The Farnesyltransferase Inhibitor Lonafarnib Induces CCAAT/Enhancer-binding Protein Homologous Protein-dependent Expression of Death Receptor 5, Leading to Induction of Apoptosis in Human Cancer Cells

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Pre-clinical studies have demonstrated that farnesyltransferase inhibitors (FTIs) induce growth arrest or apoptosis in various human cancer cells independently of Ras mutations. However, the underlying mechanism remains unknown. Death receptor 5 (DR5) is a pro-apoptotic protein involved in mediating the extrinsic apoptotic pathway. Its role in FTI-induced apoptosis has not been reported. In this study, we investigated the modulation of DR5 by the FTI lonafarnib and the involvement of DR5 up-regulation in FTI-induced apoptosis. Lonafarnib activated caspase-8 and its downstream caspases, whereas silencing of DR5 expression abrogated lonafarnib-induced apoptosis. These results indicate that lonafarnib up-regulated DR5 expression, increased cell-surface DR5 distribution, and enhanced tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Overexpression of a dominant-negative Fas-associated death domain mutant or silencing of DR5 expression using small interfering RNA attenuated lonafarnib-induced apoptosis. These results indicate that lonafarnib induces caspase-8-dependent apoptosis and that lonafarnib-induced apoptosis is mediated by the extrinsic apoptotic pathway.

Lonafarnib (LNF; also called SCH66336 and Sarasar), a non-peptide tricyclic FTI, was one of the first FTIs to undergo clinical testing and to exhibit significant activity (3). In vitro, this agent, either alone or in combination with other therapeutic agents, inhibits the growth or induces apoptosis of several types of human cancer cells (4–12). In animal models, LNF demonstrates potent oral activity in a wide array of human tumor xenograft models including tumors of colon, lung, pancreas, prostate, and urinary bladder origin (12). When LNF is combined with other chemotherapeutic agents, enhanced antitumor activity is observed (4, 6). In a phase I trial enrolling individuals with lung or aerodigestive tract cancer, a provocative clinical activity was observed when LNF was combined with paclitaxel (13). In a recent phase II trial, LNF plus paclitaxel achieved significant clinical activity with a favorable safety profile in patients with taxane-refractory/resistant metastatic non-small cell lung cancer (14), and these results were later supported by pre-clinical data in cell lines (15).

There are two major apoptotic pathways: one involves death signals transduced through death receptors (extrinsic apoptotic pathway), and the other relies on mitochondrial signals (intrinsic apoptotic pathway) (16). Both pathways are involved in an ordered activation of a set of caspases, which in turn cleave cellular substrates, leading to apoptosis. The activation of caspase-8 and caspase-9 has been documented to play a central role in FTI-induced apoptosis. Its role in FTI-induced apoptosis and the involvement of DR5 by the FTI lonafarnib are critical aspects of this pathway. Thus, understanding the mechanisms underlying FTI-induced apoptosis is of great importance for the further development of FTIs as anticancer agents.
role in mediating apoptosis signaled by death receptors and by mitochondria, respectively; however, caspase-8 can activate the caspase-9-mediated apoptotic pathway by activating or cleaving Bid protein (16).

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, death receptor 5 (DR5; also named Apo2, TRAIL receptor 2, TRICK2, or Killer/DR5), belongs to the tumor necrosis factor receptor gene superfamily, the members of which all share a similar cysteine-rich extracellular domain and an additional cytoplasmic death domain (17). DR5 localizes to the cell surface, becomes activated or oligomerized (trimerized) upon binding to its ligand TRAIL or through overexpression, and then signals an apoptotic response through caspase-8-mediated rapid activation of caspase cascades (17). DR5 has recently attracted more attention because its ligand TRAIL preferentially induces apoptosis in transformed or malignant cells, demonstrating potential as a tumor-selective apoptosis-inducing cytokine for cancer treatment (18, 19). Certain cancer therapeutic agents induce the expression of DR5 in various types of cancer cells and thus are able to augment TRAIL-induced apoptosis or to initiate apoptosis (20).

