The epidermal growth factor receptor tyrosine kinase inhibitor gefitinib sensitizes colon cancer cells to irinotecan

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Epidermal growth factor receptor (EGFR) overactivity plays a significant role in colon cancer biology and has been associated with poor clinical prognosis. Early clinical trials reported efficacy of receptor-targeted compounds, including modulation of clinical irinotecan resistance. We investigated the effects of the EGFR tyrosine kinase inhibitor gefitinib on cellular determinants of irinotecan resistance in human colon cancer cells. At non-cytotoxic concentrations, gefitinib sensitized colon cancer cells to SN-38, the active metabolite of irinotecan. Gefitinib increased the SN-38-mediated induction of protein-linked DNA single-strand breaks in a dose-dependent manner, with no alteration of topoisomerase (Topo) I protein expression or enzymatic activity. Whereas Topo IIβ protein expression was not affected by gefitinib, significant time- and concentration-dependent downregulation of Topo IIα protein and inhibition of its enzymatic function were observed, corresponding to a G_1 phase cell cycle arrest. Gefitinib significantly inhibited EGFR-associated signaling molecules, including phospho-mitogen-activated protein kinase or protein kinase C, which may account for decreases in proliferation or topoisomerase activity, respectively. Although a dose-dependent decrease of the BCRP/MXR/ABCP half-transporter was observed under gefitinib, cellular pharmacokinetics revealed no significant differences in accumulation or retention of the active SN-38 lactone using reverse-phase HPLC analysis. This study delineates mechanisms that may contribute to the synergism observed between irinotecan and EGFR inhibitors. *Anti-Cancer Drugs* 16:1099–1108 © 2005 Lippincott Williams & Wilkins.

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Introduction

Colorectal cancer (CRC) is the second-leading cause of cancer-related deaths in the developed world. Treatment options for patients with metastatic disease have improved considerably with the addition of irinotecan and oxaliplatin to 5-fluorouracil (5-FU)-based chemotherapy. However, overall treatment results remain unsatisfactory, and accelerated efforts of modulating drug resistance by combining standard and innovative therapeutic approaches are mandatory.

Irinotecan (CPT-11; 7-ethyl-10-[4-(-1-piperidino)-1-piperidino] carbonyloxy camptothecin) is established as one of the most active drugs in the first- and second-line treatment of advanced CRC. This semi-synthetic derivative of the natural plant alkaloid camptothecin is a prodrug, which is enzymatically converted by carboxylesterase to 7-ethyl-10-hydroxy-camptothecin (SN-38), its most active metabolite [1]. SN-38 possesses at least 200-fold higher cytotoxic potency *in vitro* [2], which is exerted by generating drug-stabilized covalent DNA/topoisomerase (Topo) I complexes in actively transcribed genes. These 'cleavable complexes' may accumulate, collide with moving replication forks in S phase, and ultimately lead to cell death due to replication arrest, replication fork disassembly and chromosomal fragmentation (DNA double-strand breaks). Additionally, encounters with transcription complexes may result in cytotoxic lesions throughout the cell cycle [3]. Sensitivity to camptothecin derivatives correlates positively with Topo I expression [4], cleavable complex formation [5] and tumor carboxylesterase activity [6]. *In vivo*, protein kinase C (PKC) may restore camptothecin susceptibility by phosphorylating Topo I [7], which re-induces the enzyme's DNA relaxing activity. Similarly, PKC may catalyze serine phosphorylation and activation of Topo II [8,9], a related enzyme, which may compensate for impaired Topo I function [10]. Of note, membrane transporter proteins of the ATP-binding cassette (ABC) family such as BCRP/MXR/ABCP may alter the intracellular pharmacokinetics of camptothecin derivatives [11], thereby contributing to a resistant phenotype.

Epidermal growth factor receptor (EGFR) signaling is involved in the regulation of fundamental (patho-)physiological processes including proliferation, differentiation,

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apoptosis, angiogenesis or metastasis. Overactivity of the receptor tyrosine kinase is considered a hallmark of malignancy, and has been associated with poor clinical prognosis for CRC patients in terms of chemotherapy resistance, early metastasis, and decreased disease-free and overall survival [12]. In sublethally damaged tumor cells, e.g. following conventional chemotherapy, EGFR activation may constitute a relevant survival mechanism. Targeting the EGFR network has become a rational clinical strategy with growing importance in the anti-cancer armamentarium [13]. Approximately 80% of CRC express the EGFR [14], with commonly marked intra-tumor heterogeneity. In patients with metastatic CRC refractory to either irinotecan alone or to both, 5-FU and irinotecan, the combination of anti-EGFR monoclonal antibody cetuximab and irinotecan showed significant anti-tumor activity [15]. Likewise, low-molecular-weight EGFR tyrosine kinase inhibitors (TKIs) such as the anilinoquinazoline gefitinib have been shown to enhance efficacy of conventional chemotherapy and increase radiosensitivity in various solid tumors [16]. We therefore hypothesized that EGFR blockade by tyrosine kinase inhibition (gefitinib) may influence cellular determinants of clinically relevant irinotecan resistance in human colon cancer cells. The present study describes a sensitization to irinotecan through concurrent, non-cytotoxic EGFR tyrosine kinase blockade and outlines potential molecular mechanisms.

Materials and methods

Compounds

The EGFR TKI gefitinib [Iressa; 4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholino-propoxy)-quinazoline] was provided by AstraZeneca (Macclesfield, UK). SN-38, the active metabolite of irinotecan, was purchased from Pharmacia and Upjohn (Kalamazoo, Michigan, USA).

