Treatment with Gefitinib or Lapatinib Induces Drug Resistance through Downregulation of Topoisomerase IIα Expression

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Abstract

The EGF receptor (EGFR) is therapeutically targeted by antibodies and small molecules in solid tumors including lung, colorectal, and breast cancer. However, chemotherapy remains important, and efforts to improve efficacy through combination with targeted agents is challenging. This study examined the effects of short and long durations of exposure to the EGFR- and HER2-targeted tyrosine kinase inhibitors (TKI) gefitinib and lapatinib, on induction of cell death and DNA damage by topoisomerase IIα (Topo IIα) poisons, in the SK-Br-3 HER2-amplified breast cancer cell line. Short exposure to either gefitinib or lapatinib for 1 hour did not affect the induction of apoptosis by the Topo IIα poisons doxorubicin, etoposide, and m-AMSA. In contrast, cells treated for 48 hours were resistant to all three drugs. Short exposure (1 hour) to TKI did not alter the number of DNA single- or double-strand breaks (DSB) induced, whereas longer exposure (48 hours) reduced the number of DNA DSBs and the formation of γ-H2AX foci. Both gefitinib and lapatinib reduced the expression and activity of Topo IIα at 48 hours. Studies using a cell line with inducible downregulation of Topo IIα showed that expression of Topo IIα, and not Topo IIβ, determined the number of DNA strand breaks induced by these chemotherapeutic agents. These results indicate that prolonged exposure to TKIs targeting EGFR and HER2 induce resistance to doxorubicin, etoposide, and m-AMSA through downregulation of Topo IIα. This may explain why their addition to chemotherapy regimens have not increased efficacy.

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Introduction

The development of targeted therapies marked a major advance in the systemic treatment of cancer. However, despite their success, these agents are only useful in the management of a minority of patients and cytotoxic chemotherapy remains an important treatment modality in the management of solid tumors including lung, gastric, breast, and colorectal cancers. In an effort to increase the efficacy of chemotherapy, a number of phase III studies have assessed their combination with targeted drugs in a variety of solid tumors; only a few of these combinations have been successful.

Several drugs target members of the human EGF receptor (EGFR/HER) family. This family comprises 4 membrane receptors, EGFR (HER1/erbB1), HER2 (erbB2), HER3 (erbB3), and HER4 (erbB4) and they activate key cell signaling pathways controlling cell growth, proliferation, migration, apoptosis, and resistance to cytotoxic agents (1). Malignant tumors can be driven by HER signaling through the gain of activating receptor mutations [e.g., EGFR mutations in non–small cell lung cancer (NSCLC)], receptor overexpression (e.g., HER2 amplification in breast cancer), and increased ligand secretion. In addition, EGFR mediates resistance to DNA-damaging agents, including irradiation (IR) and cisplatin, through the activation of cell survival pathways and modulation of DNA protein kinase (DNA-PK; refs. 2–5), a key protein involved in the repair of DNA damage through the non-homologous end-joining pathway.

The receptors EGFR and HER2 can be targeted using monoclonal antibodies (e.g., cetuximab and trastuzumab) or small-molecule tyrosine kinase inhibitors (TKI; e.g., gefitinib, erlotinib, and lapatinib). In vitro, TKIs sensitize cells to the cytotoxic effects of anticancer therapies through the inhibition of HER signaling (6), multidrug cotransporters (7), and DNA repair (8, 9). Despite the effectiveness of combining TKIs with DNA damaging agents in the preclinical setting, their...
translation into the clinic has not produced the results anticipated.

Drugs that target the enzyme topoisomerase IIα (Topo IIα) such as doxorubicin, epirubicin, and etoposide play an important role in the treatment of a number of cancers. Studies indicate that there are differences between the effects of the monoclonal HER2-targeted antibody trastuzumab and TKIs, when combined with standard anthracycline-containing chemotherapy in breast cancer. While trastuzumab increases the efficacy of doxorubicin and cyclophosphamide (10), the addition of gefitinib to epirubicin and cyclophosphamide (EC) is not beneficial (11). In combination with neoadjuvant EC plus docetaxel, lapatinib produced significantly fewer responses than trastuzumab (12), indicating that while targeting HER2 with a monoclonal antibody in combination with chemotherapy is efficacious, the inhibition of HER2 with a TKI is not.

