC cell lines HCC827, NCI-H522, and NCI-1651 were purchased from ATCC (LGC, Teddington, UK); PC-9 was purchased from HPA Culture (Salisbury, UK). HCC827, PC9 and NCI-H522 were grown in RPMI-1640 and NCI-H1651 was grown in ACL-4, all media were supplemented with 10% foetal bovine serum. Cells were maintained in a humidified 5% CO2 atmosphere at 37°C. All tissue culture consumables were purchased from Sigma–Aldrich (Gillingham, UK).

2.2. Drugs and chemicals

Gefitinib was purchased from Selleck (Stratech Scientific, Newmarket, UK) and AKT inhibitor 1/2 (AKTi-1/2) from Sigma–Aldrich (Gillingham, UK). AZD5363 was generously provided by AstraZeneca, (Alderley Park, UK).

2.3. Cell growth inhibition assay and drug combination studies

The cell growth inhibitory effect of gefitinib, AKTi-1/2 and AZD5363 was studied using the 96h sulforhodamine B assay (SRB) as described [15]. Drug concentrations that inhibited 50% of cell growth (IC50) were calculated for each compound in GraphPad Prism 6.0 using non-linear regression analysis and sigmoidal dose–response (variable slope) equation (Graph Pad Software, Inc., San Diego, CA).

Each drug combination was tested with two drugs delivered simultaneously at constant ratios, spanning the IC50 of each drug. In vitro combination effects on cell growth were analysed using the combination index (CI) method based on the median effect analysis according to Chou and Talalay [16]. This is founded on the assumption that the potency (Dm) and shape of dose-effect curve (m) are different for each drug. Dm and m values for both single drugs and the combination, as obtained from the median-effect equation, are entered in the CI equation to provide a quantitative estimate of drug interaction. Data analysis was performed with the CalcuSyn software version 2.0 (Biosoft, Cambridge, UK). CI values above 1.1 were considered antagonistic, values between 0.9 and 1.1 were considered additive, and CI values less than 0.9 were considered synergistic. CI can be calculated at any growth-inhibitory effect level; for our analysis CI values at ED50 level were considered (ED50 representing the effective dose of 50% inhibition). Four replicates were performed for each experiment and p values were obtained using a one-sided t-test compared with a hypothetical value of 1.

2.4. Western blotting and antibodies

Cells were lysed in RIPa lysis buffer (Thermo Scientific, Rockford, IL) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by BCA assay as standard. Western blotting was performed as described [17]. Primary antibodies used: cleaved PARP antibody human specific (1:1000), phospho-EGFR Tyr1086 (1:1000), phospho-AKT Ser473 (1:1000), phospho-ERK Thr202/Tyr204 (1:2000) and phospho-S6 Ser235/236 (1:1000); all antibodies were purchased from Cell Signalling Technology (New England BioLabs, Hitchin, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) was used as loading control (1:20,000) (Millipore, Temecula, CA). As secondary antibodies, goat anti-rabbit (1:5000) or anti-mouse (1:10,000) Ig HRP-conjugate were used (Bio-Rad, Hemel Hempstead, UK). The intensities of bands on the Western blots were determined by UN-SCAN-IT software version 6.1 (Silk Scientific, Orem, USA).

3. Results

3.1. Effects of the combination of gefitinib and the AKT inhibitor AKTI-1/2 or AZD5363 on growth inhibition of EGFR mutant and EGFR wild-type NSCLC cell lines

To evaluate the combination of gefitinib and an AKT inhibitor we selected four NSCLC adenocarcinoma cell lines which were either EGFR wild-type (NCI-H522 and NCI-H1651) or had a sensitising EGFR mutation (HCC872 and PC-9). In addition, all cell lines were