The CCAAT/enhancer-binding protein homologous protein (CHOP), also known as GADD153 (growth arrest and DNA damage gene 153), is an endoplasmic reticulum (ER) stress-induced transcription factor involved in the regulation of apoptosis, particularly ER stress-associated apoptosis (21). Recent studies have demonstrated that CHOP directly regulates DR5 expression through a CHOP-binding site in the 5-flanking region of the DR5 gene (22, 23). Certain drugs induce DR5 expression through CHOP-dependent transactivation of the DR5 gene (22–24). The mechanisms underlying FTI-mediated growth arrest and apoptosis induction are largely undefined (1). Several non-Ras targets such as RhoB and Rab geranylgeranyltransferase have been proposed (25, 26). In addition, Akt inactivation appears to play a role in apoptosis induced by a subset of FTIs (5, 27, 28), although some studies have been unable to demonstrate this role (29–31). However, DR5 has not been suggested to be involved in the biological actions of FTIs. In this study, we reveal, for the first time, a novel mechanism in human cancer cells by which FTIs, particularly LNF, induce apoptosis via the DR5 gene (22–24).

**EXPERIMENTAL PROCEDURES**

**Reagents**—LNF and its analog SCH66337 were provided by Schering-Plough Research Institute (Kenilworth, NJ). They were dissolved in Me$_2$SO at a concentration of 10 mM, and aliquots were stored at −80 °C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. Soluble human recombinant TRAIL was purchased from BIOMOL International (Plymouth Meeting, PA). The caspase inhibitors benzylxoycarbonyl-Val-Ala-Asp-fluoromethyl ketone and benzylxoycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone were purchased from Enzyme System Products (Livermore, CA). Staurosporine and other chemicals were purchased from Sigma. Rabbit polyclonal anti-DR5 antibody was purchased from ProSci Inc. (Poway, CA).

Mouse monoclonal anti-caspase-3 antibody was purchased from Imgenex (San Diego, CA). Rabbit polyclonal anti-caspase-9, anti-caspase-8, and anti-poly(ADP-ribose) polymerase antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal anti-Ras GTPase-activating protein (RasGAP) antibody B4F8 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-HDJ-2 antibody (clone KA2A5.6) was purchased from Lab Vision Corp. (Fremont, CA). Mouse monoclonal anti-Bip/GRP78 antibody was purchased from BD Transduction Laboratories.

**Cell Lines and Cell Cultures**—All cell lines were purchased from American Type Culture Collection (Manassas, VA). H460 cell lines stably expressing a dominant-negative Fas-associated death domain mutant (FADDm) were described previously (32). These cell lines were grown in monolayer culture in RPMI 1640 medium supplemented with glutamine and 5% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere consisting of 5% CO$_2$ and 95% air.

**Western Blot Analysis**—The procedures for preparation of whole cell protein lysates and Western blot analysis were described previously (32, 33).

**Detection of DR5 mRNA Expression**—DR5 mRNA was detected by reverse transcription-PCR as follows. Total RNA was isolated from cells using TriReagent (Sigma) as instructed by the manufacturer. First-strand cDNA was synthesized from 2 μg of total RNA in a volume of 20 μl containing 1 μl of avian myeloblastosis virus reverse transcriptase, 0.5 μl of dNTP (25 mM each), 0.5 μl of random primer (0.5 μg/μl), 4 μl of 5× reverse transcription buffer, and sterile H$_2$O, followed by incubation at 42 °C for 60 min and inactivation by heating at 70 °C for 15 min. cDNA was then amplified by PCR using the following primers: DR5, 5′-GACCTAGCTCCCGAGCAGAGAG-3′ (sense) and 5′-CGGCTGCAACTGTGACTC-CTAT-3′ (antisense); and β-actin, 5′-GAAAATCTTCTACTCCAT-CCATC-3′ (sense) and 5′-CTAGAAGCATTTCGGTGACAGTGGACGGGC-5′ (antisense). The 25-μl amplification mixture contained 2 μl of cDNA, 0.5 μl of dNTP (25 mM each), 1 μl each of the sense and antisense primers (20 μM each), 1 μl of Taq DNA polymerase (5 units/μl; Promega, Madison, WI), 2.5 μl of 10× reaction buffer, and sterile H$_2$O. PCR was performed for 28 cycles. After an initial step at 95 °C for 3 min, each cycle consisted of 50 s of denaturation at 94 °C, 50 s of annealing at 58 °C, and 5 s of extension at 72 °C. This was followed by an additional extension step at 72 °C for 10 min. The housekeeping gene β-actin was also amplified as an internal reference. PCR products were resolved by electrophoresis on a 4% agarose gel, stained, and directly visualized under UV illumination.

