A Novel Epidermal Growth Factor Receptor Inhibitor Promotes Apoptosis in Non–Small Cell Lung Cancer Cells Resistant to Erlotinib


Abstract
Non–small cell lung cancer (NSCLC) with activating mutations in the epidermal growth factor receptor (EGFR) responds to EGFR tyrosine kinase inhibitors such as erlotinib. However, secondary somatic EGFR mutations (e.g., T790M) confer resistance to erlotinib. BMS-690514, a novel pan-HER/vascular endothelial growth factor receptor (VEGFR) inhibitor described here, exerted antiproliferative and proapoptotic effects on NSCLC cell lines, with prominent efficacy on H1975 cells expressing the T790M mutation. In this model, BMS-690514 induced a G1 cell cycle arrest, as well as ultrastructural hallmarks of apoptosis, mitochondrial release of cytochrome c, and activation of caspases involved in the intrinsic (e.g., caspase-2, caspase-3, caspase-7, and caspase-9), but not in the extrinsic (e.g., caspase-8), pathway. Caspase inhibition conferred partial protection against BMS-690514 cytotoxicity, pointing to the involvement of both caspase-dependent and caspase-independent effector mechanisms. Transcriptome analyses revealed the up-regulation of proapoptotic (e.g., Bim, Puma) and cell cycle inhibitory (e.g., p27kip1, p57kip2) factors, as well as the down-regulation of antiapoptotic (e.g., Mcl1), heat shock (e.g., HSP40, HSP70, HSP90), and cell cycle promoting [e.g., cyclins B1, D1, and D3; cyclin-dependent kinase 1 (CDK1); MCM family proteins; proliferating cell nuclear antigen (PCNA)] proteins. BMS-690514–induced death of H1975 cells was modified in a unique fashion by a panel of small interfering RNAs targeting apoptosis modulators. Down-regulation of components of the nuclear factor-κB survival pathway (e.g., p65, Nemo/IKKγ, TAB2) sensitized cells to BMS-690514, whereas knockdown of proapoptotic factors (e.g., Puma, Bax, Bak, caspase-2, etc.) and DNA damage–related proteins (e.g., ERCC1, hTERT) exerted cytoprotective effects. BMS-690514 is a new pan-HER/VEGFR inhibitor that may become an alternative to erlotinib for the treatment of NSCLC. [Cancer Res 2007;67(13):6253–62]

Introduction
Most patients with non–small cell lung cancer (NSCLC, the most frequent cancer in males) present at an inoperable stage, with a poor prognosis (1). Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is aberrantly activated in several epithelial solid tumors, and notably in NSCLC (2). Overexpression of the gene coding for EGFR has been found in 40% to 80% of NSCLC biopsies, and has been correlated with shorter survival after surgical resection (3). Activating mutations of EGFR may render NSCLC cells independent from external growth factors (4), converting EGFR into an attractive therapeutic target (5). For the treatment of NSCLC, small-molecule EGFR tyrosine kinase inhibitors (EGFR-TKI), like erlotinib (Tarceva) and gefitinib (Iressa), have been evaluated (6). However, only erlotinib prolonged the survival of patients with recurrent NSCLC (7). In particular, a subset of patients carrying EGFR-activating mutations exhibited encouraging responses to EGFR-TKIs (8). The deletion ΔE746-A750 in exon 19 and the leucine-to-arginine substitution at position 858 (L858R) in exon 21 account for 85% of NSCLC-related EGFR mutations (9). Deletions in exon 19 are associated with better responses to EGFR-TKIs and prolonged overall survival, compared with activating mutations in exon 21 (i.e., L858R; ref. 10). Nevertheless, secondary mutations, including the threonine-to-methionine substitution at position 790 (T790M, in exon 20) and the aspartate-to-tyrosine substitution at position 761 (D761Y, in exon 19), have been reported in lesions progressing after treatment with EGFR-TKIs (11). At present, it is not clear whether the T790M mutation is present in a small fraction of tumor cells before treatment (12) or whether it arises de novo (13). Recently, it has been shown that alternative EGFR-TKI inhibitors retain the ability to promote apoptosis in gefitinib-resistant cancer cells (14).