Table 1

Sensitivity of NSCLC cell lines to gefitinib, AKTI-1/2 and AZD5363 according to their molecular profile. IC50 = concentrations of drug that inhibited growth by 50% relative to control. Values represent the mean ± SD from three independent experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR gene</th>
<th>KRAS gene</th>
<th>PIK3CA/PTEN expression</th>
<th>Gefitinib IC50 (μM)</th>
<th>AKTI-1/2 IC50 (μM)</th>
<th>AZD5363 IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H522</td>
<td>wt</td>
<td>wt</td>
<td>wt/</td>
<td>7.0 (±2.5)</td>
<td>7.2 (±1.5)</td>
<td>11.3 (±2.7)</td>
</tr>
<tr>
<td>NCI-H1651</td>
<td>wt</td>
<td>wt</td>
<td>wt/</td>
<td>8.8 (±0.9)</td>
<td>9.5 (±1.8)</td>
<td>–</td>
</tr>
<tr>
<td>HCC827</td>
<td>exon19 (E746-A750)del</td>
<td>wt</td>
<td>wt/</td>
<td>0.004 (±0.0005)</td>
<td>4.7 (±1.5)</td>
<td>–</td>
</tr>
<tr>
<td>PC-9</td>
<td>exon19 (E746-A750)del</td>
<td>wt</td>
<td>wt/</td>
<td>0.07 (±0.03)</td>
<td>4.9 (±1.4)</td>
<td>9.3 (±1.2)</td>
</tr>
</tbody>
</table>

Fig. 1. The combination of (A) gefitinib and either AKTI-1/2 or (B) AZD5363 is synergistic in EGFR mutant and EGFR wild-type NSCLC cell lines. Combination index at ED50 (effective dose of 50% response) for the four NSCLC cell lines studied. Mean is derived from four replicates, bars are standard deviations. P values were determined using one sample t-test compared with a hypothetical value of 1. CI statistically significantly >1 = synergism, CI statistically significantly <1 = antagonism, CI equal to (or not statistically significantly different from) 1 = additivity.
wild-type (wt) for those genes encoding for components of the KRAS–BRAF and PI3K–AKT pathways as reported by the COSMIC database, and expressed PTEN by Western blot analysis (data not shown) [18]. The mutation profiles and IC50 values of gefitinib, AKTi-1/2 and AZD5363 for the cell lines studied are summarised in Table 1. As expected, the EGFR wt cell lines were relatively insensitive to gefitinib alone as compared to EGFR mutant cell lines. The combination of gefitinib and AKTi-1/2 showed synergistic growth inhibition in all four cell lines. The synergistic interaction was more pronounced in the EGFR wt cell lines (NCI-H522, NCI-H1651) (Fig. 1A). We then tested the effect of gefitinib in combination with the clinically active compound AZD5363 in one EGFR wild-type (NCI-H522) and one EGFR mutant cell line (PC-9). A synergistic interaction was confirmed in both cell lines (Fig. 1B).

3.2. Effects of the combination of the EGFR inhibitor gefitinib and the allosteric AKT inhibitor AKTi-1/2 on signalling output and apoptosis

To study the effect of dual EGFR and AKT inhibition on downstream pathways, EGFR wt and EGFR mutant NSCLC cell lines were exposed to gefitinib and AKTi-1/2 at 5 × IC50 concentrations. EGFR, AKT, S6 and ERK phosphorylation and total protein levels were evaluated at an early (4 h) and late time point (24 h).

Within the PI3K–AKT–m-TOR axis, reduced levels of p-AKT and downstream p-S6 were observed in all cell lines after exposure to the combination of gefitinib and AKTi-1/2 (Fig. 2); these effects were more pronounced than those achieved by each drug alone after 4 h (p-S6) and 24 h (p-AKT and p-S6). In addition, additive reduction of p-ERK levels was observed in NCI-H522 (EGFR wt) and PC-9 cells (EGFR mutant) at 24 h (Fig. 2). After exposure to the combination, EGFR phosphorylation levels were reduced by a greater extent than gefitinib alone in both EGFR mutant cell lines, but not in EGFR wt cells (Fig. 2).

The expression levels of total EGFR, AKT, S6 and ERK proteins were not significantly altered by AKTi-1/2 and gefitinib treatment (Fig. 2).

Enhanced apoptosis, as evidenced by the increased detection of cleaved PARP (c-PARP), was observed in all four cell lines when treated with gefitinib and AKTi-1/2 in combination as compared to either drug alone (Fig. 4A and B).