**Construction of DR5 Reporter Plasmid, Transient Transfection, and Luciferase Activity Assay**—The plasmid containing a 5′-flanking region of the DR5 gene was kindly provided by Dr. G. S. Wu (Wayne State University School of Medicine, Detroit, MI). We used this plasmid as a template to amplify a series of deletion fragments of the 5′-flanking region of the DR5 gene ranging from 3070 to 120 bp upstream of the translation start site by PCR and then subcloned these fragments into the pGL3-Basic reporter vector (Promega) through KpnI and...
Role of DR5 Up-regulation in FTI-induced Apoptosis

BglII restriction sites. In the PCR amplification, the reverse primer 5’-CTTAAAGATCTGGCCGTAGGGAACGCTTATAGTC-3’ was used to make all deletion constructs. The upstream primers were 5’-CTTAGGTACCTGGCTGTCTGTCTCTACGGCCC.3’ (−3070), 5’-CTTAGGGTAATCTAGTATCTAGAGGGCGG-3’ (−373), 5’-CTTAGGTACCTTTATATTTGGCAACCCAGCTG-3’ (−240), and 5’-CTTAGGTACCCCCAGAAAACCAACACGCCCCG-3’ (−120). These constructs were named pGL3-DR5(−3070), pGL3-DR5(−240), pGL3-DR5(−373), pGL3-DR5(−240), and pGL3-DR5(−120), respectively. The reporter constructs containing a 552-bp 5’-flanking region of the DR5 gene with a wild-type or mutated CHOP-binding site, pGL3-DR5(240), respectively. The upstream primers used were 5’-CTTAGGTACCTGGCTGTCTGTCTCTACGGCCC.3’ (−3070), 5’-CTTAGGGTAATCTAGTATCTAGAGGGCGG-3’ (−373), 5’-CTTAGGTACCTTTATATTTGGCAACCCAGCTG-3’ (−240), and 5’-CTTAGGTACCCCCAGAAAACCAACACGCCCCG-3’ (−120).

To examine the effect of LNF on DR5 transactivation activity, cells were seeded in 24-well plates and cotransfected with the given reporter plasmid (0.5 μg/well) and the pCH110 plasmid (0.2 μg/well) using FuGENE 6 transfection reagent (3:1 ratio; Roche Applied Science) following the manufacturer’s protocol. Twenty-four hours later, the cells were treated with LNF. After 12 h, the cells were lysed and subjected to luciferase activity assay using a luciferase assay system (Promega), which contains an SV40 promoter, as a control. A pCH110 plasmid encoding β-galactosidase (GE Healthcare) was used in the cotransfection for normalization. These plasmids were purified with a Qiagen plasmid maxi kit (Qiagen Inc.).

Detection of Cell-surface DR5—In this study, cell-surface DR5 expression was analyzed by both flow cytometry and immunofluorescent staining. The procedure for direct antibody staining and subsequent flow cytometric analysis of cell-surface protein was described previously (35). The mean fluorescence intensity that represents antigenic density on a per cell basis was used to represent DR5 expression levels. Phycoerythrin (PE)-conjugated mouse monoclonal anti-human DR5 (clone DJR2-4), anti-human DR4 (clone DJR1), anti-human decoy receptor 1 (clone DJR3), anti-human decoy receptor 2 (clone DJR4-1), and anti-human Fas (clone DX2) antibodies and PE-conjugated mouse IgG1 isotype control (MOPC21/P3) were purchased from eBioscience (San Diego, CA). For immunofluorescent staining, cells were plated overnight on coverslips in 24-well plates and treated with different concentrations of LNF. Cells were then fixed in 68 mM Pipes, 25 mM Hepes, 15 mM EGTA, and 3 mM MgCl2 with 3.7% formaldehyde, 0.05% glutaraldehyde, and 0.5% Triton X-100 for 10 min; washed three times with phosphate-buffered saline; and then blocked for 15 min with 10% normal goat serum. Next, cells were incubated with PE-conjugated mouse monoclonal anti-DR5 antibody (clone DJR2-4) diluted 1:15 in 5% normal goat serum for 1 h at room temperature. After the cells were washed three times, coverslips were removed from wells, inverted, and placed on slides. Images were acquired using a ×63 Zeiss Plan-Apochromat oil lens (numerical aperture = 1.4) mounted on a Zeiss LMS 510 confocal laser scanning microscope.