In vitro cytotoxicity

The parental wild-type (HCT-8/wt) and 4-fold SN-38 resistant (HCT-8/SN-38) cell lines have been described previously [17]. For drug sensitivity assessment by the sulforhodamine B (SRB) assay [18], exponentially growing human colon cancer HCT-8/wt and HCT-8/SN-38 cells were cultured in 96-well microtiter plates (Falcon, Becton-Dickinson, Heidelberg, Germany). After 24 h attachment, cells were exposed to either drug-free medium with the corresponding volumes of diluents or to various concentrations and schedules of gefitinib and/or SN-38 as indicated. Subsequently, cells were washed, re-fed with medium and incubated for a total of 4–5 cell doubling times. Finally, cells were fixed with 10% trichloric acid, washed twice with PBS and stained with SRB for spectrophotometric absorbance assessment at 570 nm using an MRX 96-well Microplate Reader (Dynatech, Deckendorf, Germany) and Riowin 10 software (Microtec, Niederwanger, Switzerland). Drug concentrations that inhibited cell growth by 50% (IC_{50}) were determined from semilogarithmic dose–response plots.

Cell cycle analysis (BrdU labeling)

Cells were exposed to increasing concentrations of gefitinib for 24 h at 37°C, with BrdU labeling (50 μmol/l; Sigma-Aldrich, Taufkirchen, Germany) during the last 30 min of treatment. They were then trypsinized, washed with PBS, fixed in 100% ice-cold methanol and stained with FITC-conjugated anti-BrdU antibody (BD Biosciences, Heidelberg, Germany) under UV protection. Nuclei were counterstained with propidium iodide (Sigma) and DNA content was determined in an Epics XL flow cytometer (Beckman-Coulter, Krefeld, Germany). Finally, the numbers of cells in G_1, S and G_2/M phases of the cell cycle were calculated using Mod Fit LT cell cycle analysis software (Verity, Software House Inc., Topsham, Maine, USA).

Immunoblot analysis of total cell lysates and nuclear extracts

Whole-cell extracts were obtained after treatment with 0-, 0.25-, 1-, 2- or 4-fold IC_{50} gefitinib and/or SN-38 for various time periods, as indicated. In a subset of experiments, cells were stimulated with 80 nM 4α-phorbol 12-myristate 13-acetate (PMA; Sigma) for 20 min or with 1–100 ng/ml human recombinant EGF (Upstate Biotechnology, Lake Placid, New York, USA) for 10 min prior to cell lysis. Nuclear extracts from gefitinib-pretreated cells were prepared according to the methods of Sullivan *et al.* and Danks *et al.* [19,20]. For immunoblot analyses, 50–100 μg of cellular or nuclear protein was dissolved by 7.5–20% SDS–PAGE, electroblotted onto nitrocellulose and incubated with the respective primary antibodies (Ab): sheep polyclonal (p) IgG anti-EGFR, mouse monoclonal (m) IgG to cyclin E (Upstate Biotechnology), IgG1 to tyrosine phosphorylated (activated) EGFR, Stat3, c-myc (BD, Biosciences) or $p27^{kip1}$ (Sigma); IgG2a to breast cancer resistance protein (BCRP; Chemicon, Temecula, California, USA) or cyclin D1; IgG2b to $p21^{WAF1/Cip1}$ (Sigma), rabbit pAb to mitogen-activated protein kinase (MAPK) or phosphorylated p44/42 MAPK (Thr202/Tyr204; Cell Signaling, Beverly, Massachusetts, USA), PKCα (Upstate), Topo I or Topo II, mouse mAb to Topo IIα or β (TopoGEN, Columbus, Ohio, USA) or phosphorylated Topo IIα (Research Diagnostics, Flanders, New Jersey, USA). Protein loading was assessed with a mouse IgG1 against β-actin (Sigma) or Histone H1 (Upstate Biotechnology). Binding of specific secondary antibodies was visualized with enhanced chemiluminescence (ECL) detection using a Protein A–horseradish peroxidase complex (Amersham Biosciences, Arlington Heights, New Jersey, USA). For each sample, at least three independent experiments were performed.

Immunocytochemistry

Cells were cultured and treated on chamber slides (Lab-Tek II; Nalge Nunc, Illinois, Naperville, USA) as indicated. They were subsequently washed, fixed in 2% glutaraldehyde in cacodylate buffer for 24 h and transferred to TBS. For retrieval of total EGFR, a 15-min pretreatment with 0.1% pronase E (Sigma) was realized. After protein blocking for 5 min (TissueGnost; Merck, Darmstadt, Germany), slides were incubated with a primary mouse mAb against EGFR (DakoCytomation, Hamburg, Germany) or phosphorylated EGFR (BD) for 12 h at 4°C. The ChemMate detection system (Dako) was used and color reactions were performed using a neufuchsin-based substrate-chromogen solution (Dako) and Hemalaun counterstaining (Merck). Specimens were mounted in Immu-Mount (ThermoShandon, Pittsburg, Pennsylvania, USA) and subjected to microscopic examination.

Protein-linked DNA (double-strand) breaks (PLDBs)

To evaluate the effect of gefitinib on the SN-38-mediated induction of PLDBs *ex vivo*, DNA of mid-logarithmic phase cells was labeled with 0.1 μCi/ml [^{14}C]dThy (DuPont, Bad Homburg, Germany; specific activity 62.8 mCi/mmol) during 24 h incubation with increasing concentrations of gefitinib (0-, 1- and 4-fold IC_{50}) at 37°C. Cells were harvested, washed in PBS and re-incubated for 30 min in pre-warmed (37°C) [^{14}C]dThy-free medium containing 0.1 to 10 μmol/l SN-38 or solvent alone. After drug exposure, cells were washed in pre-warmed PBS, lysed (1.25% SDS, 5 mM EDTA, 0.4 mg/ml sheared salmon sperm DNA, adjusted to pH 8.0/1 ml/10^6 cells, and KCI–SDS coprecipitation assays were immediately performed using 4 M KCl as described [21]. Results were calculated as the percentage of precipitable [^{14}C]dThy-labeled DNA of drug-treated cells/total [^{14}C]-labeled DNA using a Tri-Carb 2100TR liquid scintillation analyzer (Perkin-Elmer, Rodgau-jügesheim, Germany). The reaction products were separated on a 1.0% agarose gel (4.6 V/cm) and stained with 0.5 μg/ml ethidium bromide.