EGFR signaling is linked to multiple intracellular pathways that inhibit apoptosis and promote survival and proliferation. Upon ligand-induced activation, EGFR generates phosphotyrosine sites for the recruitment of Ras and phosphatidylinositol-3 kinase, setting off the classic mitogen-activated protein kinase and Akt pathways (15). Moreover, several members of the signal transducer...
and activator of transcription (STAT) family of transcriptional factors (e.g., STAT1, STAT3, STAT5a, and STAT5b) can be activated by EGFR (16). Interestingly, the inhibition of STAT3 promotes apoptosis in cells expressing mutant EGFR (17).

Apoptosis is an accurately regulated program by which vertebrates eliminate superfluous, ectopic, and damaged cells (18, 19), as well as one of the mechanisms through which chemotherapeutics kill cancer cells (20). The cell death program is at least partially suppressed during oncogenesis, thus favoring chemotherapy and radiotherapy resistance (21, 22). Apoptosis can be distinguished from other cell death subroutines by means of morphologic criteria, including chromatin condensation (pyknosis) and nuclear fragmentation (karyorrhexis; refs. 23, 24). Cells that succumb to apoptosis eventually break down into membrane-enclosed bodies, which, in vivo, are engulfed by resident phagocytic cells and usually fail to elicit inflammatory/immune responses (23, 25).

Apoptosis may be executed via the extrinsic pathway, which emanates from the extracellular environment and is propagated along the “death receptors → death-inducing signaling complex → caspase-8” axis (26–28), or through an intracellular cascade of events that involves mitochondria (intrinsic pathway; refs. 29, 30). In this case, proapoptotic signals from several subcellular compartments (e.g., the nucleus, the endoplasmic reticulum, etc.) are transmitted to mitochondria where they promote mitochondrial membrane permeabilization, often through the activation of proapoptotic proteins of the Bcl-2 family (22, 31). After mitochondrial membrane permeabilization, cytotoxic proteins that normally reside in the intermembrane space are liberated into the cytosol, and can either favor directly the activation of the caspases, as does cytochrome c (32), or promote apoptosis in a caspase-independent fashion (33), as do apoptosis-inducing factor (34–36) and endonuclease G (37).

In the present article, we report the characterization of a novel panHER/vascular endothelial growth factor receptor (VEGFR) inhibitor, BMS-690514, that exerts antiproliferative and proapoptotic effects on NSCLC cell lines, in particular on cells that are resistant to erlotinib due to the T790M mutation. In addition, we describe the mechanisms through which BMS-690514 exerts is cytostatic and cytotoxic effects.

Materials and Methods

Cell lines, culture, and treatments. A549 cells [wild-type (wt) EGFR and p53] were grown in F12-K medium containing t-galactosamine, supplemented with 10% FCS, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. H1299 cells (wt EGFR, inactive p53 R175H), H1650 cells (bearing a deletion in exon 19 of the EGFR gene, i.e., ΔE746-A750, and wt p53), and H1975 cells (EGFR L858R/T790M and wt p53) were maintained in RPMI 1640 with GlutaMAX supplemented with 10% FCS and antibiotics (as above). For proliferation and cytotoxicity assays based on 96-well plates, all cell lines were cultured in DMEM/F12 (1:1) with t-glutamine but no phenol red supplemented with 10% FCS and antibiotics (as above). For proliferation and cytotoxicity assays, 1 × 10^5 to 3 × 10^5 cells were seeded in 12-well plates 12 h before the treatment with BMS-690514 or erlotinib (Tarceva, from Sequoia). When the sequential administration of BMS-690514 and CDDP was investigated, cells were treated with 5 μmol/L BMS-690514 for 24 h (48 h) before the administration of 10 μmol/L CDDP for additional 48 h (24 h) or vice versa. In both cases, treatments lasted a total of 72 h, after which plates were analyzed for cell proliferation.