At clinically relevant concentrations of TKIs, despite the initial inhibition of EGFR and HER2 phosphorylation and their downstream signaling, continued exposure results in reactivation of AKT signaling and tumor growth (13–15). This process occurs in HER2-amplified tumors and is driven by the dephosphorylation of HER2, which increases the secretion of HER ligands, shifts the dephosphorylation equilibrium of HER3, and promotes the formation of alternative dimers by HER receptors (13–15). These observations may explain why schedule is important when combining TKIs with chemotherapy, with intermittent scheduling being more efficacious than continuous combined treatment (19, 20).

To study whether duration of exposure to the TKIs gefitinib or lapatinib altered the efficacy of the Topo IIα-targeted drugs doxorubicin, etoposide, and m-AMSA, the ability of these drugs to induce cell death were investigated in the HER2-amplified SK-Br-3 breast cancer cell line.

Materials and Methods
Cell line and tissue culture
SK-Br-3 cells were obtained from Cancer Research UK, Clare Hall Laboratories, London Research Institute, London, UK, and immediately expanded upon receipt and stored in liquid nitrogen. The cell line was not authenticated following receipt, but cells were cultured for less than 3 months following resuscitation and were maintained in McCoy’s 5A medium modified (Sigma-Aldrich) supplemented with 2 mmol/L l-glutamine (Autogen Bioclear), 100 units/mL penicillin and streptomycin (Sigma-Aldrich), and 10% fetal calf serum (FCS; Autogen Bioclear).

The HTETOP cell line is a human fibrosarcoma HT1080 cell line in which Topo IIα expression can be depleted by the addition of tetracycline. The creation of this cell line has been described previously (21). Cells were maintained in Dulbecco’s Modified Eagle’s Media (DMEM), 10% FCS, 1 mmol/L sodium pyruvate (Sigma-Aldrich, UK), 2 mmol/L l-glutamine, 4% MEM amino acid solution (Gibco), and 200 μg/mL G418 (Sigma-Aldrich).

Drug treatment
All drugs were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mmol/L and then in cell culture media to reach the final drug concentration. Clinical-grade gefitinib (Iressa/ZD1839 AstraZeneca) was used at a concentration of 5 μmol/L and lapatinib at 2 μmol/L (Tykerb/GW572016 GSK). Doxorubicin, etoposide, m-AMSA, doxycycline, and N-acetylcycteine (NAC; Sigma-Aldrich) were used at the concentrations indicated. Cells were exposed to doxorubicin or etoposide for 2 hours and m-AMSA for 1 hour, following which media were replaced with fresh media containing vehicle control or TKI as required. In cells treated for 48 hours, the media were replaced at 23 and 47 hours with fresh media ± TKI.

Western blotting
Cells were treated as required, washed twice in cold PBS, pH 7.3 (Sigma-Aldrich), scraped, collected, and pelleted by centrifugation at 1500 rpm at 4°C. The cell pellet was resuspended in 60 to 300 μL of lysis buffer (Celllytic, Sigma-Aldrich) containing phosphatase (PhosStop) and protease inhibitor cocktails (Roche) and incubated on ice for 15 minutes. Protein concentrations were determined using a protein assay (Bio-Rad Laboratories). Proteins (25–50 μg) were separated using pre-cast 7% Tris acetate gels (Invitrogen) and transferred onto Immobilon-P membranes (Millipore), at room temperature. Primary antibodies were anti-EGFR antibody, anti-pEGFR1173, anti-HER2, anti-pHER21221, anti-HER3, anti-pHER31289, anti-AKT, anti-AKT473 (Cell Signaling Technology), anti-α-tubulin (Sigma-Aldrich), anti-topoisomerase IIα (kind gift from Ian Hickson, Weatherall Institute of Molecular Medicine, Oxford, UK), and anti-topoisomerase IIβ (BD Biosciences). Secondary antibodies against mouse and rabbit were used (Cell Signaling Technology) and immunoreactive bands visualized using chemiluminescence detection (ECL system, Amersham Biosciences).