Topo I unwinding activity of pBR322 plasmid DNA

In order to evaluate the effect of gefitinib on the SN-38-mediated inhibition of Topo I enzymatic activity, 0.3 μg pBR322 plasmid DNA (Roche, Mannheim, Germany) and serial dilutions of nuclear extract protein from gefitinib pre-treated cells (0-, 1- and 4-fold IC_{50}) or 2 U of purified Topo I enzyme (positive control) were incubated at 37°C for 30 min in a final volume of 20 μl drug-free medium or in 50 μmol/l SN-38. Reaction products, supercoiled and relaxed DNA topological forms, were separated on a 1.0% (w/v) agarose gel (4.6 V/cm) and stained with 0.5 μg/ml ethidium bromide; three independent experiments were performed in duplicate. SN-38, gefitinib and L-15 alone had no effect on the pBR322 plasmid DNA at the concentrations used (data not shown). Being conducted in the absence of ATP on which Topo II is dependent, this assay is highly specific for Topo I: within 30 min at 37°C, 1 U of Topo I catalyzes 50% ATP-independent relaxation of 0.5 μg supercoiled DNA.

Topo II decatenation of kinetoplast kDNA

Intermolecular strand passage activity of Topo II was assessed using kinetoplast kDNA according to the method of Melendy and Ray [22]. Reactions were initiated by adding 40–1280 ng nuclear extract or 2 U of purified Topo IIα (positive control). After 30 min incubation at 37°C, reactions were terminated by supplementation of 5 μl 5% sarkosyl and 50% glycerol in bromophenol blue on ice. Reaction products were subjected to electrophoresis at 120 V for 0.5 h in 1% (w/v) agarose gels containing 0.1 μg/ml ethidium bromide.

Reverse-phase (RP)-HPLC analysis of SN-38 concentrations

Exponentially growing cells were exposed to SN-38 with or without gefitinib using various schedules as indicated. To analyze intracellular SN-38 concentrations by HPLC, cells were lysed and deproteinized with 3 ml ice-cold methanol/acetonitrile (1:1, v/v), covered with nitrogen and frozen at −20°C for 12 h. After 15 min centrifugation at 1000 *g*, supernatants were evaporated in a Speed Vac Plus concentrator (Savant Instruments, Holbrook, New York, USA), and pellets were suspended in 500 μl methanol/water (3:7, v/v), covered with nitrogen and stored at −20°C for 2 h. Then, samples were centrifuged at 21 000 *g* for 5 min at 4°C and 100 μl each applied to RP columns (4.6 × 250 mm) packed with nucleosil (C_{18}) 5-μm particles (Machery and Nagel, Düren, Germany) at 20°C. Sample injections were performed using a Waters 717 Plus Autosampler and absorbance of the column effluent was monitored using a Waters 470 Scanning Fluorescence Detector (Waters, Eschborn, Germany; excitation: 370 nm, emission: 525 nm). The solvent mixtures were as follows: potassium hydrogen phosphate buffer (6.5 mmol/l, containing 0.05% EDTA), acetonitrile and methanol (67:20:13, v/v/v; solvent A or 55:27:18, v/v/v; solvent B), adjusted to pH 4.0 with phosphoric acid. For elution of SN-38, a linear gradient from A (100%) at time point 0 to B (100%) after 10 min was used, using 10 min equilibration with solvent A prior to the next analysis. The flow rate was maintained at 1 ml/min using Waters model 510 chromatography pumps, the column temperature was 40°C. Absorbance of the column effluent was monitored with a variable UV detector (Waters Lambda-Max 481 LC) at 254 nm. Peak areas were calculated using a Waters Millennium Chromatography Manager. The linearity of SN-38 detection was $r^2 = 0.998$ in the range of 0.005–50 μmol/l, the limit of detection was 20 pmol/l and the limit of quantitation was 50 pmol/l (data not shown).

Statistics

Differences between mean values were analyzed for significance using the paired two-tailed Student's *t*-test for independent samples; $P < 0.05$ was considered statistically significant. Results are given as mean values ± SE from at least three independent experiments performed on different days.

Results

EGFR tyrosine kinase inhibition with gefitinib sensitizes resistant colon cancer cells to SN-38

SN-38-mediated cytotoxicity was assessed repetitively via SRB assays in both wild-type and HCT-8/SN-38 cells in order to confirm or adjust the levels of irinotecan resistance. The IC_{50}s of SN-38 (24 h drug exposure) and gefitinib (2 [or 24] h exposure) were 0.007 and 63 [or 13.74] μmol/l in HCT-8/wt and 0.029 and 45 [or 12.68] μmol/l in HCT-8/SN-38 cells, respectively. Similar results were obtained using the more sensitive colony formation assay (data not shown). When coadministered with SN-38, non-cytotoxic concentrations of gefitinib (0.25-fold IC_{50}) sensitized HCT-8/SN-38 cells to irinotecan: IC_{50} (SN-38, 24 h) under gefitinib (120 h) = 0.008 μmol/l; $n = 8$; $P = 0.002$. A drug sensitization was also observed in the wild-type: IC_{50} (SN-38, 24 h) under gefitinib (0.25-fold IC_{50}, 120 h) = 0.002 μmol/l; $n = 8$; $P = 0.001$ (Fig. 1A). Neither withdrawal from EGF stimuli nor EGF coadministration at concentrations ranging from 1 to 100 ng/ml affected experimental results (data not shown).