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Fig. 2. The combination of gefitinib and AKTi-1/2 affects intracellular mediators of signal transduction. (A) Immunoblots for p-EGFR, EGFR, p-AKT, AKT, pS6, S6, p-ERK and ERK. Cells were treated with gefitinib and AKTi-1/2 at five times the IC50. Representative blots of at least two independent Western blotting analyses; GAP-DH was used as a loading control. (B) Histograms show levels of p-EGFR, p-AKT, pS6 and p-ERK expressed relative to loading control GAP-DH and normalised to the non-treated control.
3.3. Effects of the combination of the EGFR inhibitor gefitinib and the pan-AKT kinase inhibitor AZD5363 on signalling output and apoptosis

In order to confirm the findings of previous experiments and affirm clinical relevance, we studied the combination of gefitinib and AZD5363, a pan-AKT kinase inhibitor currently in phase I trials. An EGFR mutant (PC-9) and EGFR wt cell line (NCI-H522) were exposed to 5 × IC50 of gefitinib alone or in combination with increasing doses of AZD5363, within a clinically relevant concentration range (1–10 μM), for 4 and 24 h.

Contrary to the allosteric AKT inhibitor AKTi-1/2, which inhibits phosphorylation of AKT, AZD5363 determined increased AKT phosphorylation at all dose levels (Fig. 3). This finding is consistent with the reported mechanism of AKT inhibition by AZD5363 [19]. Downstream of AKT, an additive reduction of p-S6 levels were confirmed in both cell lines when exposed to the combination of gefitinib and AZD5363, at all dose levels, for 4 and 24 h (Fig. 3). Total protein expression levels of EGFR, AKT, S6 and ERK were not affected.

An increase in apoptosis, as evidenced by increased detection of c-PARP, was confirmed in both the cell lines when AZD5363 was added to gefitinib (Fig. 4C and D).

4. Discussion

This study demonstrated significant synergistic inhibition of cell growth with the combination of gefitinib and either AKTi-1/2 or AZD5363 in EGFR mutant and EGFR wt NSCLC cell lines. An increased in apoptosis was also observed across all cell lines. These effects were associated with reduced signalling output through AKT and key downstream proteins such as S6. S6 is a ribosomal protein which is involved in the regulation of cell size, cell proliferation, and glucose homeostasis [20]. Although S6 is regarded as a downstream target of the PI3K–AKT signalling, the stimulation of S6 phosphorylation by RAS–ERK signalling via an independent mechanism has also been described [21], indicating that both the PI3K–AKT and RAS–ERK pathways converge downstream of ERK and AKT to independently regulate S6 [5]. This may explain why dual inhibition of EGFR and AKT caused an additive reduction of p-S6 levels.
Interestingly, the additive effect on AKT and S6 phosphorylation levels achieved by the combination of EGFR and AKT inhibitors were observed not only in EGFR mutant cell lines, which are sensitive to EGFR TKI inhibition, but also EGFR wt cells. Thus, EGFR-TKI insensitive NSCLC tumours expressing wild-type EGFR may still be dependent to some extent on EGFR, a hypothesis supported by an independent line of in vitro studies using siRNA-mediated knockdown models of EGFR [22,23]. Concomitant blockade of AKT, along with EGFR, may be crucial in achieving an antiproliferative effect on EGFR wt cell lines, where AKT phosphorylation appears to be refractory to EGFR TKI inhibition [13]. Here we describe how, in EGFR wt cell lines, as well as in EGFR mutant cells, phosphorylation of AKT was decreased by the addition of AKTI-1/2 to gefitinib, resulting in a more profound inhibition than that achieved by AKTI-1/2 alone. Downstream of AKT, an additive reduction of p-S6 levels was also observed when AKTI-1/2 or AZD5363 were added to gefitinib.

Synergistic effects with a different combination of inhibitors of EGFR and AKT inhibitors, i.e. MK2206, have been reported by other in vitro models of NSCLC [14]. This study has extended previous findings to include AZD5363, a pan-AKT kinase inhibitor currently in clinical development. Importantly, for the first time to our knowledge, this study demonstrated reproducible modulation of intracellular signalling, such as phosphorylation of S6, and an increased apoptotic cell death across EGFR mutant and EGFR wild-type cell lines, which may contribute to the synergy of the combination.

5. Conclusion

Our findings have clinical relevance and indicate that dual inhibition of EGFR and AKT may be a useful up-front strategy for patients with both EGFR mutant and EGFR wild-type NSCLC. The combination of EGFR and AKT inhibitors should be further evaluated in hypothesis-testing clinical trials to improve outcomes of patients with NSCLC.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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