Detection of Apoptosis—The amounts of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) formed during apoptosis were measured using a Cell Death Detection ELISA plus kit (Roche Applied Science) according to the manufacturer’s instructions. Caspase activation and substrate cleavage were detected by Western blot analysis as described above. In addition, we also counted floating cells in the medium as another indicator of apoptotic cell death. Because the albumin in the serum attenuates the FTI effect in culture (31), we performed these experiments in 0.1% serum-containing medium to increase induction of apoptosis.

Cell Survival Assay—Cells were seeded in 96-well cell culture plates and treated on the 2nd day with the indicated agents. At the end of treatment, the cell number was estimated by the sulforhodamine B assay as described previously (36).

Small Interfering RNA (siRNA)-mediated Gene Silencing—High purity control (non-silencing) siRNA oligonucleotides that target sequence 5’-AAATTCCTGCGAGTGCACGT3’– was purchased from Qiagen Inc. Caspase-8, DR5, and CHOP siRNA duplexes that target sequences 5’-AACTACAGAAAAGTTATACCT3’, 5’-AAGCCTTTGTGTGT-GTGC3’ (32, 37), and 5’-AAGAACCACAGAGGUAACAAAC3’ (22), respectively, were synthesized by Qiagen Inc. The additional DR5 siRNA-2, which targets sequence 5’TAGGTTGACCG-TAGCTTTAGA3’ (22), was also synthesized by Qiagen Inc. Transfection of siRNA was performed as described previously (32). Gene silencing effects and caspase activation were evaluated by Western blot analysis, whereas DNA fragmentation was measured using the Cell Death Detection ELISA plus kit as described above.

Statistical Analysis—Cell survival and apoptosis (i.e., DNA fragmentation) between two groups were analyzed with twosided unpaired Student’s t tests when the variances were equal or with Welch’s corrected t test when the variances were not equal using GraphPad InStat Version 3 software. The assumption for use of the t tests was calculated and suggested by the same software. All means ± S.D. from triplicate or quadruplicate samples were calculated with Microsoft Excel Version 5.0 software. In all statistical analyses, results were considered to be statistically significant at p < 0.05.

