Cancer Therapy: Preclinical

AZD8931, an Equipotent, Reversible Inhibitor of Signaling by Epidermal Growth Factor Receptor, ERBB2 (HER2), and ERBB3: A Unique Agent for Simultaneous ERBB Receptor Blockade in Cancer

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Abstract

Purpose: To test the hypothesis that simultaneous, equipotent inhibition of epidermal growth factor receptor (EGFR; erbB1), erbB2 (human epidermal growth factor receptor 2), and erbB3 receptor signaling, using the novel small-molecule inhibitor AZD8931, will deliver broad antitumor activity in vitro and in vivo.

Experimental Design: A range of assays was used to model erbB family receptor signaling in homodimers and heterodimers, including in vitro evaluation of erbB kinase activity, erbB receptor phosphorylation, proliferation in cells, and in vivo testing in a human tumor xenograft panel, with ex vivo evaluation of erbB phosphorylation and downstream biomarkers. Gefitinib and lapatinib were used to compare the pharmacological profile of AZD8931 with other erbB family inhibitors.

Results: In vitro, AZD8931 showed equipotent, reversible inhibition of EGFR (IC50, 4 nmol/L), erbB2 (IC50, 3 nmol/L), and erbB3 (IC50, 4 nmol/L) phosphorylation in cells. In proliferation assays, AZD8931 was significantly more potent than gefitinib or lapatinib in specific squamous cell carcinoma of the head and neck and non-small cell lung carcinoma cell lines. In vivo, AZD8931 inhibited xenograft growth in a range of models while significantly affecting EGFR, erbB2, and erbB3 phosphorylation and downstream signaling pathways, apoptosis, and proliferation.

Conclusions: AZD8931 has a unique pharmacologic profile providing equipotent inhibition of EGFR, erbB2, and erbB3 signaling and showing greater antitumor activity than agents with a narrower spectrum of erbB receptor inhibition in specific preclinical models. AZD8931 provides the opportunity to investigate whether simultaneous inhibition of erbB receptor signaling could be of utility in the clinic, particularly in the majority of solid tumors that do not overexpress erbB2.

The erbB receptor family is composed of four related receptor tyrosine kinases [epidermal growth factor receptor (EGFR, erbB1), erbB2 (human epidermal growth factor receptor 2, HER2), erbB3 (HER3), and erbB4 (HER4)]. ErbB2 lacks ligand-binding capacity and erbB3 is intrinsically inactive as a kinase. There are two main ligand classes: the first bind specifically to EGFR whereas the second includes the new differentiation factors, or heregulins, which bind erbB3 and erbB4 (1). In cancer, activation of erbB2 may arise by (a) receptor overexpression inducing homodimerization and (b) receptor heterodimerization with another family member, of which erbB3 is considered to be the preferred and most oncogenic partner (2).

Homodimerization and/or heterodimerization of erbB receptors results in the phosphorylation of key tyrosine residues in the intracellular domain and leads to the stimulation of numerous intracellular signal transduction pathways involved in cell proliferation and survival (3, 4). The deregulation of erbB family signaling promotes proliferation, invasion, metastasis, angiogenesis, and tumor cell survival and has been described in many human cancers, including those of the lung, head and neck, and breast (5, 6).

The erbB family therefore represents a rational target for anticancer drug development, and several agents targeting EGFR or erbB2 are now clinically available, including...
Translational Relevance

The erbB receptor family has an important role in driving many cancers. In this report, we show that a novel small-molecule inhibitor, AZD8931, has equipotent activity against signaling by three members of this family (epidermal growth factor receptor, erbB2, and erbB3). We further show that this simultaneous, potent inhibition delivers greater antitumor activity in experimental models than standard agents such as gefitinib or lapatinib. This has important implications for therapeutic interventions in the clinic in the majority of solid tumors, which do not contain amplified erbB2 or mutated epidermal growth factor receptor genes.