Comet assays
Single-cell gel electrophoresis (comet) assays were used to assess total DNA strand breaks (alkaline assay) and DNA double-strand breaks (DSB; neutral assay). To examine strand break induction, cells were processed immediately following incubation with the Topo II poison under investigation. Cells were washed twice in ice-cold PBS, trypsinized (Autogen Bioclear), pelleted, and frozen in 10% FCS and DMSO and stored at −80°C. For the alkaline and neutral comet assays, cells were processed as described previously (22, 23), except that in the neutral comet assay, an extra step of protein digestion with proteinase K (Roche) for 2 hours at 37°C was added following lysis. Slides were visualized using an inverted fluorescence microscope and captured with a digital
camera. For the alkaline comet assay, 25 cells were analyzed and 50 cells for the neutral comet assay from each of 2 duplicate slides. The tail moment for each cell was calculated using the Komet 5.5 Analysis software (Andor Technology, Kinetic Imaging).

Measurement of γ-H2AX foci
Cells were grown in 8-well LabTek II chamber slides (Nalge Nunc) and treated with gefitinib as documented above for the required duration. Following incubation with Topo II poison, the media were removed, replaced with fresh media ± TKI and cells left for the required duration, with TKI replacement every 24 hours. Slides were then processed and analyzed as described previously (24). Images were visualized with a Zeiss LSM510 fluorescence microscope (100× oil immersion objective), equipped with a cooled charge-coupled device (CCD) camera and 2 detector channels with 488 nm Argon ion and 543 nm HeNe excitation lasers. Foci were counted in 50 cells from each slide and expressed as mean number of foci per cell from 3 independent experiments.

Fluorescence-activated cell sorting (FACS) was used to conduct cell-cycle analysis, measure intracellular doxorubicin, and apoptosis. Following drug treatment, cells were collected by trypsinization and processed as documented below. Samples were analyzed using a flow cytometer (CyAn ADP) and histograms or dot plots constructed using Summit v4.3 software (USA).

Cell-cycle analysis
Cells were fixed in cold 70% (v/v) ethanol and incubated at −20°C. Before analysis cells were pelleted, resuspended in a solution of 50 μg/mL propidium iodide, 0.1 mg/mL RNase A, 0.05% Triton X-100, and PBS (all Sigma-Aldrich), and incubated for 40 minutes at 37°C. Cells were pelleted and resuspended in ice-cold PBS for analysis. DNA content was estimated by gating histograms generated in the FL-2 area variable.

Measurement of doxorubicin fluorescence
Following collection, cells were resuspended in ice-cold PBS, stored on wet ice, and analyzed within 30 minutes. Doxorubicin is naturally fluorescent, this was measured using the FL-2 channel, histograms constructed of FL-2 area and the mean fluorescence intensity calculated as described previously (25). The fluorescence from controls treated with gefitinib alone was used to normalize the data.

Annexin V measurement
Following collection, cells were stained with fluorescein isothiocyanate (FITC) labeled Annexin V as per the manufacturer’s instructions (ApoTox Detection Kit, BioVision). Instead of using propidium iodide, Sytox Red dead cell stain (Invitrogen) was used, as propidium iodide emits fluorescence in the same spectrum as doxorubicin. Unstained and apoptosis controls were used to set boundaries between live, necrotic, early, and late apoptotic cells.

Trypan blue assessment of cell viability
Cell pellets were resuspended in media and mixed thoroughly by repeated pipeting to produce a single-cell suspension. Fifty microliters of cell suspension was mixed 1:2 with 50 μL of 0.4% trypan blue stain (Sigma-Aldrich) for 1 to 2 minutes to identify dead cells.

Topoisomerase II activity assay
Topo II activity was assessed using a decatenation assay (TopoGen). Following treatment with gefitinib for the required duration, nuclear extracts were obtained as per the manufacturer’s protocol and the protein concentration determined. The same concentration of nuclear proteins was then examined in accordance with the manufacturer’s protocol. Images were visualized with a dual-intensity ultraviolet transilluminator and images captured with a digital camera.

Quantitation and statistical analysis
Quantitation of Western blot analyses and decatenation assays were conducted using ImageJ (U.S. NIH); data are presented as mean ± SEM for at least 3 independent experiments. Values are expressed as a fraction of the control value that is assigned the value 1.0. Statistical analysis was conducted with paired t test, one- and two-way ANOVA using GraphPad Prism v5 software. P < 0.05 was considered significant.

Results

Effect of gefitinib exposure on the induction of apoptosis by doxorubicin, etoposide, and m-AMSA

Treatment with gefitinib (5 μmol/L) inhibits EGFR, HER2, and HER3 signaling within 1 hour but with continued exposure, HER3 signaling is reactivated by 48 hours (13). To investigate whether the duration of exposure to gefitinib altered chemotherapy-induced cell death, Annexin V expression was measured in SK-Br-3 cells treated with gefitinib for 1 or 48 hours, before exposure to the Topo IIα poisons doxorubicin, etoposide, and m-AMSA.