For transmission electron microscopy, H1975 cells were treated for 48 h with 5 μmol/L BMS-690514, then fixed and processed as previously reported (39).

Cell proliferation assays. Cell proliferation was quantified by means of a colorimetric assay based on the reduction of the colorless tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (WST-1, from Roche) to formazan (which exhibit an absorbance peak around 450 nm), according to the manufacturer's instructions.

Small interfering RNA transfections. Detailed information on the small interfering RNAs (siRNA) can be found in Supplementary Materials and Methods. For 96-well plates based proliferation assays, A549 and H1975 cells were transfected while seeded, as follows. siRNA (300 pmol) dissolved in 2.1 mL of serum-free, antibiotic-free, DMEM/F12 (1:1) with t-glutamine but no phenol red were mixed with 63 μL of HiPerFect transfection reagent (Qiagen) dissolved in 2.1 mL of the same medium and allowed to stand at room temperature for 30 min. Thereafter, transfection complexes were added to 2.35 × 10^5 cells suspended in 7.8 mL of DMEM/F12 (1:1) with t-glutamine but no phenol red supplemented with 10% FCS and antibiotics. The resulting cell suspension was seeded into a 96-well plate (3,500 cells in 100 μL of medium per well). Transfected cells were cultured for 48 h before the administration of BMS-690514 (25 and 70 μmol/L for H1975 and A549 cells, respectively) or CDDP (70 μmol/L for both cell lines). Finally (after a total of 72 h), plates were analyzed for cell proliferation.

For FACS analysis, H1975 cells were reverse transfected after a slightly modified protocol. One hundred picomoles of siRNA (unrelated, caspase-2, p65) dissolved in 92 μL of serum-free, antibiotic-free RPMI 1640 with GlutaMAX were mixed with 8 μL of HiPerFect transfection reagent and allowed to stand at room temperature for 30 min. Then, transfection complexes were added to 10^5 cells suspended in 1.9 mL of RPMI 1640 with GlutaMAX supplemented with 10% FCS and antibiotics. The resulting suspension was seeded into six-well plates. After 48 h (to allow for protein down-regulation), cells were harvested and transferred to 12-well plates (30 × 10^5 to 40 × 10^5 per well). Upon adhesion to the plate, cells were retransfected with the siRNAs originally used (to grant for persistent down-regulation) by means of the HiPerFect transfection reagent (Qiagen), in this case following the manufacturer's instructions. Simultaneously, cells were treated or not with 5 μmol/L BMS-690514. After an additional 48 h, cells were subjected to cytfluorimetric analysis of viability and ΔΨ_m dissipation.

Data treatment and statistical analysis. All experiments were done in duplicates or triplicates, and repeated at least twice. Data are reported as means ± SE. Statistical significance was evaluated by means of paired Student’s t-test. The assay-independent indicator Δ was introduced to allow for normalization among different assays, and to analyze the effects of siRNAs per se separately from their influence on drug-induced cell death. A precise mathematical definition of Δ may be found in Supplementary Materials and Methods.

Cytoloiomeric analysis and immunofluorescence. Cells were stained with the following probes to assess apoptosis-associated modifications: propidium iodide (1 μg/mL, Sigma-Aldrich) for viability and dihydroethyloxacarbonylamine iodide [DQO]_3] (40 nmol/L, Molecular Probes-Invitrogen) for ΔΨ_m dissipation (40). Caspase-3 activation was measured by staining with an FITC-conjugated monoclonal antibody specific for active caspase-3 (Becton Dickinson). After overnight fixation of the cells in glacial 70% ethanol, DNA content was quantified by staining with propidium
iodide (50 μg/mL) and RNase (10 μg/mL, Roche) for 30 min at room temperature. All cytofluorimetric analyses were done using a FACSScan equipped with Cell Quest software (Becton Dickinson).