gefitinib and lapatinib. More recently, the importance of the composition of functional erbB dimeric units in tumor cell signaling has become apparent in diverse systems modeling both ligand-dependent and ligand-independent drives. Careful profiling of all four erbB receptors has differentiated their molecular function (7), and erbB3 has been found to have a central role in the transduction of signals to the phosphatidylinositol 3-kinase pathway, thus mediating cell survival signals for EGFR, erbB2, and potentially erbB4 (8). This is supported clinically by a range of studies linking erbB3 expression to poor survival in several differentiated their molecular function (7), and erbB3 has been found to have a central role in the transduction of signals to the phosphatidylinositol 3-kinase pathway, thus mediating cell survival signals for EGFR, erbB2, and potentially erbB4 (8). This is supported clinically by a range of studies linking erbB3 expression to poor survival in several differentiated their molecular function (7), and erbB3 has been found to have a central role in the transduction of signals to the phosphatidylinositol 3-kinase pathway, thus mediating cell survival signals for EGFR, erbB2, and potentially erbB4 (8). This is supported clinically by a range of studies linking erbB3 expression to poor survival in several differentiated their molecular function (7), and erbB3 has been found to have a central role in the transduction of signals to the phosphatidylinositol 3-kinase pathway, thus mediating cell survival signals for EGFR, erbB2, and potentially erbB4 (8). This is supported clinically by a range of studies linking erbB3 expression to poor survival in several.

Materials and Methods

Reagents. The free base of AZD8931 is disclosed in PCT patent publication number WO2005/028469 and can be prepared using the methods described therein. The synthesis of gefitinib and lapatinib has been previously described (14, 15).

Isolated kinase assays. The intracellular kinase domains of human EGFR and erbB2 were cloned and expressed in the baculovirus/Sf21 system. The inhibitory activity of AZD8931, gefitinib, and lapatinib was determined with ATP at Km concentrations (0.4 mmol/L for erbB2 and 2 mmol/L for EGFR) using the ELISA method previously described (16).

Cell lines. The majority of cell lines in this publication were either used within 6 mo of purchase from a cell bank and/or have been authenticated by short tandem repeat (STR) to an available profile (see Supplementary Materials and Methods for details). The human squamous cell carcinoma of the head and neck (SCCHN) cell line panel (20 lines) was obtained from the European Collection of Cell Cultures (Sigma), the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Life Technologies Bethesda Research Laboratories Ltd., and the Ludwig Institute for Cancer Research. The cells were cultured in DMEM/Ham's F-12 (phenol red free; Sigma) containing 10% FCS (Sigma) and 1% t-glutamine (Life Technologies Bethesda Research Laboratories Ltd.).

The human NSCLC cell line panel (16 lines) was obtained from Professor K. Nishio (PC-9, National Cancer Centre Hospital, Tokyo, Japan) and Drs. Frederic Kaye, Herbert Oie, and Bruce Johnson (NCI-H3255, NIH, MD) with all the other lines from the ATCC. The cells were grown in RPMI 1640 (phenol red free; Sigma) containing 10% FCS and 1% L-glutamine (Life Technologies). The majority of cell lines in this publication were either used within 6 mo of purchase from a cell bank and/or have been authenticated by short tandem repeat (STR) to an available profile (see Supplementary Materials and Methods for details). The human squamous cell carcinoma of the head and neck (SCCHN) cell line panel (20 lines) was obtained from the European Collection of Cell Cultures (Sigma), the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Life Technologies Bethesda Research Laboratories Ltd., and the Ludwig Institute for Cancer Research. The cells were cultured in DMEM/Ham's F-12 (phenol red free; Sigma) containing 10% FCS (Sigma) and 1% t-glutamine (Life Technologies Bethesda Research Laboratories Ltd.). For both panels, cells were harvested after 4 d of incubation or at 70% to 80% confluence.

The BT474c cell line was subcloned from the human breast cell line BT474 provided by Professor J. Baselga (Vall d'Hebron University Hospital, Barcelona, Spain).

In vitro EGFR phosphorylation studies. KB cells were grown in RPMI 1640 containing 10% FCS for 72 h, serum starved (0% FCS) for 24 h, and then incubated with AZD8931, gefitinib, or lapatinib for 90 min before stimulating for 5 min with 15 ng/mL recombinant EGF (R&D systems) to increase EGFR phosphorylation to 90% of max (ED90) to allow interassay comparison. The cells were lysed in RIPA buffer [50 mmol/L tris (pH 7.4), 1% NP40, 0.25% deoxycholate, 150 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, and Roche protease inhibitor cocktail] and EGFR phosphorylation was measured by a sandwich ELISA (R&D systems).

In vitro erbB2 and erbB3 phosphorylation studies. MCF-7 cells (ATCC; HTB-22) were grown in RPMI 1640 containing 10% FCS for 72 h, serum starved (2.5% stripped serum) for 24 h, and incubated with compound for 90 min before stimulating for 5 min with 35 ng/mL (ED90) HER2 (Heregulin; R&D systems). Cells were lysed in a radiomunoprecipitation assay buffer (Sigma radiomunoprecipitation assay buffer with protease inhibitors) and analyzed using the human phospho-erbB2 and phospho-erbB3 DuoSet ELISA kits (R&D systems).