In cells treated with drug-free media (DFM) for the duration of the experiment (72 hours), 17.1% ± 3.8% were identified as undergoing apoptosis. Following treatment with doxorubicin (5 μmol/L), etoposide (50 μmol/L), or m-AMSA (5 μmol/L), this increased to 59.5% ± 3.0%, 43.0% ± 8.1%, and 56.5% ± 4.7%, respectively (Fig. 1A). Treatment with gefitinib for 1 hour did not increase the level of apoptosis over DFM and did not alter the induction of apoptosis by doxorubicin, etoposide, or m-AMSA (Fig. 1A). In contrast, in cells treated with gefitinib for 48-hour doxorubicin, etoposide and m-AMSA failed to increase apoptosis over that induced by gefitinib for 48 hours alone. These results indicate that gefitinib treatment for 48 hours renders cells resistant to the cytotoxic effects of doxorubicin, m-AMSA, and etoposide.

HER Inhibitors Induce Resistance to Topoisomerase II Poisons
Effect of gefitinib exposure on the induction of γ-H2AX foci by doxorubicin, etoposide, and m-AMSA

Doxorubicin, m-AMSA, and etoposide target Topo IIα producing DNA strand breaks (26). These can be assessed by measuring γ-H2AX foci, which are formed when the H2AX protein is phosphorylated at sites flanking a DNA DSB. The formation of these foci signal the presence and location of a DNA DSB, recruiting DNA repair proteins (27).

Initial experiments determined the time at which γ-H2AX formation peaked following exposure to the Topo II poison. Representative images at the peak time of γ-H2AX formation are shown in Fig. 1B and C. The induction of foci over a 24-hour period is observed in Supplementary Fig. S1. Following exposure to doxorubicin (0.5 μmol/L) for 2 hours, γ-H2AX foci formation peaked 6 hours later at 61.1 ± 7.0 foci/cell (Fig. 1C).

In cells pretreated with gefitinib for 1 hour, foci formation peaks earlier at 4 hours at 48.3 ± 9.6 foci/cell (P < 0.05) when compared with doxorubicin alone. In cells pretreated with gefitinib for 48 hours, foci formation also peaks at 6 hours at a level of 7.3 ± 3.3 (P < 0.05) foci/cell (Fig. 1C).

The treatment of cells for one or 48 hours with gefitinib did not alter the formation of γ-H2AX foci in response to IR (2 Gy; Fig. 1B and C), indicating that gefitinib for 48 hours does not inhibit γ-H2AX formation per se.

At a clinically relevant concentration of etoposide (15 μmol/L) for 2 hours, γ-H2AX foci expression peaked 2 hours later, at a level of 64.4 ± 11 foci/cell with 54.5 ± 3.9 foci/cell (P < 0.05) in cells treated with gefitinib for 1 hour before etoposide. Following gefitinib treatment for 48 hours, the peak number of foci is observed at time zero at 9.6 ± 3.0 foci/cell (P < 0.05; Fig. 1C). Following m-AMSA (0.5 μmol/L) for 1 hour, γ-H2AX foci induction peaked 4 hours later at 39.3 ± 3.5 foci/cell with 29.1 ± 4.5 foci/cell (P < 0.05) in cells exposed to gefitinib for 1 hour before m-AMSA exposure. Following gefitinib pretreatment for 48 hours, the peak of foci induction is observed straight after drug removal at 7.1 ± 3.9 foci/cell (P < 0.05; Fig. 1C). These results show that in cells treated with gefitinib for 48 hours, significantly fewer DNA DSBs are formed in response to the 3 Topo IIα-targeted agents investigated, yet no significant differences are observed in response to IR, under the same conditions.