RESULTS

LNF Induces Caspase-8-dependent Apoptosis—Our previous study has shown that LNF induces apoptosis, particularly in low serum culture medium (31). Here, we examined further the effects of LNF on caspase activation and its involvement in LNF-induced apoptosis in human lung cancer cells. In both H157 and H1792 human lung cancer cell lines, LNF increased the levels of the proforms of caspase-8, -9, and -3; increased the levels of their cleaved forms; and induced DNA fragmentation. Because caspase-8 activation is a critical event in the extrinsic apoptotic signaling pathway, we tested...
Whether caspase-8 activation is required for LNF-induced apoptosis. LNF significantly increased the amount of DNA fragments \((p < 0.001)\), but in the presence of the pan-caspase inhibitor benzoxycarbonyl-Val-Ala-Asp-fluoromethyl ketone or the caspase-8 inhibitor benzoxycarbonyl-Ile-Glu(O\text{Me})-Thr-Asp(O\text{Me})-fluoromethyl ketone, LNF failed to increase the levels of DNA fragments \((p < 0.001)\) (Fig. 1B), indicating that LNF-induced apoptosis is caspase-dependent, particularly caspase-8-dependent. To further demonstrate the importance of caspase-8 activation in LNF-induced apoptosis, we silenced the expression of caspase-8 and then examined its impact on LNF-induced apoptosis. As shown in Fig. 1C, caspase-8 levels were dramatically decreased in H1792 cells transfected with the caspase-8 siRNA in comparison with caspase-8 levels in those cells transfected with the control siRNA. By measuring cell survival, we found that LNF caused significantly less cell death in caspase-8 siRNA-transfected cells than in control siRNA-transfected cells (Fig. 1D). Accordingly, we detected cleaved forms of caspase-8, caspase-3, and poly(ADP-ribose) polymerase (Fig. 1C) and increased amounts of DNA fragments (Fig. 1E) in control siRNA-transfected cells but not in caspase-8 siRNA-transfected cells after exposure to LNF. These results clearly indicate that silencing of caspase-8 expression inhibits LNF-induced apoptosis, further supporting the essential role of caspase-8 activation in LNF-induced apoptosis. Used as control treatments, TRAIL, a cytokine known to trigger the extrinsic apoptotic pathway, failed to decrease cell survival (Fig. 1D) and exhibited attenuated effects on the cleavage of caspase-8, caspase-3, and poly(ADP-ribose) polymerase (Fig. 1C) in caspase-8 siRNA-transfected cells, whereas staurosporine, a small molecule known to induce apoptosis through the mitochondrial apoptotic pathway, was equally active in inducing cell death (Fig. 1D) and cleavage of caspase-3 and poly(ADP-ribose) polymerase in both control and caspase-8 siRNA-transfected cells, although caspase-8 cleavage was inhibited in caspase-8 siRNA-transfected cells (Fig. 1C).

**LNF Induces DR5 Expression**—Because caspase-8 activation is an essential step in the extrinsic death receptor-mediated apoptotic pathway, we next tested whether LNF affects the expression of death receptors. By Western blot analysis, we found that LNF increased DR5 expression in a time- and concentration-dependent manner (Fig. 2, A and B). Specifically,
Role of DR5 Up-regulation in FTI-induced Apoptosis

LNF-induced DR5 expression occurred at 8 h, reached a peak at 24 h (H1792 cells) or 48 h (H157 cells), and was sustained up to 48 h (H1792 cells) or 72 h (H157 cells) post-LNF treatment (Fig. 2A). The highest levels of DR5 expression were observed with 10 \( \mu M \) LNF; however, 2.5 \( \mu M \) LNF was sufficient to increase DR5 expression relative to untreated cells (Fig. 2B). Therefore, it appears that, at clinically achievable and tolerable concentrations (2–5 \( \mu M \)) (39, 40), LNF induces rapid but sustained up-regulation of DR5 expression. Moreover, we found that LNF also increased DR5 expression in other human lung cancer cell lines, as shown in Fig. 2C, as well as in other types of cancer cell lines, including breast (MCF-7), cervical (HeLa), prostate (DU145), head and neck (38 and SqCC/Y1), and colon (HCT116) (Fig. 2D). We also compared the effects of LNF on DR5 induction in HCT116(p53\(^{+/-}\)) and HCT116(p53\(^{-/-}\)) cell lines and found that LNF up-regulated DR5 expression in both cell lines (Fig. 2D). These findings indicate that DR5 induction by LNF commonly occurs in human cancer cells. We noted that LNF also increased DR4 expression; however, it did not occur in all tested cell lines (data not shown). Thus, we focused our subsequent studies on DR5 induction.

To determine whether DR5 induction by LNF occurs at the transcriptional level, we detected DR5 mRNA levels in cells exposed to different concentrations of LNF using reverse transcription-PCR. As shown in Fig. 2E, LNF at concentrations ranging from 2.5 to 10 \( \mu M \) increased DR5 mRNA levels in a concentration-dependent manner. Moreover, we examined the effect of LNF on DR5 promoter activity and observed that LNF significantly increased luciferase activity in cells transfected with a luciferase reporter plasmid carrying a DR5 5′-flanking region (i.e. pGL3-DR5(−3070)), but not in cells transfected with a reporter plasmid driven by the SV40 promoter (i.e. pGL3-SV40-Luc) (Fig. 2F), indicating that LNF enhances DR5 transcription. Collectively, these results clearly show that LNF up-regulates DR5 expression at the transcriptional level.