The MCF-7 cl24 line was created by transfecting parental MCF-7 cells with the full-length erbB2 receptor. For details, see Supplementary Materials and Methods. For functional studies, MCF-7 cl24 cells were grown in DMEM with 10% FCS for 72 h, incubated with compound for 4 h, fixed in 3.3% formaldehyde, and immunostained...
with a phospho-erbB2 primary antibody (Santa Cruz Biotechnology) followed by an Alexa Fluor 488 secondary antibody (Molecular Probes). ErbB2 phosphorylation was quantified using a laser scanning fluorescence microplate cytometer (Acumen Explorer).

**In vitro drug sensitivity testing—cell viability assay.** To determine their antiproliferative activity against cell lines grown in vitro, AZD8931, gefitinib, and lapatinib were tested in a panel of NSCIC and SCCHN cell lines. Cells were incubated for 96 h with a suitable range of concentrations of drug to ensure accurate estimation of the inhibitor concentration required to give 50% growth inhibition (Gl50, typically between 0.001-10 μmol/L). Viable cell number was determined by 4 h of incubation with MTS Colorimetric Assay reagent (Promega) and absorbance measured at 490 nm on a spectrophotometer (Molecular Devices). Each experiment was carried out in triplicate for each drug concentration and data are presented as geometric means. Sensitivity groupings of Gl50 data were <1 μmol/L (classed as sensitive), 1 to 7 μmol/L (classed as intermediate), and >7 μmol/L (classed as resistant).

**In vitro drug sensitivity testing—erbB receptor inhibition.** PE/CA-PJ41, PE/CA-PJ49, DOK, and FaDu cells at 80% confluence were dosed with compound for 90 min and then lysed in radioimmunoprecipitation assay buffer. Phospho-EGFR, phospho-erbB2, and phospho-erbB3 levels were measured using the erbB family multiplexed phospho-EGFR, phospho-erbB2, and phospho-erbB3 levels were analyzed in tumor samples using the multiplex MesoScale Discovery kit or by ELISA as described above. Pharmacodynamic effects were also examined by immunohistochemistry (IHC). Antigen retrieval was done on formalin-fixed, paraffin-embedded tumor sections and the following primary antibodies used were as follows: Ki67 (DAKO M7240, 1:100), phospho-extracellular signal-regulated kinase (ERK; CST #4376, 1:50), cleaved caspase-3 (CST #9661, 1:100), phospho-EGFR (CST #4407, 1:100), phospho-erbB2 (CST #2243, 1:50), phospho-erbB3 (in-house antibody, 1:50), phospho-AKT (CST #3787, 1:50), M30 (Roche 2140349, 1:100), total erbB2 (DAKO Herceptest). A polymer detection system (DAKO Envision +K4007) was used for secondary detection and sections were counterstained with Carazzi’s hematoxylin. Semiquantitative scoring was carried out using Image Analysis.

**In vivo methods.** Swiss nude (nu/nu genotype; AstraZeneca) and severe combined immunodeficient (Charles River) mice were housed in negative-pressure isolators (PFI Systems Ltd.) at Alderley Park, United Kingdom. Experiments were conducted on 8- to 12-wk-old female mice in full accordance with the UK Home Office Animal (Scientific Procedures) Act 1986. All human tumor xenografts were established by s.c. injecting 0.1 mL tumor cell suspension (between 4 × 10^6 and 9 × 10^7 cells) mixed 1:1 with Matrigel. The BT474c cell line was subcloned with Matrigel (Becton Dickinson), with the exception of LoVo, which did not include Matrigel, and Calu-3, which was grown with Matrigel (Becton Dickinson), with the exception of PFI Systems Ltd. For the complete summary of xenograft models, treatment and IHC protocols, pharmacodynamic sampling, image analysis details, and statistical methods see Supplementary Materials and Methods.