Effect of gefitinib exposure on the induction of DNA DSBs by doxorubicin, etoposide, and m-AMSA

The previous results show a reduction in γ-H2AX foci following doxorubicin, etoposide, or m-AMSA in cells treated with gefitinib for 48 hours. This is due to a reduction in DNA damage and not the inhibition of γ-H2AX foci production by gefitinib. To investigate this further, the nature of the DNA strand breaks induced by these drugs was examined using the alkaline and neutral comet assays. The single-cell gel electrophoresis (comet) assay allows the assessment of DNA damage directly, in contrast to a surrogate marker such as γ-H2AX.
Treatment of cells with gefitinib for 1 hour did not alter the total number of DNA strand breaks induced by doxorubicin, etoposide, or m-AMSA at all drug concentrations investigated (Fig. 2A). In contrast, treatment with gefitinib for 48 hours significantly reduced the numbers of DNA strand breaks induced by doxorubicin and etoposide but not m-AMSA (Fig. 2A). In addition, following gefitinib treatment for 48 hours, a dose-dependent increase in the numbers of DNA strand breaks was observed with etoposide, which did not occur with doxorubicin (Fig. 2A).

The neutral comet assay showed that treatment of cells with gefitinib for 1 hour did not alter the ability of all 3 Topo IIα poisons to induce DNA DSBs. In contrast, continuous treatment of cells with gefitinib for 48 hours significantly decreased the production of DNA DSBs by doxorubicin, etoposide, and m-AMSA (Fig. 2B).

Both the neutral comet assay and measurement of γ-H2AX foci show that in cells treated with gefitinib for 48 hours, m-AMSA induced significantly fewer DNA DSBs. The Olive tail moment in the alkaline comet assay measures both single and double DNA strand breaks and this assay shows that m-AMSA is able to induce the same number of DNA strand breaks irrespective of TKI treatment. Therefore, the difference in strand break induction observed between the alkaline comet assay compared with measurement of γ-H2AX foci and neutral comet assay indicates that in cells treated with gefitinib for 48 hours, m-AMSA induces single-strand breaks, explaining why the tail moment is not significantly altered, despite the fall in DSBs induced under these conditions.

m-AMSA can produce DNA strand breaks through the production of reactive oxygen species (ROS; ref. 28), which are also induced by gefitinib (13). To investigate whether an increase in ROS accounted for the differences in DNA strand break induction observed with the alkaline and neutral comet assays with m-AMSA, the effect of the free radical scavenger NAC was investigated.

The addition of NAC did not significantly affect the induction of DNA strand breaks m-AMSA in cells treated with gefitinib for 48 hours. However, a small but significant reduction in total DNA strand breaks induced by etoposide (Fig. 2C) was observed following the addition of NAC. Therefore, following gefitinib treatment for 48 hours, a small proportion of etoposide-induced DNA strand breaks are due to ROS; this is not the case with m-AMSA.

The dependence on Topo IIα expression by doxorubicin, etoposide, and m-AMSA for the induction of DNA strand breaks

Etoposide, doxorubicin, and m-AMSA interact with both Topo II isoforms to produce DNA strand breaks...
but the reported targeting of Topo IIα relative to Topo IIβ varies depending upon the method of assessment (29–32). To investigate the effects of the Topo II poisons used in this study on the formation of Topo IIβ–induced DNA strand breaks, a cell system in which Topo IIα expression can be reduced was used. The HTETOP cell line is derived from a human fibrosarcoma cell line in which endogenous Topo IIα has been disrupted and a tetracycline-controlled exogenous Topo IIα transfected. This allows the suppression of Topo IIα expression by the addition of tetracycline (21).

Treatment of cells with 1 μg/mL doxycycline for 24 hours reduced but did not abolish Topo IIα expression (Fig. 3A). The ability of doxorubicin, etoposide, and m-AMSA to induce DNA strand breaks following reduction in Topo IIα expression was investigated using the alkaline comet assay in cells treated with and without doxycycline. A significant reduction (74%–95%) in the production of DNA strand breaks by doxorubicin (Fig. 3B), etoposide (Fig. 3C), and m-AMSA (Fig. 3D) in cells treated with doxycycline compared with untreated cells was observed. These results indicate that DNA strand breaks produced by doxorubicin, etoposide, and m-AMSA primarily occur through targeting of Topo IIα.

**Effect of gefitinib exposure on levels of intracellular doxorubicin**

As shown above, in cells treated with gefitinib for 48 hours, levels of apoptosis induced by doxorubicin, etoposide, and m-AMSA are reduced because of significant reduction in their ability to produce DNA DSBs. Resistance to Topo II poisons can be produced by a number of mechanisms including a reduction in intracellular drug concentration, decreased Topo IIα expression or activity, and/or cell-cycle arrest (28). Topo IIα poisons are substrates of the ATP-binding cassette ABC transporters and resistance to TKIs can also occur through this mechanism (7, 28). To investigate the effects of gefitinib on intracellular doxorubicin concentration, intracellular doxorubicin fluorescence was measured using FACS. A 17.4% ± 1.1% decrease in doxorubicin fluorescence was observed in cells treated with gefitinib for 48 hours compared with cells treated with doxorubicin alone, with a 4.5% ± 2.1% decrease observed in cells treated with gefitinib for 1 hour (Fig. 4A). These data confirm that doxorubicin is not completely extruded from cells treated with gefitinib for 48 hours and the resistance to Topo IIα poisons cannot be explained solely by reduced intracellular drug concentration.