For immunofluorescence studies, cells were fixed with paraformaldehyde (4% w/v in PBS) followed by staining with antibodies for the detection of cytochrome c (mouse monoclonal IgG anti-cytochrome c; BD PharMingen) and active caspase-3 [rabbit polyclonal anti-caspase-3 (Asp175); Cell Signalling Technology]. Nuclei were counterstained with 10 μg/mL Hoechst 33342 (Molecular Probes-Invitrogen). Primary antibodies were revealed either with goat anti-rabbit IgG conjugated to Alexa 488 (green) or with goat anti-mouse IgG conjugated to Alexa 568 (red) from Molecular Probes-Invitrogen. Fluorescence microscopy determinations were done by means of a Leica IRE2 microscope equipped with a Leica DC300F camera.

**Analysis of protein expression.** Protein samples of H1975 cells were prepared in lysis buffer, according to standard established protocols (41). Extracted proteins (50 μg/lane) were separated by 12% SDS-PAGE and subjected to immunoblotting using mouse monoclonal IgG1 antibodies specific for caspase-2 (Santa Cruz Biotechnology), active caspase-8, phosphorylated EGFR (Cell Signalling Technology), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon International) and rabbit polyclonal antibodies against caspase-3, caspase-7, active caspase-9 (Cell Signalling Technology), total EGFR, or the p65 subunit of NF-κB (Santa Cruz Biotechnology). Membranes were then incubated with secondary goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Southern Biotechnology). Membranes were then incubated with secondary goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Southern Biotechnology) before revelation with ECL Detection kit and Hyperfilm X-ray films (both from Amersham Pharmacia).

**Results and Discussion**

BMS-690514 kills erlotinib-resistant NSCLC cells by apoptosis. BMS-690514 is a novel specific inhibitor of EGFR, exhibiting an *in vitro* IC50 of ~5 nmol/L. At higher concentrations, it acts also on other members of the EGFR family (i.e., ErbB2 and Erb-B4, with an IC50 of ~20 and ~60 nmol/L, respectively) as well as on VEGFR2 (IC50 of ~50 nmol/L). BMS-690514 inhibits the proliferation of NSCLC cell lines, showing a marked effect on the H1975 cell line that expresses an EGFR mutation (T790M) that is associated with clinical resistance against erlotinib. The antiproliferative effect of BMS-690514 on H1975 cells was obtained at an IC50 of ~1 μmol/L, which is 10-fold lower than the IC50 of erlotinib (Fig. 1A). It was associated with a marked increase in cells manifesting signs of apoptosis including the dissipation of the mitochondrial transmembrane potential (ΔΨm, as measured by staining with the potential-sensitive probe DiOC6(3)) and the permeabilization of the plasma membrane (as measured by staining with the vital dye propidium iodide). H1975 cells (EGFR L858R/T790M, wt p53) were far more sensitive to BMS-690514 than H1650 (EGFR ΔE746-A750, wt p53), H1299 (wt EGFR, inactive p53), and A549 (wt EGFR, wt p53) NSCLC cell lines (Fig. 1B).

Transmission electron microscopy revealed that H1975 cells transit from a normal morphology (Supplementary Fig. S1A) to full-blown apoptosis when treated with BMS-690514. After an initial swelling of the Golgi apparatus (Supplementary Fig. S1B) and the accumulation of pseudomyelinic figures in the cytoplasm (Supplementary Fig. S1C and S1D), the three defining hallmarks of apoptosis, namely nuclear condensation (pyknosis, Supplementary Fig. S1E) and fragmentation (karyorrhexis, Supplementary Fig. S1F), and apoptotic blebbing (Supplementary Fig. S1G) became manifest. Then, the dying cells either were phagocytosed by yet alive neighbors (Supplementary Fig. S1H) or underwent secondary necrosis (Supplementary Fig. S1I). In conclusion, BMS-690514 induces the apoptotic demise of H1975 NSCLC cells.