**Results**

AZD8931 shows potent in vitro inhibition of EGFR and erbB2 tyrosine kinases. The activities of AZD8931, lapatinib, and gefitinib were assessed against constitutively active, isolated cytosolic fragments of EGFR and erbB2 in enzyme assays containing Km concentrations of ATP (Table 1). AZD8931 was the most potent inhibitor of both EGFR and erbB2 enzymes. Although gefitinib was also a potent inhibitor of EGFR, as expected, it was 18-fold less
active against erbB2. Lapatinib was more active against erbB2 than EGFR. The broader kinase selectivity of these agents was assessed using a diverse panel of serine/threonine, tyrosine, and lipid kinases. Outside of the erbB kinase family, none of the compounds showed significant activity at 10 μmol/L (data not shown). Specifically for AZD8931, this translated to a >1,000-fold selectivity for 206 of these enzymes with >100-fold selectivity for MNK1 and Flt3. More detailed ATP competition analyses confirmed that AZD8931 is a reversible, ATP competitive inhibitor (data not shown).

AZD8931 shows greater inhibition of ligand-stimulated EGFR phosphorylation in cells than gefitinib or lapatinib. KB (human buccal carcinoma) cells express high levels of EGFR with extremely low levels of erbB2 and erbB3, so when stimulated with EGFR ligands, the rapid activation and phosphorylation of EGFR occurs primarily through homodimerization. This assay thus determined the cellular inhibition of EGFR signaling without interference from other erbB family members. As expected, gefitinib displayed potent inhibition of EGFR phosphorylation [IC50, 11 nmol/L; 95% confidence interval ratio (CIR), 1.601]; however, AZD8931 was found to be significantly more potent (IC50, 4 nmol/L; 95% CIR, 1.377; P = 0.0025; Fig. 1). Lapatinib showed weaker EGFR inhibitory activity than either AZD8931 or gefitinib (IC50, 33 nmol/L; 95% CIR, 2.913) and was significantly less potent than AZD8931 (P = 0.0014; Fig. 1).

**Table 1. Structure and isolated kinase activities of AZD8931, gefitinib, and lapatinib**

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Structure</th>
<th>EGFR kinase activity [geometric mean IC50, (μmol/L); 95% CIR; P value from comparison with AZD8931]</th>
<th>ErbB2 kinase activity [geometric mean IC50, (μmol/L); 95% CIR; P value from comparison with AZD8931]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZD8931</td>
<td><img src="image" alt="AZD8931 Structure" /></td>
<td>0.012; 1.354</td>
<td>0.014; 2.074</td>
</tr>
<tr>
<td>Gefitinib</td>
<td><img src="image" alt="Gefitinib Structure" /></td>
<td>0.018; 1.307; 0.022</td>
<td>0.335; 4.271; 0.004</td>
</tr>
<tr>
<td>Lapatinib</td>
<td><img src="image" alt="Lapatinib Structure" /></td>
<td>0.302; 1.119; &lt;0.001</td>
<td>0.093; 3.012; 0.005</td>
</tr>
</tbody>
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AZD8931 also potently inhibits erbB2- and erbB3-mediated signaling in vitro. To better understand the erbB2 pharmacology of these agents in cells with a low level of EGFR (MCF-7), we explored their activity in two assay formats. The first, in parental MCF-7 cells, used heregulin to drive activation of erbB2 through heterodimerization with erbB3. The second modeled ligand-independent erbB2 signaling through spontaneous homodimerization as a consequence of the overexpression of erbB2 in MCF-7 cl24 cells.

In HRG-stimulated parental MCF-7 cells, gefitinib, as expected, was the least potent agent for erbB2 (IC\textsubscript{50}, 24 nmol/L; CIR, 2.827) and erbB3 (IC\textsubscript{50}, 95 nmol/L; CIR, 2.180) phosphorylation (Fig. 1). Lapatinib showed inhibition of erbB2 and erbB3 phosphorylation with IC\textsubscript{50} values of 9 nmol/L (CIR, 1.952) and 13 nmol/L (CIR, 2.270), respectively (Fig. 1). However, AZD8931 was the most potent inhibitor of erbB2 (IC\textsubscript{50}, 3 nmol/L; CIR, 1.817) and erbB3 (IC\textsubscript{50}, 4 nmol/L; CIR, 1.890) phosphorylation and was significantly more potent that either gefitinib or lapatinib for both erbB2 (P = 0.0016 and P = 0.0082) and erbB3 (P = 0.0002 and P = 0.007) phosphorylation, respectively.

In contrast to the ligand-driven parental MCF-7 cellular system, in the ligand-independent MCF-7 cl24 cells, lapatinib (IC\textsubscript{50}, 5 nmol/L; CIR, 1.36) was the most potent inhibitor of erbB2 phosphorylation compared with AZD8931 (IC\textsubscript{50}, 59 nmol/L; CIR, 1.29; P < 0.001) or gefitinib (IC\textsubscript{50} value of >300 nmol/L; data not shown).