LNF Increases Cell-surface DR5 Levels—It is known that DR5 functions as a cell-surface protein (17). Thus, we were interested in determining whether LNF increases cell-surface DR5 levels. In this study, we treated cells with LNF, stained the cells with PE-conjugated anti-DR5 antibody, and analyzed PE-positive cells by flow cytometry and confocal microscopy. By flow cytometry, we detected a dramatic increase in fluorescent intensity in both H1792 and H157 cells treated with LNF compared with cells exposed to the Me\(_2\)SO control (Fig. 3A). The mean fluorescent intensities were 22.82, 60.01, and 142.71 in H1792 cells treated with the Me\(_2\)SO control, 5 \( \mu M \) LNF, and 10 \( \mu M \) LNF, respectively, and 29.44, 67.70, and 109.96 in H157 cells, respectively. By confocal microscopy, we observed low levels of cytoplasmic DR5 protein in control cells without cell-surface staining of DR5. After treatment with LNF, we observed clear DR5 staining on the cell surface, particularly at 5 and 10 \( \mu M \) (Fig. 3B), indicating that LNF induces cell-surface localization of DR5. Collectively, these data clearly demonstrate that LNF increases cell-surface DR5 levels.

In addition, we determined whether LNF alters the levels of other death or TRAIL receptors. H1792 cells expressed cell-surface DR4 and Fas, but very low levels of decoy receptors 1 and 2. Upon treatment with either 5 or 10 \( \mu M \) LNF, the levels of these surface receptors were not further increased (supplemental Fig. S1). Thus, it is clear that LNF does not affect the levels of...
other death or TRAIL receptors at the cell surface. Collectively, these data further suggest the importance of DR5 up-regulation in LNF-induced apoptosis.

**LNF Induces DR5 Expression through a CHOP-dependent Mechanism**—To determine how FTIs increase DR5 expression at the transcriptional level, we examined the effects of LNF on the transactivation of reporter constructs with different lengths of DR5 5′-flanking regions (Fig. 4A) to identify the region responsible for LNF-mediated DR5 transactivation. In this transient transfection and luciferase assay, LNF failed to increase the luciferase activity of the construct pGL3-DR5(−120) while significantly increasing the luciferase activity of pGL3-DR5(−373), pGL3-DR5(−420), and pGL3-DR5(−3070) (Fig. 4A), indicating that the region between −240 and −373 contains essential element(s) responsible for LNF-induced DR5 transactivation. We identified a CHOP-binding site in this region, which has been demonstrated to be responsible for DR5 up-regulation by several cancer therapeutic agents (22–24). Thus, we further compared the effects of LNF on the transactivation of reporter constructs carrying wild-type and mutated CHOP-binding sites. We also included constructs carrying mutated NF-κB- and Elk-binding sites as controls (Fig. 4B). As shown in Fig. 4B, LNF increased the luciferase activity of the constructs carrying the wild-type DR5 promoter region or the DR5 promoter region with a mutated NF-κB- or Elk-binding site. However, LNF failed to increase the luciferase activity of the construct carrying the DR5 promoter region with a mutated CHOP-binding site. These results clearly indicate that the CHOP-binding site in the DR5 promoter region is required for LNF-mediated DR5 transactivation.

We next examined whether LNF actually modulates the expression of CHOP. By Western blot analysis, we detected a time-dependent DR5 induction accompanied by CHOP up-regulation in cells exposed to LNF, both of which occurred at 3 h and were sustained up to 24 h post-LNF treatment (Fig. 4C). The cleavage of caspase-8, caspase-3, and poly(ADP-ribose) polymerase was detected at 12 h after LNF treatment (Fig. 4C). Thus, the up-regulation of both CHOP and DR5 appears to be an early event that occurs before induction of apoptosis. By blocking LNF-induced CHOP expression using CHOP siRNA, we detected that DR5 induction by LNF was also accordingly diminished (Fig. 4D), indicating that LNF-induced DR5 up-regulation is secondary to CHOP induction. We conclude that LNF induces CHOP-dependent DR5 expression.