**Effect of gefitinib on cell-cycle distribution and topoisomerase II expression**

Another mechanism of resistance to Topo IIα poisons is cell-cycle arrest and consequent reduction in Topo IIα expression (28). In untreated proliferating cells, 62.8% ± 0.9% were in the G0–G1 phase of the cell cycle. Following continuous exposure to gefitinib, this increased to 83.6% ± 1.9%, 83.6% ± 2.5%, and 79.7% ± 2.6% at 24, 48, and 72 hours, respectively, with a corresponding decrease in cells in the S and G2 cell-cycle phases (Fig. 4B).

Figure 3. Doxorubicin, etoposide, and m-AMSA are dependent upon Topo IIα to induce DNA strand breaks. A, Western blotting was used to assess the expression of Topo IIα and Topo IIβ in the HTETOP cell line ± 1 μg/mL doxycycline for 24 hours. Relative densitometry values ± SEM are indicated for Topo IIα from 4 separate experiments. After incubation for 24 hours, cells were treated with 5 μmol/L doxorubicin for 2 hours (B), 150 μmol/L etoposide for 2 hours (C), or 5 μmol/L m-AMSA for 1 hour (D). Cells were then collected and DNA strand breaks assessed using the alkaline comet assay. Data are presented as mean ± SEM of 3 independent experiments. *, P < 0.05 compared with cells treated with doxorubicin, etoposide, or m-AMSA alone.
Topo II expression is regulated by the cell cycle, with levels increasing during S-phase and falling in G0–G1 and is a mechanism through which resistance to Topo II poisons can be mediated (28). Gefitinib reduced the expression of Topo IIα within 24 hours with little protein detectable at 48 hours, whereas the expression of Topo IIβ remained unchanged (Fig. 4C). These results indicate that cells treated with gefitinib for 48 hours enter G0–G1 cell-cycle arrest and downregulate Topo IIα expression. This may contribute to decreased efficacy of doxorubicin, etoposide, and m-AMSA.

Effect of lapatinib on the production of DNA strand breaks by doxorubicin, etoposide, and m-AMSA

Like gefitinib, the dual EGFR and HER2 TKI lapatinib also fails to inhibit Akt signaling following continuous exposure for 48 hours, AKT was reactivated, though to a lesser extent than observed with gefitinib (Fig. 5B). Lapatinib for 48 hours also results in a G0–G1 cell-cycle arrest (Supplementary Fig. S2).

Lapatinib for 48 hours increased apoptosis compared with untreated cells and induced resistant to doxorubicin, etoposide, and m-AMSA. Both etoposide and m-AMSA were unable to increase apoptosis over that produced by lapatinib alone and, in the case of doxorubicin, less apoptosis was observed (Fig. 5C). Doxorubicin and etoposide induced significantly fewer DNA strand breaks in cells treated with lapatinib for 48 hours, compared with cells treated with either Topo II poison alone or following lapatinib for 1 hour (Fig. 5D). As found with gefitinib, exposure to lapatinib did not significantly alter the induction of DNA strand breaks by m-AMSA (Fig. 5D). In addition, like gefitinib, pre-treatment of cells with lapatinib for 48 hours significantly reduced the numbers of DNA DSBs induced by doxorubicin, etoposide, and m-AMSA using the neutral comet assay (Fig. 5E).

Effect of gefitinib and lapatinib on topoisomerase IIα expression and activity

As discussed above, resistance to Topo II poisons can be induced by a reduction in Topo II expression or its
activity. Western blotting shows that Topo IIα expression decreased in cells treated with either gefitinib (5 μmol/L) or lapatinib (2 μmol/L) for 48 hours (Fig. 6A). A decatenation assay was used to investigate whether the observed reduction in Topo IIα expression following prolonged exposure to TKI translated into a reduction in cellular Topo II activity. Cells treated with either gefitinib or lapatinib for 48 hours contained reduced decatenation activity compared with untreated cells or those treated with TKI for 1 hour (Fig. 6B–D). Given that only Topo IIα expression decreased following TKI treatment, the reduction in the ability of nuclear extracts to decatenate DNA is due to a reduction in Topo IIα activity within cells treated with gefitinib or lapatinib for 48 hours.