Fig. 2. Scatter plots for NSCLC (A and B) and SCCHN (C and D) cell line panels—comparing AZD8931 versus gefitinib (A and C) and AZD8931 versus lapatinib (B and D). A panel of 20 head and neck tumor cell lines [KYSE-30, OE21, PE/CA-PJ15, PE/CA-PJ34 (clone C12), PE/CA-PJ41 (clone D2), PE/CA-PJ49, DOK, Detroit562, RPMI2650, SCC-4, SCC-25, CAL 27, SW579, FaDu, Hs 840.T, KB, KYSE-450, and HNS] and a panel of 16 NSCLC cell lines [PC-9, Calu-3, NCI-H2073, NCI-H1623, NCI-H522, NCI-H2085, NCI-H2030, NCI-H1703, NCI-H1291, NCI-H2135, NCI-H1975, NCI-H23, NCI-H1650, NCI-H1437, NCI-H2055, and NCI-H1666] were tested. Following a 96-h incubation with drug, GI\textsubscript{50} (\mu mol/L) was determined using the MTS Colorimetric Assay reagent as per the manufacturer’s instructions. Each experiment was carried out on three occasions for each drug concentration and data are presented as geometric means. The line on each plot represents the line of equality. Points above the line imply that AZD8931 is more potent as the GI\textsubscript{50} for AZD8931 is less than that of the comparator compound. Sensitivity groupings of GI\textsubscript{50} data were as follows: <1 \mu mol/L classed as sensitive, 1 to 7 \mu mol/L classed as intermediate, and >7 \mu mol/L classed as resistant.
AZD8931 shows a distinct pattern of tumor cell growth inhibition in NSCLC and head and neck squamous cell carcinoma cell panels. The activity of AZD8931, gefitinib, and lapatinib against the NSCLC cell lines differed greatly (>100,000×) across the panel, ranging from relative insensitivity (GI\textsubscript{50}, >10 μmol/L) in PC-9 cells harboring an EGFR activating mutation [exon 19 (Del E746-A750)]. Importantly, the activity profiles of gefitinib and lapatinib in these assays were distinct, with AZD8931 having a combined spectrum of activities. AZD8931 was statistically significantly more potent than gefitinib in PC-9 (P = 0.0001), NCI-H11975 (P = 0.009), NCI-H1703 (P = 0.020), and NCI-H2085 (P = 0.050) cell assays (Fig. 2A). Moreover, AZD8931 was significantly more potent than lapatinib in PC-9 (P < 0.0001) and NCI-H3255 (P = 0.0003) cell assays (Fig. 2B). Lapatinib was found to be most potent, relative to other NSCLC cells, in the Calu-3 cell line, which is consistent with its expression of high levels of erbB2. In addition to PC-9, several other cell lines harbored somatic EGFR mutations in their kinase domains including NCI-H3255 (L858R), NCI-H1650 (Del E746-A750), and NCI-H11957 (L858R, T790M). Lapatinib is significantly less potent than gefitinib and AZD8931 in the EGFR inhibitor-sensitive cell lines (NCI-H3255 and PC-9), suggesting weaker EGFR inhibitory activity of lapatinib under these conditions.

A similar spread in the activity of AZD8931, gefitinib, and lapatinib was seen in a panel of SCCHN cell lines, ranging from the highly sensitive KYSE-450 and HN5 cells (<1 nmol/L) to the insensitive Hs840.T, Hep-2, and Sc-9 cells (>10 μmol/L). As seen in the NSCLC panel, some SCCHN cell lines were significantly more sensitive to AZD8931 than to either gefitinib (DOK, P = 0.002; PE/CA-PJ41, P = 0.006; PE/CA-PJ49, P = 0.03; Fig. 2C) or lapatinib (DOK, P = 0.0001; FaDu, P = 0.003; HN5, P = 0.03; Fig. 2D). These data clearly show that AZD8931 is consistently the most potent erbB family inhibitor in these cell lines under these conditions.

Molecular analysis in selected SCCHN lines revealed that AZD8931, gefitinib, and lapatinib all showed inhibition of EGFR, erbB2, and erbB3 phosphorylation (PE/CA-PJ41, Fig. 3A; PE/CA-PJ49, Fig. 3B; DOK, Fig. 3C; and FaDu, Fig. 3D). In these cell lines, AZD8931 showed greater potency against phospho-EGFR, phospho-erbB2, and phospho-erbB3 than either lapatinib or gefitinib (all P values are <0.05 when comparing to AZD8931 except for gefitinib FaDu phospho-EGFR and lapatinib PJ49 phospho-erbB2). Lapatinib was more potent against phospho-erbB2 than phospho-EGFR in all lines.