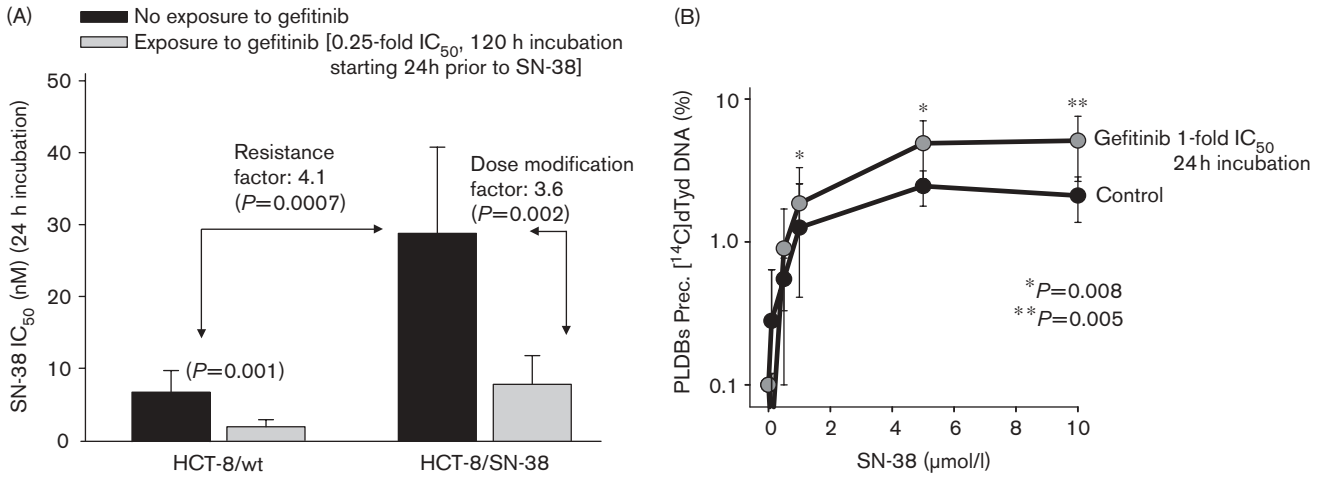
Gefitinib increases the net formation of SN-38-induced PLDBs

Pre-treatment of HCT-8/SN-38 cells with gefitinib resulted in a significantly higher induction of Topo I-related PLDBs by increasing concentrations of SN-38, as reflected in the relative quantity of precipitated [^{14}C]dThy-labeled DNA fragments (Fig. 1B), e.g. for 10 μM SN–38: 5.1 ± 0.87 (1-fold IC_{50} gefitinib) and 15.9 ± 0.44 (4-fold IC_{50} gefitinib) versus 2.42 ± 0.29% (untreated; $P = 0.005$ and $P < 0.0001$, respectively). Conversely, Topo I protein expression levels (Fig. 2A) and Topo I unwinding activity of pBR322 plasmid DNA (Fig. 2B) remained unaltered after 24 h exposure to gefitinib.

Gefitinib induces downregulation of Topo IIα expression, phosphorylation and enzymatic activity

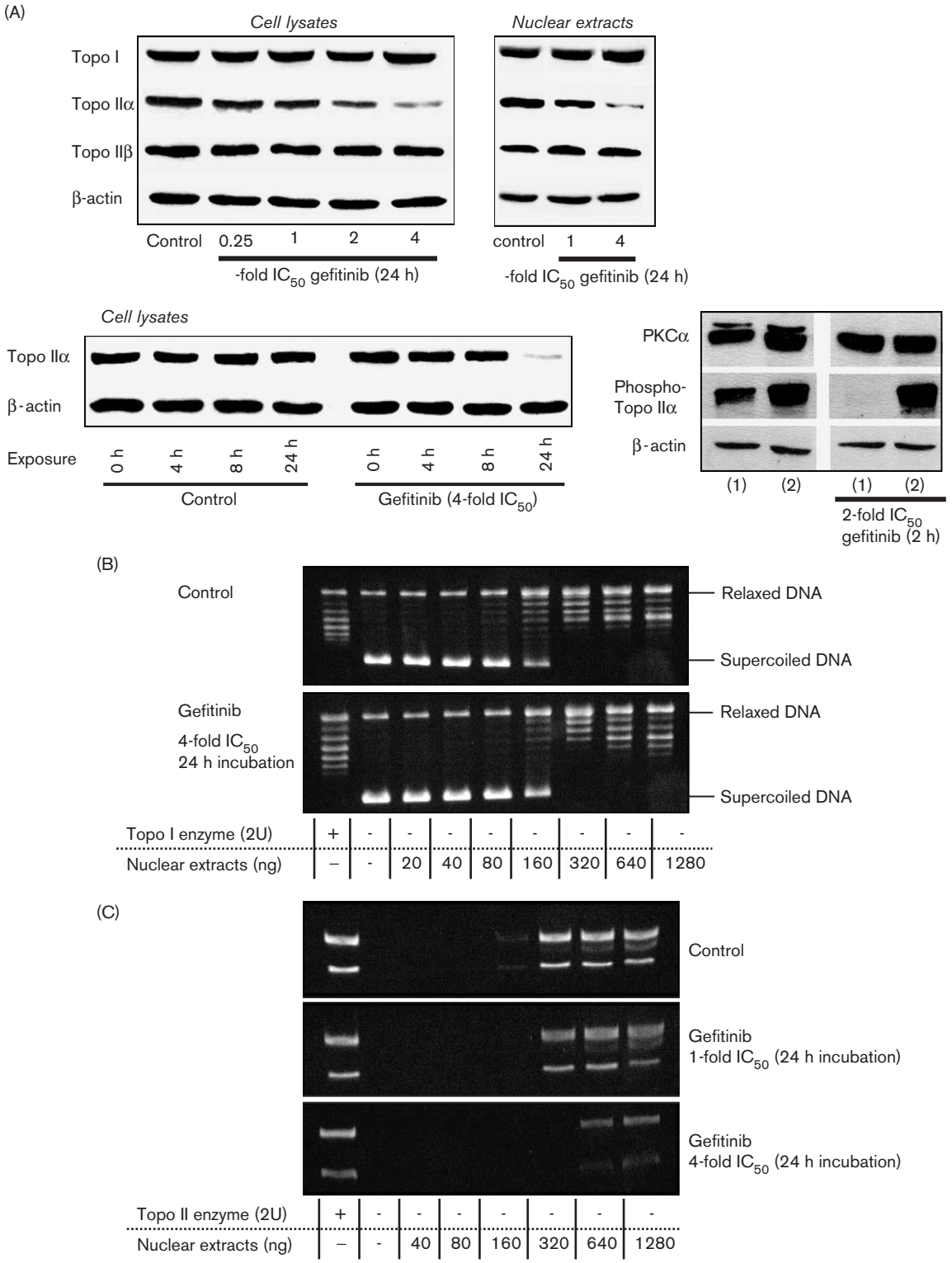
We observed a time- and concentration-dependent downregulation of Topo IIα protein in both total cell lysates and nuclear extracts under gefitinib, which was preceded by a decrease in specific phosphorylation of the enzyme (Fig. 2A). Interestingly, PKCα, which has been implicated in topoisomerase activation, was slightly decreased in the presence of gefitinib. Consistently, gefitinib caused a dose-dependent downregulation of Topo II enzymatic activity in nuclear extracts from pretreated cells as assessed by specific decatenation of kinetoplast kDNA (Fig. 2C). In contrast, exposure to increasing concentrations of gefitinib showed no effect on Topo IIβ expression in specific immunoblotting of cell lysates or nuclear extract protein (Fig. 2A).