**Cellular effects of BMS-690514: cell cycle inhibition and induction of the mitochondrial caspase activation pathway.** To characterize the mechanisms through which BMS-690514 kills H1975 cells, we determined its transcriptional effects at a preapoptotic stage, measured at 24 h (at a dose of BMS-690514, 5 μmol/L, which kills 50% of the cells after 48 h of incubation) or at 48 h (at a dose of BMS-690514, 1 μmol/L, which kills 50% of the cells after 72 h of incubation). All data obtained by microarray analysis have been submitted to Array Express at the European Bioinformatics Institute with the accession number E-TABM-239 (42). At 24 h, BMS-690514 caused the up-regulation of ~250 genes and the down-regulation of ~500 genes by a factor >2, with a profile that was markedly different from that induced by CDDP (Fig. 2A; see also Supplementary Table S1). Hence, part of the changes in the transcriptome are specific for BMS-690514 and do not reflect a general property of apoptosis in H1975 cells. Of note, BMS-690514 suppressed the expression of several cell cycle–associated proteins (cyclins B1, D1, and D3; CDK1; members of the minichromosome maintenance family, i.e., MCM-3, MCM-4, MCM-6, MCM-7, and MCM-10; PCNA), whereas it induced two CDK1 inhibitors CDKN1B (p27kip1) and CDKN1C (p57kip2; Fig. 2B). In the same conditions, BMS-690514 led to a G1 cycle arrest (Fig. 3A) that preceded the overt induction of apoptosis recorded after 72 h (Fig. 1B). This has important consequences on the antitumor response mediated by the DNA-damaging agent CDDP. Because CDDP preferentially kills proliferating cells, it loses its cytotoxic activity when added to short-term BMS-690514–pre-treated (and hence G1 arrested) cells. In contrast, BMS-690514 mediates cytotoxic/antiproliferative effects even after pretreatment with CDDP, as shown in experiments in which CDDP and BMS-690514 were added in different orders to H1975 cells (Fig. 3B).

BMS-690514 influenced several genes that are involved in the mitochondrial cell death pathway. It induced several transcripts whose products can induce mitochondrial membrane permeabilization, namely the prosapoptotic BH3-only proteins from the Bcl-2 family Bim (by a factor of 3) and Bbc3/Puma (by a factor of 2.5) as well as p53AP1 (by a factor of 2). In addition, BMS-690514 down-regulated several cytoprotective proteins: the antiapoptotic Bcl-2 homologue Mcl-1 and several members of the HSP family (HSP40, HSP70, HSP90; Fig. 2B). In this context, it may be noteworthy that HSP90 promotes the stability of mutant EGFR and that the use of HSP90 inhibitors (like the geldanamycin derivative 17-AAG) may represent a novel approach for the therapy of NSCLC (43).

BMS-690514 induced signs of the mitochondrial apoptosis pathway. Thus, H1975 cells treated with BMS-690514 manifested the release of cytochrome c and subsequent caspase-3 activation, as determined by two-color immunofluorescence staining, immunoblotting, and cytofluorimetric quantification (Fig. 4A–C). BMS-690514–activated caspases that contribute to the mitochondrial pathway (including caspase-2, caspase-3, caspase-7, and caspase-9; Fig. 4B), yet failed to induce the proteolytic maturation of caspase-8, which characterizes the extrinsic pathway of apoptosis (Fig. 4B). Blockade of caspase activation by the broad-spectrum inhibitor 11-A.V. Gavai, P. Chen, D. Norris, et al. Preclinical antitumor activity of BMS-690514, a pan-HER/VEGFR2 kinase inhibitor [abstract]. American Chemical Society National Meeting, 2007.