Given that CHOP is an ER stress-associated protein (21), we further examined whether LNF alters the expression levels of BiP/GRP78, another key protein marker of ER stress (21). As shown in Fig. 4C, under the same conditions used to test CHOP and DR5, LNF did not alter the levels of BiP/GRP78 from 3 to 16 h post-treatment. At 24 h, LNF only slightly increased the BiP/GRP78 levels. Together, these data clearly show that LNF increases the levels of CHOP, but not BiP/GRP78.

**Induction of DR5 Contributes to LNF-induced Apoptosis**—It is well known that DR5 activation (e.g. ligation with TRAIL) recruits and activates caspase-8 via the adaptor molecule FADD, leading to induction of apoptosis (18, 19). One common strategy to disrupt death receptor-induced apoptosis is to use a dominant-negative FADDm, which prevents death receptors from recruiting caspase-8 (41). To determine whether DR5 up-regulation contributes to LNF-induced apoptosis, we compared the effects of LNF on apoptosis induction in vector-transfected H460 cells (H460/V1) and FADDm-transfected cells (H460/Fm6 and H460/Fm16). These cell lines were equally sensitive to staurosporine in terms of decreasing cell survival. However, both H460/Fm6 and H460/Fm16 cell lines were significantly less sensitive to TRAIL in comparison with H460/V1 cells (Fig. 5A). LNF effectively increased the number of floating (dead) cells (Fig. 5B) and the levels of DNA fragments (Fig. 5C) in H460/V1 cells. However, these effects were significantly diminished in H460 cells expressing FADDm (i.e. H460/Fm6 and H460/Fm16; p < 0.05), indicating that FADDm overexpression abrogates the ability of LNF to induce apoptotic cell death, suggesting that the death receptor-mediated extrinsic apoptotic pathway is critical for LNF-induced apoptosis.

To further decipher the role of DR5 in LNF-induced apoptosis, we knocked down DR5 gene expression using siRNA and then examined its impact on LNF-induced apoptosis. In both H1792 and H157 cells, DR5 siRNA transfection dramatically decreased the basal levels of DR5 expression and, more importantly, abolished LNF-induced DR5 expression as detected by Western blot analysis (Fig. 5D). Furthermore, in cells transfected with DR5 siRNA, LNF-induced DNA fragmentation was significantly suppressed compared with cells transfected with control siRNA (Fig. 5E). To avoid possible off-target effects of siRNA, we also used a second DR5 siRNA (i.e. DR5 siRNA-2) that targets a different sequence of the DR5 gene to reproduce the aforementioned results. Similarly, blockage of DR5 induction by silencing DR5 using DR5 siRNA-2 significantly inhibited LNF-induced DNA fragmentation (supplemental Fig. S2). Collectively, these results further support the critical role of the DR5-mediated extrinsic apoptotic pathway in LNF-induced apoptosis.

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**FIGURE 3. LNF increases cell-surface DR5 levels.** A, H1792 and H157 cell lines were exposed to the indicated concentrations of LNF for 24 h. The cells were then harvested, stained with PE-conjugated DR5 antibody, and analyzed by flow cytometry. The shaded peak represents cells stained with a matched PE-conjugated IgG isotype control (PE-IgG). The open peaks were cells stained with PE-conjugated anti-DR5 antibody. DMSO, MeSO, B; H1792 cells were treated with the indicated concentrations of LNF for 16 h and then stained with PE-conjugated DR5 antibody. DR5 expression was visualized under a confocal microscope.