Fig. 1



Effect of gefitinib on the *in vitro* cytotoxicity of SN-38. (A) SRB assay. Modulation of cellular resistance to SN-38 (arrows) by non-cytotoxic concentrations of gefitinib (0.25-fold IC_{50}). IC_{50}s of SN-38 (24 h incubation) were assessed by SRB proliferation assays in the presence or absence of 0.25-fold IC_{50} gefitinib; spectrophotometric absorbance was measured at a wavelength of 570 nmol/l. At least three independent experiments were performed in duplicate. Similar results were obtained using colony formation assays (data not shown). (B) The generation of SN-38-mediated Topo I-specific PLDBs was examined in cleavable complex formation assays with HCT-8/SN-38 cells.

Fig. 2

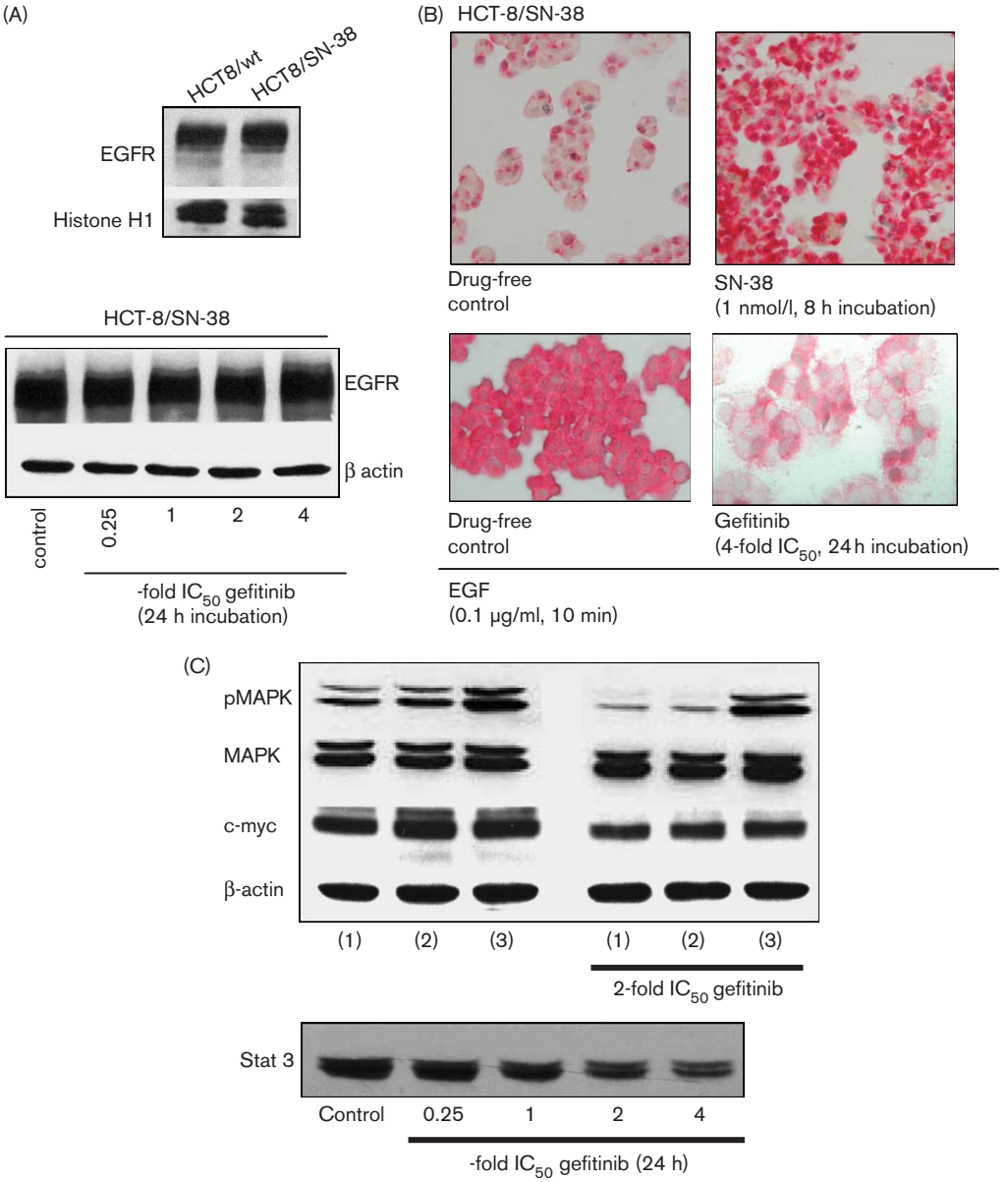


Effects of gefitinib on Topo I, IIα and IIβ in HCT-8/SN-38 cells. (A) Changes in protein expression of Topo I, IIα and IIβ related to gefitinib exposition at various concentrations and time points were assessed by immunoblot analyses of total cell lysates or nuclear extracts, as indicated. Additionally, the expression of PKCα and phosphorylation of Topo IIα were evaluated in the presence or absence of gefitinib. Lanes: (1) control; stimulation with (2) EGF (0.1 μg/ml, 10 min). Nuclear extracts from gefitinib-pretreated cells were examined for (B)Topo I-relaxing activity of pBR322 plasmid DNA or (C) Topo II decatenation of kinetoplast kDNA.