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Z-VAD-fmk (Fig. 4B and C) retarded cell death induced by BMS-690514 (Fig. 1B). On the contrary, Z-VAD-fmk failed to prevent the dephosphorylation of EGFR promoted by BMS-690514; it also did not (or very slightly) affect the BMS-690514–mediated downregulation of EGFR (Fig. 4D). This correlated with the transcriptional effects of BMS-690514 on various chaperons of the HSP family, some of which have been implicated in the internalization and degradation of EGFR (43).

**siRNA screening of cell death modulators reveals the importance of Bcl-2 family proteins and NF-κB for BMS-690514–induced killing.** To further explore the mechanisms accounting for BMS-690514 cytotoxicity, we transfected a panel of ~40 validated siRNAs into H1975 cells and determined their influence on the antiproliferative effects mediated by BMS-690514. To this aim, transfected cells were treated with BMS-690514 for 24 h, before the assessment of proliferation by a tetrazolium salt reduction assay. The effect of each siRNA was evaluated by comparing the residual proliferation, as measured upon the treatment with BMS-690514, to the level observed in untreated (transfected) cells (Fig. 5A and B). Results were then compared with the negative control provided by an irrelevant, “unrelated” siRNA. In H1975 cells, several siRNAs attenuated the cytotoxic effects of BMS-690514. This applies in particular to siRNAs targeting proapoptotic members of the Bcl-2 family.
Figure 2. Microarray analysis of the transcriptome of H1975 cells treated with BMS-690514 or CDDP. H1975 cells were treated with either 5 μmol/L (1 μmol/L) BMS-690514 and 50 μmol/L (25 μmol/L) CDDP for 24 h (48 h) and subjected to RNA isolation. Total RNA was then retrotranscribed into double-stranded cDNA followed by T7 RNA polymerase–mediated linear amplification, labeling, and hybridization to a human whole genome 44k oligonucleotide array. Samples from treated cultures were compared with cRNAs obtained through the same procedure from untreated cells. A, hierarchical cluster analysis of genes, the transcription of which is modified in a statistically significant fashion (P < 10^{-5}) in treated cells compared with untreated controls. Each row represents the combination of two dye-swap experimental samples, and each column a single gene. The table (B) reports some examples of the differential transcriptional effects promoted by BMS-690514 and CDDP. This table is an excerpt from Supplementary Table S1, which contains a complete list of genes that are transcriptionally modulated by BMS-690514 by a factor higher than 2. n.s., nonstatistically significant P value (>10^{-5}).
(Puma, Bax, Bak, Bid, Bad), sessile proapoptotic mitochondrial proteins (ANT3, PBR), as well as caspases and their activators (caspase-2, Apaf-1; Fig. 5C). In contrast, the knock down of components of the NF-κB–activating system (p65, Nemo/IKKg, TAB2) sensitized H1975 cells to death mediated by BMS-690514 (Fig. 5C). It is important to note that the profile of siRNA-mediated modulation of BMS-690514 cytotoxicity, as obtained in H1975 cells, was rather specific and hence very different from that observed for another cell death inducer (CDDP) on the same cell line, or that observed for a cell line bearing wt EGFR (A549) treated with either BMS-690514 or CDDP (Fig. 5C). Using a different readout, namely the cytofluorimetric quantification of dying [DiOC6(3)low] and dead (propidium iodide positive) cells, we could confirm that the knock down of caspase-2 reduced the lethal effect of BMS-690514 on H1975 cells, whereas that of the NF-κB subunit p65 sensitized to BMS-690514 (Fig. 6A and B). Similarly, inhibition of the NF-κB–activating kinase IKK1 with BAY 11-7082 sensitized to cell death induction by BMS-690514 (Fig. 6C). Altogether, these data validate the notion that BMS-690514 kills through a mitochondrial pathway that is controlled by the NF-κB system.