**Discussion**

There is a clear rationale for the combination of HER-targeted TKIs with chemotherapy based upon the correlation between EGFR expression and resistance to chemotherapy (34, 35), the ability of transfected EGFR to confer chemotherapy resistance in cancer cell lines (36) and the observation that EGFR is phosphorylated in response to chemotherapy (6, 37).
The TKIs gefitinib, lapatinib, and erlotinib inhibit EGFR, HER2, HER3, and PI3K/AKT signaling within 1 hour, but with continuous exposure over 48 hours PI3K/AKT signaling reactivates despite continued inhibition of EGFR and HER2. This has been implicated as a mechanism of resistance to these drugs (13, 16). We hypothesized that the induction of cell death by chemotherapy drugs would be different in cells exposed to TKI for a short duration (1 hour, when AKT signaling is inhibited), compared with longer exposure (48 hours, when AKT signaling is reactivated) and that this may explain why combinations of DNA-damaging drugs with TKIs have proved successful in vitro, when exposure to TKI tends to be short, but not in the clinic when exposure is continuous.

The results presented here show that the treatment of cells continuously with gefitinib or lapatinib for 48 hours renders them resistant to the cytotoxic effects of the Topo II poisons, doxorubicin, etoposide, and m-AMSA. This resistance is modulated through the downregulation of Topo IIα expression and activity, thereby inhibiting the production of DNA DSBs by these drugs. Despite this, total DNA strand break induction by m-AMSA is unaffected indicating an increase in single-strand break induction in conditions when DNA DSB induction is inhibited.

Doxorubicin and m-AMSA target both Topo II isoforms equally, whereas etoposide shows a preference for Topo IIα (29). However, cytotoxicity assays indicate that etoposide and doxorubicin are dependent upon Topo IIα expression to induce cell death (30), whereas resistance to m-AMSA can be produced by the knockdown of Topo IIβ (31, 32). To investigate the role of Topo IIα in mediating the production of DNA strand breaks by doxorubicin, etoposide, and m-AMSA, a cell system in which Topo IIα expression can be knocked down was used. This showed that all 3 Topo II poisons were dependent on Topo IIα expression to produce DNA strand breaks.
In addition to reduced expression of Topo II protein, resistance to Topo IIα poisons can occur through decreases in the nuclear concentration of Topo II and alterations in phosphorylation (28). As Topo IIα down-regulation is observed following treatment with both gefitinib and lapatinib, which differ in the level of AKT/HER3 reactivation, this is not the mechanism of the alteration in Topo IIα expression.

Topo IIα expression is regulated by the cell cycle, with increased expression detected in proliferating cells in the S and G2–M phases of the cell cycle. The observed down-regulation of Topo IIα in this study may be due to the induction of cell-cycle arrest by TKIs, or due to TKIs modulating Topo IIα expression. The precise mechanisms through which cell-cycle arrest is mediated by TKIs are yet to be fully elucidated, although the dephosphorylation of the forkhead transcription factor FOXO3a in response to the inhibition of AKT phosphorylation has been implicated (38). In SK-Br-3 cells, gefitinib induces the transport of unphosphorylated FOXO3a back into the nucleus. This in turn increases the expression of the cell-cycle control protein p27kip1, producing G1 cell-cycle arrest (38) and reducing the gene and protein expression of forkhead transcription factor, FOXM1 (39). FOXM1 has been shown to bind to the promoter of the mouse Topo IIα gene and activate its transcription (40).

Overexpression of FOXM1 has been linked to the overexpression of HER2 in both breast cancer cell lines and tissue, and the knockdown of HER2 reduces the expression of FOXM1 (39). Lapatinib reduces the expression of FOXM1 within 12 hours, with G1 cell-cycle arrest occurring later, indicating that the decrease in expression is not due to cell-cycle arrest (39). This is therefore a possible mechanism through which gefitinib could reduce the expression of Topo IIα.