AZD8931 inhibits the growth of EGFR-sensitive and erbB2-sensitive human tumor xenograft models. The ability of AZD8931 to inhibit human tumor xenograft growth in mice was tested in a variety of models with different sensitivities to agents targeting either EGFR or erbB2. Oral dosing of AZD8931 significantly inhibited BT474c (breast), Calu-3 (NSCLC), LoVo (colorectal), FaDu (SCCHN), and PC-9 (NSCLC) tumor xenograft growth.
(Fig. 4A, B, C, D, and E), showing that AZD8931 is active in xenograft tumor models responsive to EGFR inhibition alone (LoVo and PC-9) or EGFR or erbB2 inhibition (BT474c, Calu-3, and FaDu). No xenograft model selectively dependent on erbB2 alone was available. Furthermore, we found that lapatinib was not significantly active in LoVo (Supplementary Table S1) and was only modestly active in the EGFR mutant model PC-9 (Fig. 4E), even at maximum well-tolerated dose. In contrast, AZD8931 inhibited PC-9 tumor volume by 145% at a dose (6.25 mg/kg bid), well below its maximum well-tolerated dose (50 mg/kg bid; Supplementary Table S1;
Fig. 5. Pharmacodynamic effects of AZD8931, lapatinib, and gefitinib. Pharmacodynamic effects in (A) BT474c xenografts following AZD8931 acute treatment (100 mg/kg, p.o. qd × 4 d) assessing phospho-EGFR, phospho-erbB2, phospho-erbB3, phospho-AKT, phospho-ERK, and Ki67 (all by IHC). B, box plot of M30 expression (log % positive nuclei, assessed by IHC) in BT474c following AZD8931 acute treatment (100 mg/kg, p.o. qd × 4 d). C, FaDu following AZD8931 (50 mg/kg, p.o., bid × 21 d), lapatinib (100 mg/kg, p.o, bid × 21 d), or gefitinib (150 mg/kg, p.o, qd × 21 d) treatment. Phospho-EGFR, phospho-erbB2, and phospho-erbB3 were analyzed in tumor samples using a prototype multiplex MesoScale Discovery kit. The percentage inhibition of phosphorylation was compared with control expression. The percentage inhibition of tumor growth is indicated for each treatment condition; D, box plot of cleaved caspase-3 expression by IHC (log % positive nuclei) in LoVo following AZD8931 (50 mg/kg, p.o. bid × 13 d), lapatinib (100 mg/kg, p.o. bid × 13 d), or gefitinib (150 mg/kg, p.o. qd × 13 d) treatment. E, pharmacokinetic/pharmacodynamic relationship in LoVo xenograft. AZD8931 was dosed at 50 mg/kg (single dose) and a time course after acute dosing for 48 h was evaluated. Phospho-EGFR levels were determined by ELISA from frozen tumor samples. Total plasma drug levels and the percentage inhibition of phospho-EGFR compared with control at each time point are plotted.
Fig. 4E). Lapatinib was significantly active in the EGFR and erbB2 inhibitor–sensitive xenograft models BT474c, Calu-3, and FaDu, which is consistent with an erbB2-selective mode of action. A summary of all antitumor data generated across these five models can be seen in Supplementary Table S1.

AZD8931 causes pharmacodynamic changes in proliferation and apoptosis markers in human tumor xenograft models. Having determined the antitumor activity profile of these compounds, we evaluated their in vivo mechanism of action using specific EGFR, or EGFR- and erbB2-driven models. Using the BT474c xenograft model as a high erbB2-expressing example, pharmacodynamic analysis by IHC showed significant inhibition of EGFR ($P < 0.04$), erbB2 ($P = 0.024$), and erbB3 ($P < 0.001$) phosphorylation with AZD8931 compared with control (Fig. 5A). Furthermore, a significant reduction in the downstream signaling biomarker p-AKT ($P = 0.002$) and a significant decrease in Ki67 expression ($P < 0.001$) by IHC ex vivo analysis were also observed with AZD8931 (Fig. 5A). A reduction in the expression of p-ERK was also detected with AZD8931 compared with vehicle control xenografts, but this did not reach statistical significance.