Concluding remarks. Clinical data indicate that EGFR-targeted therapies may provide advantages over traditional chemotherapeutic regimens for the treatment of advanced NSCLC. However, objective responses are limited to a subset of patients who invariably develop resistance against currently available EGFR-TKIs such as erlotinib and gefitinib. Novel EGFR-TKI overcoming drug resistance might represent important weapons in the fight against cancer.

As shown here, BMS-690514 was able to arrest proliferation and/or to induce apoptosis in all tested NSCLC cell lines, although at different levels of potency. Importantly, BMS-690514 exhibited a prominent effect on cells affected by the T790M mutation of EGFR, which confers resistance against erlotinib. The proapoptotic effects of BMS-690514 were only partially counteracted by the pan-caspase inhibitor Z-VAD-fmk, suggesting the involvement of caspase-dependent as well as of caspase-independent mechanisms in BMS-690514–mediated killing. Accordingly, BMS-690514 was
able to elicit the proteolytic maturation of several caspases involved in the mitochondrial apoptotic pathway (e.g., caspase-2, caspase-3, caspase-7, and caspase-9), as determined by immunoblotting. On the other hand, caspase-8, which is specifically linked to the activation of death receptors, was not activated. Moreover, siRNA-mediated down-regulation of caspase-2 provided partial protection against BMS-690514–induced cell death. It has been previously reported that caspase-2 is necessary for the death of PC12 cells and sympathetic neurons induced by withdrawal of obligate trophic factors (44–46). Interestingly, our results point

![Figure 4](image_url)

**Figure 4.** Molecular pathways activated by the administration of BMS-690514 to H1975 cells. A. H1975 cells were treated or not with 10 μmol/L BMS-690514 for 48 h, followed by immunofluorescence staining to visualize cytochrome c (Cyt c), active caspase-3 (Casp-3a), and nuclei (with Hoechst 33342). Bars, 10 μm. B. H1975 cells were treated with the indicated concentration of BMS-690514 for 72 h, in the absence or presence of 25 μmol/L Z-VAD-fmk. Total cell lysates were then separated on SDS-PAGE and analyzed by immunoblotting with antibodies against total caspase-2, caspase-3, and caspase-7 as well as against active caspase-8, caspase-9, and GAPDH (loading control). The activation of caspases coincides with a reduction in the intensity of the procaspase band (P) paralleled by an increase of the band corresponding to the active fragment(s) (A). C. H1975 cells were incubated with BMS-690514 at the indicated concentration (in the absence or presence of 25 μmol/L Z-VAD-fmk) for 24 h, followed by the cytofluorometric quantification of caspase-3 activation. Columns, means of duplicate experiments; bars, SE. **, significant (P < 0.05) differences. D. H1975 protein extracts obtained as in (B) were analyzed by immunoblotting with antibodies specific for total (T) or phosphorylated (p) EGFR as well as for GAPDH (loading control).
Figure 5. siRNA-mediated modulation of BMS-690514 effects in H1975 and A549 cells. H1975 cells were transfected with the indicated siRNAs for 48 h, then left untreated (A) or incubated with 25 μmol/L BMS-690514 (B and C) or 25 μmol/L CDDP (B and C) for additional 24 h before the colorimetric assessment of cell proliferation/viability. A, gray columns, proliferation of cells transfected with the indicated siRNAs for 72 h normalized to that of untransfected cells. Negative Δ values indicate antiproliferative effects of siRNAs per se. EG5 down-regulation is known to arrest proliferation and is used here as a positive control for transfection. **, significantly different (P < 0.05) from unrelated control. UNR, unrelated siRNA. B, residual proliferation of cells transfected with the indicated siRNA for 48 h and then treated with 25 μmol/L BMS-690514 (black columns) or 25 μmol/L CDDP (white columns) for additional 24 h, normalized to that of cells transfected with the same siRNA for 48 h and then left untreated. Positive and negative Δ values indicate protective and sensitizing effects, respectively. C, for each siRNA, data are Δ values obtained as illustrated in (B) with BMS-690514 (first panel) and CDDP (second panel). siRNAs have been ordered from sensitizing to protective effects with respect to BMS-690514. The same order is then kept in the other panels. D, the same experimental approach was applied here to A549 cells. Red and green columns, negative and positive Δs, respectively, which differ in a statistically significant fashion from unrelated control (P < 0.05). White columns, nonsignificant values (P > 0.05). Dashed lines, boundaries of statistical significance from unrelated controls. Bars, 3 SE. The mathematical definition of Δ values and details on siRNAs are provided in Supplementary Materials and Methods.
again to the involvement of caspase-2 in the lethal pathways emanating from inhibited transmembrane growth factor receptors.