Gefitinib inhibits SN-38-induced EGFR phosphorylation and downstream signaling events

HCT-8 cells exhibit high expression levels of EGFR, which were unaltered in the resistant subline or under exposure to gefitinib (Fig. 3A). Treatment with SN-38 at subtoxic concentrations significantly increased EGFR phosphorylation, both in the wild-type and the resistant cell line. Conversely, concomitant exposure of HCT-8/SN-38 cells to non-cytotoxic concentrations of gefitinib significantly decreased EGFR phosphorylation levels (Fig. 3B and data not shown) and inhibited downstream signaling molecules such as MAPK or Stat3 in a time- and concentration-dependent manner (Fig. 3C and data not shown). Of note, gefitinib was potent to inhibit EGFR-related signaling even in the presence of 100 ng/ml EGF, one of the most important activating ligands of the EGF receptor.

Fig. 3



Effect of gefitinib on EGFR-related cell signaling. (A) EGFR expression levels at baseline or under increasing concentrations of gefitinib in HCT-8/wt and HCT-8/SN-38 cells. (B) Changes in EGFR phosphorylation in response to treatment with SN-38 or EGF were assessed in the presence or absence of gefitinib. (C) Selected downstream effectors of EGFR signaling in HCT-8/SN-38 cells in the presence or absence of gefitinib. Lanes: (1) control; stimulation with (2) EGF (0.1 μg/ml, 10 min), (3) PMA (0.5 μg/ml, 20 min). All observed effects displayed dose and time dependency (as indicated and unpublished observations).

Effects of gefitinib on the cell cycle: EGFR tyrosine kinase inhibition induces G_1/S phase block and downregulation of cyclin E

Whereas SN-38 is known to induce S-phase arrest, submicromolar concentrations of gefitinib induced a G_1-phase arrest as determined by BrdU incorporation and fluorescence activated cell sorting FACS (Fig. 4A). The TKI showed no significant effect on the expression of the cell cycle regulatory proteins $p21^{WAF1}$, $p27^{kip1}$ or cyclin D1 in serial Western blot analyses of HCT-8/SN-38 total cell lysates. However, cyclin E protein concentration decreased markedly between 6 and 24 h of treatment with gefitinib (Fig. 4B and data not shown).

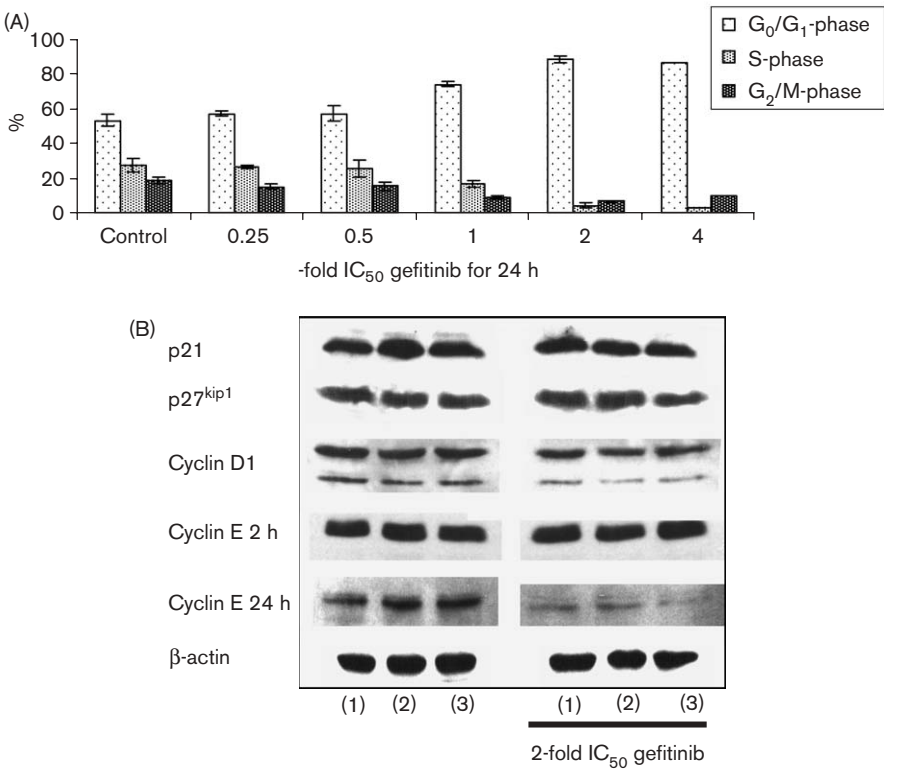
Cellular pharmacokinetics of SN-38: gefitinib downregulates BCRP/MXR/ABCP without affecting intracellular SN-38 concentrations

ABC protein family members have been shown to mediate *in vitro* drug resistance to camptothecin derivatives [23]. We observed a concentration-dependent downregulation of the BCRP/MXR/ABCP multidrug transporter in response to EGFR tyrosine kinase inhibition. Surprisingly, however, RP-HPLC analyses of intracellular concentrations of the active SN-38 lactone revealed no significant differences in drug accumulation (0–30 min) or retention (30–90 min) under gefitinib treatment (Fig. 5): exposure of HCT-8/SN-38 cells to 1 μM SN-38 with or without gefitinib (1-fold IC_{50} for at least 24 h): 164 ± 12 versus 180 ± 25 pmol/l/5 × 10^6 cells.