Apoptosis was assessed by M30 evaluation. The M30 antibody recognizes a specific caspase cleavage site within cytokeratin (CK) 18 that is not detectable in the native CK18 of normal cells, and is a measure of an early apoptotic event (19). AZD8931, versus vehicle control xenografts, caused a significant induction of the M30 apoptosis marker ($P = 0.002$) at 1 h after four doses, which recovered to near control levels at 4 h after the fourth dose (Fig. 5B).

Pharmacodynamic activities for AZD8931, gefitinib, and lapatinib were also compared using the FaDu (Fig. 5C) and LoVo (Fig. 5D) xenograft models, which do not over-express erbB2. AZD8931, gefitinib, and lapatinib all showed significant inhibition of EGFR and erbB2 phosphorylation in the FaDu xenograft model ($P < 0.001$; Fig. 5C). AZD8931 ($P < 0.003$) and gefitinib ($P = 0.03$) were also able to inhibit erbB3 phosphorylation significantly compared with control. In contrast, lapatinib did not significantly inhibit the phosphorylation of erbB3 in this model. Apoptosis was then assessed in the LoVo model, and AZD8931 showed significant induction of apoptosis compared with control as measured by the levels of cleaved caspase-3 ($P < 0.001$; Fig. 5D). Furthermore, AZD8931 also showed a significantly greater proapoptotic effect compared with gefitinib ($P = 0.003$) and lapatinib ($P < 0.001$). The pharmacokinetic and pharmacodynamic relationship for AZD8931 was assessed using the LoVo xenograft model (Fig. 5E). There was a direct relationship between the total plasma levels of AZD8931 and the inhibition of EGFR phosphorylation observed.

These in vivo data support our in vitro findings characterizing AZD8931 as an orally bioavailable, equipotent inhibitor of EGFR, erbB2, and erbB3 signaling and that this inhibition leads to effects on downstream signaling intermediates, proliferation, and apoptosis markers. Taken together, the data show that AZD8931 has activity in a broader range of in vitro and in vivo systems than either gefitinib or lapatinib alone.

**Discussion**

Through careful characterization and the selection of discriminatory models, we have shown that AZD8931 is an effective simultaneous inhibitor of EGFR, erbB2, and erbB3 receptor signaling, particularly when erbB2 is not overexpressed. The results also show the pharmacological profile of AZD8931 to be distinct from those of gefitinib and lapatinib. Furthermore, AZD8931 clearly inhibits proliferative and survival signaling pathways (p-ERK and p-AKT) as well as inducing significant inhibition of proliferation (Ki67) and induction of apoptosis (M30 and cleaved caspase-3).

erbB3 has six known phosphotyrosine docking sites for the p85 subunit of phosphatidylinositol 3-kinase (7), which is a potent mediator of tumor cell survival and resistance to cancer therapies, partly through its involvement in the activation of AKT (20). The effect of AZD8931 on apoptosis may therefore be driven through its ability to inhibit the activation of erbB3 significantly.

One of the challenges to the development of novel inhibitors of erbB family signaling as anticancer agents is to establish meaningful distinction from the clinically active EGFR and erbB2 selective tyrosine kinase inhibitors. We have therefore carried out extensive characterization of AZD8931 and compared its activity to the selective EGFR inhibitor gefitinib and to lapatinib, which has been described as a dual EGFR and erbB2 inhibitor (21).