BMS-690514 sensitized NSCLC cells to the antiproliferative and proapoptotic activity of CDDP, the most frequently chemotherapeutic agent used for the treatment of NSCLC. This synergistic effect was only seen when BMS-690514 was administered after CDDP, and not vice versa. The fact that CDDP lost its effects on BMS-690514–pretreated cells can be explained by the G1-arresting effect of BMS-690514, which reduces the fraction of cells in the S phase (the preferential target of CDDP toxicity). Altogether, these results point to the possibility of performing combination chemotherapies with improved efficacy, using an appropriate schedule of sequential drug administrations.

siRNA-mediated knock down of several proteins with an established role in apoptosis indicates that BMS-690514 activates the intrinsic, mitochondrial pathway leading to cell death. BMS-690514 did not induce any death receptors nor their ligands (and did not promote the proteolytic activation of caspase-8), yet changed the balance between antiapoptotic and proapoptotic members from the Bcl-2 family (by reducing Mcl-1 and inducing Bim, Puma, and Bad) at the transcriptional level. Accordingly, siRNA-mediated depletion of several BH3-only proteins from the

![Figure 6. Modulation by caspase-2 and NFκB of BMS-690514 effects on H1975 cells. A, H1975 cells were transfected with the indicated siRNAs for 48 h and then treated or not with 5 μmol/L BMS-690514 for additional 72 h. Finally, cells were labeled with DiOC6(3)/propidium iodide (PI) and analyzed by flow cytometry. B, proteins were extracted from cells transfected as in (B) and analyzed by immunoblotting with antibodies specific for p65, caspase-2 (CASP-2), and GAPDH (loading control). C, H1975 cells were cultured for 72 h in the presence of increasing concentrations of BMS-690514, in association with the p65 pharmacologic inhibitor BAY 11-7082 (10 μmol/L). Then, cells were subjected to cytofluorometric analyses of DiOC6(3)/PI staining. Numbers, percentage of cells in each quadrant. Results are representative of three independent experiments.](image-url)
Bcl-2 family (Puma, Bad) or of two of the proapoptotic multidomain members of the family (Bax or Bak, but in particular the combination of both) reduced the cytotoxic effect of BMS-690514. Finally, cells treated with BMS-690514 manifested signs of mitochondrial membrane permeabilization, including the partially caspase-dependent release of cytochrome c and Δψm dissipation.

Surprisingly, our siRNA screening revealed that the down-regulation of ERCC1, which in NSCLC constitutes both a positive prognostic marker and a predictive factor for the sensitivity to CDPD-based adjuvant chemotherapy (47), reduced the lethal effects of BMS-690514. Similarly, we found that the depletion of other proteins involved in the DNA damage response or telomere maintenance (FANC, FAN3D, Chk1, and hTERT) also reduced BMS-690514 cytotoxicity, through hitherto unexplored mechanisms. This suggests that NSCLC that overexpress ERCC1 or other proteins involved in the DNA damage response or telomere effects of BMS-690514. Finally, cells treated with BMS-690514 manifested signs of apoptosis.

Cancer Res 2007; 67: (13). July 1, 2007 6262 www.aacrjournals.org

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*Cancer Res* 2007;67:6253-6262.

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