Discussion

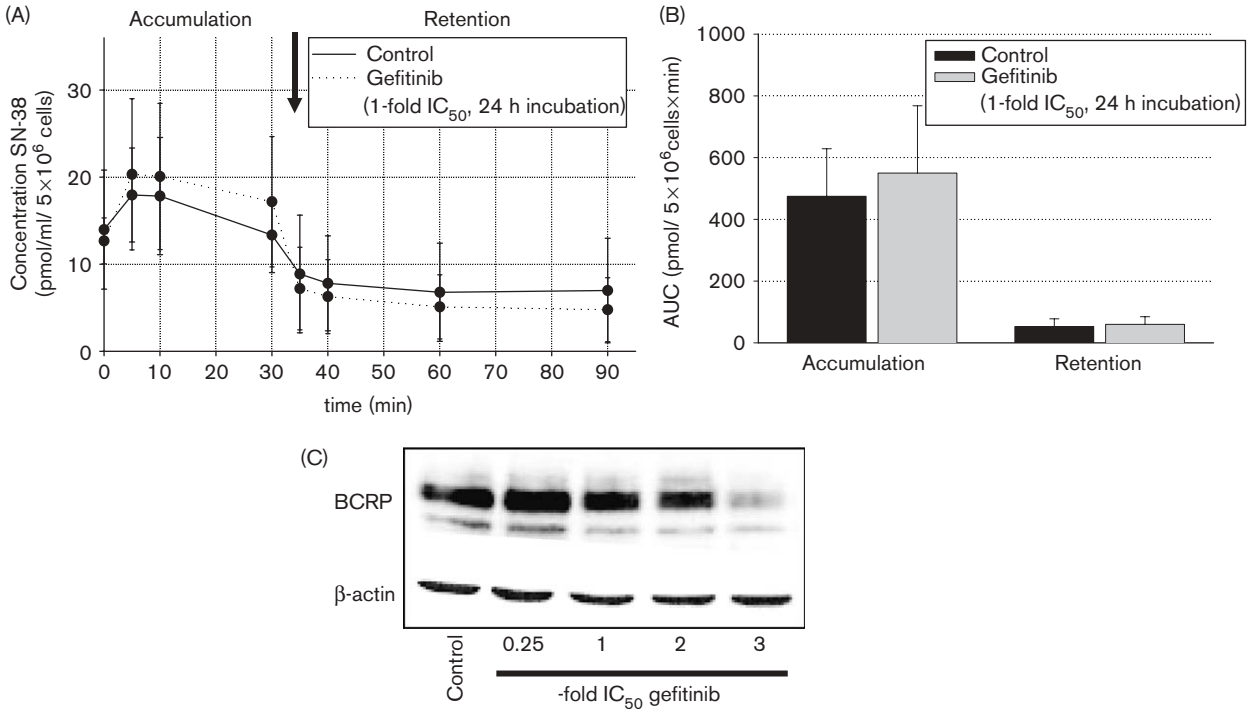
We have investigated the effects of the EGFR TKI gefitinib on molecular determinants of resistance to irinotecan (SN-38) in the HCT-8 human colon cancer cell line. Gefitinib is an anilino-quinazoline derivative, which inhibits EGFR tyrosine kinase activity with high affinity by competitively blocking its ATP-binding domain. At higher concentrations, gefitinib has been shown to inhibit erbB2 and KDR tyrosine kinases as well as Raf or MEK-1 serine-threonine kinases, amongst others (IC_{50} > 10 μmol/l [24]). While clinical and laboratory data have suggested a role for EGFR antagonists in the modulation of cancer therapy activity and resistance, the mechanisms of these interactions have largely remained elusive. Presumably, in cells expressing functional EGFR, survival responses after sublethal cell damage may involve

Fig. 4



Effects of gefitinib on the cell cycle in HCT-8/SN-38 cells. (A) Cell cycle distribution under increasing concentrations of gefitinib: FACS. (B) Effect of gefitinib (2-fold IC_{50} for 2 h or as indicated) on cell cycle regulatory proteins. Lanes: (1) control; stimulation with (2) EGF (0.1 μg/ml, 10 min) or (3) PMA (0.5 μg/ml, 20 min).

Fig. 5



Pharmacokinetics. SN-38 lactone accumulation and retention in HCT-8/SN-38 cells under gefitinib coadministration represented as (A) time–concentration curves and (B) AUCs, respectively. Represented are results from four independent experiments in duplicate. (C) Expression analysis of the camptothecin resistance associated breast cancer resistance protein (BCRP/MXR/ABCP) under 24 h treatment with gefitinib: immunoblot analysis of HCT-8/SN-38 total cell lysates.

EGFR-mediated processes, whose abrogation leads to accelerated cell death. Previously, EGFR activation has been reported in response to ionizing radiation, cisplatin or doxorubicin [25]. Here, we demonstrate phosphorylation of the EGF receptor by irinotecan (SN-38; Fig. 3) and restoration of decreased SN-38-mediated cytotoxicity under concomitant EGFR blockade (Fig. 1) [26]. While gefitinib effected SN-38 sensitization in both wild-type and 4-fold SN-38 resistant cells, effects were more distinct in the resistant cell line (Fig. 1, arrows, and data not shown), suggesting additional molecular mechanisms. Consistently, we have observed specific effects of gefitinib on molecular determinants of sensitivity to irinotecan.

Topo I is the cellular target of camptothecin derivatives, and intracellular enzyme levels have been correlated to *in vivo* cytotoxic efficacy [27] and *in vitro* resistance acquisition [28]. In our hands, Topo I expression and enzymatic activity in HCT-8 cells decreased slightly during resistance induction to SN-38, which was paralleled by a decrease in the formation of SN-38-mediated Topo I-specific protein-linked DNA double-strand breaks, the critical cytotoxic lesions of camptothecin derivatives (data not shown). Whereas treatment with gefitinib did not detectably modify Topo I expression or activity levels in the resistant subline (Fig. 2A), it significantly augmented the net formation of SN-38-induced DNA strand breaks (Fig. 1B), which might be due to alterations in the persistence time (i.e. the formation rate or stability) of the Topo I/SN-38/DNA ternary complexes. Of note, Topo II has been shown to complement impaired Topo I function [10] and tumor cells that develop resistance to one class of topoisomerase inhibitors may display increased expression or activity of the collateral enzyme [10,28]. Iterative exposure of HCT-8 cells to SN-38 did not appreciably affect Topo II protein levels or enzymatic function in immunoblotting or kDNA decatenation assays, respectively (data not shown). In contrast, gefitinib reduced intracellular levels of activated (phosphorylated) Topo IIα in specific Western blotting, decreased the enzyme's catalytic activity, and exerted a significant time- and concentration-dependent down-regulation of both nuclear and cytosolic Topo IIα protein in HCT-8/SN-38 cells (Fig. 2A). These observations may reflect several mechanisms. For instance, an inhibition of enzymes implicated in Topo IIα activation (e.g. PKCα) or enhanced phosphatase activity may account for the demonstrated loss of phosphorylated Topo IIα under gefitinib treatment. Interestingly, gefitinib caused the