Topo II activity is controlled through phosphorylation, with the phosphorylating site at ser1106 within the catalytic domain of Topo IIα important in conferring sensitivity to etoposide and m-AMSA. Amino acid substitution at this site produces a reduction in the formation of etoposide-induced cleavable complexes (41). This site is phosphorylated by the serine/threonine kinase casein kinases 1δ and 1ε (42), with casein kinase 1δ shown to be inhibited by gefitinib in vitro (43). Together, these data support the modulation of Topo II activity and its expression by TKIs, aside from their effects on the cell cycle, phosphorylated HER3 and AKT.

Despite a reduction in Topo IIα expression following gefitinib treatment for 48 hours, etoposide is still able to induce a concentration-dependent increase in both single and double DNA strand breaks as detected by both the alkaline and neutral comet assays. This indicates that the reduction in strand breaks induction is not just attributable to a reduction in the expression of Topo IIα but may be due to reduced affinity of etoposide for Topo II, which is overcome by higher concentrations of the drug. This observation is in contrast to both doxorubicin and m-AMSA, as the neutral comet assay does not show a concentration-dependent increase in the number of DSBs produced by either drug in cells treated with gefitinib for 48 hours, indicating that higher drug concentrations are unable to increase DNA DSB production.

These differences may be explained by the targeting of different sites on Topo IIα. Etoposide binds to Topo II at 2 sites, one contained in the catalytic core and the other in the ATP-binding N-terminal domain (44). To form a DSB, an etoposide molecule is required to bind each of the 2 Topo II molecules that make up the Topo II homodimer, with single-strand breaks formed if only one Topo II molecule is targeted (45). Both doxorubicin and m-AMSA exert base preferences within the Topo II–DNA complex and target the Topo II protein when it is bound to DNA at a single site (26, 28).

Finally, in cells treated with either lapatinib or gefitinib for 48 hours, the total number of DNA strand breaks induced by m-AMSA is not significantly reduced, despite the inhibition of DNA DSB induction. A number of studies examining the mechanism of action of Topo II poisons have identified that m-AMSA differs to the more commonly clinically used Topo II poisons doxorubicin, etoposide, and epirubicin, which target the forkhead transcription factor FOXO3a in response to the inhibition of DNA DSB induction. A number of studies examining the mechanism of action of Topo II poisons have identified that m-AMSA differs to the more commonly clinically used Topo II poisons doxorubicin, etoposide, and epirubicin. These differences include the induction of a greater number of single-stranded DNA breaks for each DSB (46, 47), targeting of Topo IIβ to produce DNA strand breaks (31, 32), the ability to produce strand breaks despite ATP depletion (48) and targeting Topo II mutants that are resistant to other Topo II poisons, to induce single-stranded DNA breaks (49).

The influence of scheduling on the efficacy of combining TKIs with traditional cytotoxic chemotherapy drugs is gaining recognition. Not only does scheduling appear to be important, but also the signaling dependency of a particular tumor. Lee and colleagues showed that in the HER2-amplified breast cancer cell line MDA-MB-453, erlotinib scheduled for 24 hours before doxorubicin reduced apoptosis compared with doxorubicin alone, doxorubicin before erlotinib, or both drugs simultaneously (50). In contrast, in the triple-negative (HER2, estrogen, and progesterone receptor negative) breast cancer cell line BT-20, erlotinib scheduled before doxorubicin induced the greatest level of apoptosis compared with the schedules of both drugs continuously or doxorubicin followed by erlotinib (50). They show that it is not the transient inhibition of EGFR itself that is important, but the ability of prolonged inhibition of signaling by TKI to modulate the EGFR signaling pathway.

The results presented here show that the TKIs gefitinib and lapatinib render cells resistant to the cytotoxic effects of doxorubicin, etoposide, and m-AMSA through the induction of cell-cycle arrest, inhibition of Topo II activity, and downregulation of Topo IIα expression. Whether the effect on Topo IIα expression is merely due to the induction of cell-cycle arrest or a direct effect on Topo IIα requires further investigation. The in vitro data presented here may have important clinical implications in the scheduling of drugs used in the management of cancer...
and indicate that TKIs may significantly affect cellular response to Topo II poisons.

Disclosure of Potential Conflicts of Interest
A. Makris has honoraria from speakers bureau as the Speaker for GSK-sponsored meetings. D. Hochhauser is conducting a clinical study in collaboration with AstraZeneca but has not received any personal remuneration. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

References

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Performed additional experiments addressing the reviewers’ comments: K. Kiakos
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