Our in vitro models included EGFR-driven cell line panels (NSCLC and SCCHN) in which AZD8931 was shown to inhibit proliferation significantly in a substantial proportion of cell lines. Furthermore, AZD8931 was shown to be equipotent or significantly more potent than the comparators in the majority of cell lines. These include two NSCLC cell lines (PC-9 and NCI-H3255) that carry the well-characterized EGFR-activating mutations known to confer sensitivity to gefitinib both preclinically (22, 23) and clinically (24–27). Both AZD8931 and gefitinib showed strong activity in these EGFR mutant cell lines, whereas lapatinib was significantly less potent. Under our experimental conditions and at maximum well-tolerated dose, lapatinib did not inhibit tumor growth or had a significantly lower antitumor effect against the EGFR-driven xenograft models (LoVo and PC-9, respectively) when compared with AZD8931 ($P \leq 0.01$). Mechanism of action analysis showed that the in vitro potency of AZD8931 is associated with an equipotent and simultaneous inhibition of EGFR, erbB2, and erbB3 phosphorylation, whereas gefitinib and lapatinib showed a less potent inhibitory activity against all three erbB receptors, with gefitinib being selective for EGFR compared with erbB2 and erbB3, and lapatinib being generally selective for erbB2 and erbB3 compared with EGFR.
This differential profile for AZD8931 was further exemplified by activity against EGF-stimulated KB cells (to evaluate inhibition of EGFR phosphorylation without interference from erbB2 or erbB3), HRG-stimulated MCF-7 cells (to evaluate inhibition of erbB2 and erbB3 phosphorylation without interference from EGFR), and finally, the MCF-7 cells engineered to overexpress erbB2 (MCF-7 cl24, to evaluate inhibition of erbB2 phosphorylation without interference from EGF or erbB3). These cell systems allowed an accurate assessment of AZD8931, gefitinib, and lapatinib potency based on their ability to inhibit receptor phosphorylation effectively when activated through ligand-driven homodimerization and heterodimerization or ligand-independent erbB2 overexpression. In EGF and HRG ligand–driven cell systems, AZD8931 is the most potent inhibitor of the three compounds tested. Interestingly, the data in MCF-7 cl24 cells show that when erbB2 is very highly expressed, and most likely homodimerized, lapatinib is more potent than AZD8931. Furthermore, in the NSCLC cell panel, lapatinib was found to be most active in the Calu-3 cells, consistent with the expression of high levels of erbB2 in that line, and equipotent to AZD8931 in the high erbB2-expressing xenograft models (Calu-3 and BT474c). Therefore, our data support the conclusion that HRG-induced erbB2/3 heterodimers favor AZD8931 whereas ligand-independent erbB2 homodimers favor lapatinib. These findings suggest that the erbB2 kinase active site may adopt slightly different conformations in homodimers and heterodimers, which differentially favor binding of these compounds. Thus, AZD8931 has been shown to have activity as an equipotent tyrosine kinase (TK) inhibitor against EGFR, erbB2, and erbB3 signaling across all these cellular models.

These preclinical observations are consistent with the growing knowledge about both gefitinib and lapatinib. For example, recent clinical data for first-line clinically selected (adenocarcinoma histology and never or ex-light smokers—a population enriched for EGFR mutation–positive tumors) NSCLC patients has shown significantly improved progression-free survival for gefitinib mono-therapy compared with carboplatin/paclitaxel. However, the effect was not constant over time, initially favoring carboplatin/paclitaxel and then favoring gefitinib, potentially driven by differences in progression free survival outcomes for patients with EGFR mutation–positive (gefitinib benefit) and EGFR mutation–negative (carboplatin/paclitaxel benefit) tumors (27). Lapatinib in combination with the aromatase inhibitor letrozole showed clinical benefit in erbB2-positive breast cancer similar to that seen with the erbB2 antibody trastuzumab in combination with another aromatase inhibitor, anastrozole (28, 29). Furthermore, a recent study in metastatic breast cancer detailed improved efficacy of paclitaxel in combination with lapatinib compared with paclitaxel in combination with placebo only in the erbB2-positive patient subset (30). In contrast, the expected activity in NSCLC for an EGFR inhibitor, particularly in patients with EGFR sensitivity characteristics, was not observed with lapatinib (31, 32), indicating that in clinical use, lapatinib shows selectivity for inhibition of erbB2 over EGFR. A similar clinical profile to that seen for lapatinib is also emerging for the so-called pan-erbB irreversible inhibitor neratinib (33, 34), which has shown a potent pan-erbB preclinical inhibitory profile in vitro (35, 36). This discrepancy suggests that standard in vitro methods may not be appropriate for pharmacological evaluation of such compounds when irreversible chemical reactivity with target molecules can drive the pharmacodynamics to breadth and degrees of inhibition not recapitulated in vitro. Our preclinical systems, particularly in vitro, were designed to define more rigorously a pan-erbB inhibitory profile (known EGFR, erbB2, and erbB3; erbB4 has not been profiled).

In view of the above, we believe that combined pharmacological inhibition of EGFR, erbB2, and erbB3 signaling has not yet been tested in the clinic, and that AZD8931 is an agent able to test the hypothesis that combined inhibition of signaling through these molecules could be of clinical utility in cancer, particularly in the majority of solid tumors that do not contain amplified erbB2 or mutated EGFR genes.

Disclosure of Potential Conflicts of Interest

All authors are employees or former employees of AstraZeneca and hold stock in the company. Mark Hickinson, Konstantina Grosios and Donald Ogilvie are former employees of AstraZeneca.

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AZD8931, an Equipotent, Reversible Inhibitor of Signaling by Epidermal Growth Factor Receptor, ERBB2 (HER2), and ERBB3: A Unique Agent for Simultaneous ERBB Receptor Blockade in Cancer

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