loss of a shifted band, presumably corresponding to the phosphorylated form of PKCα in specific Western blotting (Fig. 2A). This could result from suppression of EGFR-induced phosphatidylinositol 3-kinase (PI3K), 3-phosphoinositide-dependent kinase 1 (PDK1) or phospholipase C-γ1 signaling, which have been implicated in PKC activation [29,30]. Theoretically, the transient phosphotyrosine interaction between Topo IIα and its substrate DNA or the binding and hydrolysis of ATP, upon which Topo II (but not Topo I) activity relies, could be altered in the presence of the TKI, thereby affecting the enzyme's function. Finally, either transcriptional or translational repression as well as enhanced protein degradation may account for the observed reduction in Topo IIα protein levels, concomitant with a decrease in total enzyme activity. Both, Topo IIα and the *erbB2* oncogene are located on 17q21–22 and are transcriptionally co-regulated [31]. In our hands, *erbB2* receptor levels were not significantly altered under gefitinib administration (data not shown), which indicates, but does not unequivocally prove, a role for post-transcriptional regulation (i.e. translational suppression or increased targeting of Topo IIα for proteasomal degradation [32]). Importantly, Topo IIα, but not Topo IIβ or I, protein expression is tightly regulated throughout the cell cycle, with about 15-fold higher protein levels in late S phase than in G_1 phase. Whereas SN-38 is known to induce S-phase arrest, gefitinib induced a concentration-dependent G_1-phase cell cycle arrest associated with a decreased proliferative (S phase) fraction. We therefore analyzed the expression levels of regulators of the G_1/S phase transition under treatment with the EGFR TKI. Interestingly, we found a significant downregulation of cyclin E after 6–24 h (Fig. 4), while $p21^{WAF1}$, $p27^{kip1}$ or cyclin D1 expression did not display significant changes in serial Western blot analyses of HCT-8/SN-38 total cell lysates. However, a potential re-distribution of these cell cycle kinases between the nuclear and cytoplasmic compartments has not been studied, and could contribute to the noted effects. The observation that gefitinib may both prevent cells from entering S-phase and increase the cytotoxicity of a presumably 'S-phase-specific' [33,34] compound is striking at first glance. It may endorse the increasing evidence of replication-independent cytotoxic mechanisms of camptothecin derivatives, i.e. transcription encounter lesions [3,35, 36]. Interestingly, decreased Topo IIα protein levels and activity have been reported following antibody blockade of EGFR's heterodimeric partner *erbB2*, resulting in cell cycle independent modulation of sensitivity to cytotoxic compounds [37]. Gefitinib effectively reduced EGFR-related cell signaling in colorectal cancer cells, as determined by inhibited MAPK phosphorylation as well as a moderate decrease in levels of the Stat3 transcription factor, thereby attenuating the receptor's anti-apoptotic and pro-proliferative stimuli in response to DNA damage (Fig. 3B and data not shown).

A critical aspect of emerging drug resistance is the expression of transmembrane efflux pumps such as the ABC half-transporter ABCG2 (BCRP/MXR/ABCP) [11,38]. Our data demonstrate a dose-dependent downregulation of BCRP protein levels in response to gefitinib (Fig. 5), but interestingly fail to demonstrate significant differences in cellular pharmacokinetics with gefitinib. While other groups have correlated BCRP, MDR1 or P-glycoprotein inhibition with increased intracellular concentrations of SN-38 and reversal of *in vivo* resistance to camptothecin derivatives [39–41], this is the first specific evaluation of concentrations of the active SN-38 lactone form in this context. Of note, HCT-8/SN-38 cells displayed no cross-resistance to other chemotherapeutic agents such as 5-FU, raltitrexed or multitargeted antifolate (MTA), which are also substrates of membrane transporters, or to oxaliplatin. HCT-8/wt and HCT-8/SN-38 cells display normal carboxylesterase activity at baseline (736.7 μM/h/mg; courtesy of Dr Y. Rustum, Roswell Park Cancer Institute), which catalyzes the *in vivo c*onversion of irinotecan to SN-38. The primary use of SN-38 in our cell system is therefore justifiable for modeling physiologically relevant irinotecan kinetics.

In conclusion, apart from EGFR activation by irinotecan [26], which was counteracted by the small-molecule TKI, we have identified downregulation of Topo IIα protein levels, specific phosphorylation and enzymatic activity as well as an increase in the formation of Topo I-specific protein-linked DNA double-strand breaks as potential mediators of restored drug activity. We have further demonstrated a gefitinib-induced downregulation of the BCRP transmembrane drug transporter, which has been associated with *in vitro* resistance to camptothecin derivatives. Although intracellular concentrations of the active SN-38 lactone were not measurably affected in our model, the observed effects on this – and potentially other – important drug transporter systems may prove beneficial in the clinics. Recent results indicate that gefitinib increases the oral bioavailability of irinotecan [39,42]. Whereas the infusional combination of irinotecan and EGFR-targeted monoclonal antibodies has been approved for CRC therapy [15], the oral formulation of both drugs may provide a clinically meaningful benefit for cancer patients. Together with previous observations [42–44], our data warrant further studies of combination therapy with gefitinib and oral irinotecan in patients with CRC, who are refractory to irinotecan-based regimens.

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