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**FIGURE 4. LNF-induced DR5 up-regulation is CHOP-dependent.** A, Luciferase reporter activity of reporter constructs with different lengths of DR5 5′-flanking regions. B, Western blot analysis of CHOP and BiP/GRP78 expression. Western blotting was performed on cell extracts under a reducing condition and the blots were probed with antibodies against CHOP and BiP/GRP78, respectively. LNF-treated H460 cells (H460/V1) and FADDm-transfected cells (H460/Fm6 and H460/Fm16) served as controls. C, a schematic diagram of the region responsible for LNF-mediated DR5 transactivation. D, CHOP siRNA-1 and CHOP siRNA-2 were transfected into H460 cells. E, Western blot analysis of CHOP expression. Western blotting was performed on cell extracts under a reducing condition and the blots were probed with antibodies against CHOP.
Role of DR5 Up-regulation in FTI-induced Apoptosis

Combination of LNF and TRAIL Enhances Induction of Apoptosis—Because LNF increases cell-surface DR5 expression, we speculated that LNF would cooperate with exogenous TRAIL to augment induction of apoptosis if the induced DR5 is functional. Thus, we examined the effect of the combination of LNF with exogenous human recombinant TRAIL on apoptosis in two human lung cancer cell lines. As shown in Fig. 6, A, 5 μM LNF alone did not apparently induce cleavage of caspase-8, caspase-3, poly(ADP-ribose) polymerase, and RasGAP, whereas TRAIL at the tested doses, particularly 10 and 20 ng/ml, caused only weak cleavage of the caspases and their substrates. Notably, the combination of LNF and TRAIL induced obvious cleavage of not only caspase-8, but also caspase-3 and its substrate poly(ADP-ribose) polymerase, as indicated by the dramatic decreases in their proforms (uncleaved forms) and/or increase in the cleaved bands. LNF alone did not affect RasGAP levels, and TRAIL alone at 20 and 30 ng/ml caused only weak cleavage of RasGAP. However, the combination of LNF with TRAIL, even at 10 ng/ml TRAIL, induced cleavage of RasGAP (Fig. 6A). Therefore, it appears that LNF cooperates with TRAIL to enhance activation of caspase-8 and its downstream caspase-3.

In addition, both LNF alone (1–5 μM) and TRAIL alone (25 ng/ml) did not increase or only slightly increased the amount of DNA fragments; however, the combination of the two agents induced a striking increase in DNA fragment levels, which were apparently greater than the sums of the levels caused by each single agent alone in both cell lines (Fig. 5B). For example, in H1792 cells, LNF at 5 μM increased DNA fragmentation by <0.2 arbitrary units, whereas TRAIL at 25 ng/ml increased DNA fragmentation by ∼0.3 arbitrary units. However, the combination of these two agents increased DNA fragmentation by ∼1.4 arbitrary units. Therefore, it appears that LNF synergizes with TRAIL to induce apoptosis in human cancer cells.

DISCUSSION

Although FTIs were historically developed as anti-Ras agents, it is now generally agreed that FTIs exert their antitumor activity independently of their inhibition of Ras farnesylation (1, 2, 25). In this study, we have demonstrated that induction of DR5 and its mediated activation of the extrinsic apoptotic pathway play critical roles in LNF-induced apoptosis in human lung cancer cells due to the following findings. First, LNF activated caspase-8, which is required for LNF-induced apoptosis because inhibition of caspase-8 activation by either a caspase-8 inhibitor or siRNA-mediated caspase-8 silencing abolished LNF-induced apoptosis. Second, overexpression of FADDm abrogated LNF-induced caspase activation and apoptotic cell death, suggesting that the activation of the extrinsic apoptotic pathway is critical for LNF-induced apoptosis. Finally,
LNF primarily induced DR5 expression (including an increase in cell-surface DR5), whereas DR5 silencing using DR5 siRNA attenuated LNF-induced caspase activation and apoptosis, indicating that DR5 induction contributes to LNF-induced apoptosis. Thus, our study is the first to demonstrate that an FTI induces DR5-mediated, caspase-8-dependent apoptosis in human cancer cells.

DR5 expression is regulated through p53-dependent and -independent mechanisms (42, 43). In our study, most of the cell lines used for examining DR5 up-regulation, including H1792, H157, H1299, H226, DU145, HeLa, and SqCC/Y1, have mutant or deleted p53 (44–47). Because LNF still increased DR5 expression in these cell lines (Fig. 2), we conclude that LNF induces DR5 expression independently of p53. This is further supported by our observation that LNF up-regulated DR5 expression with equal potency in both HCT116 and p53 knock-out HCT116 cell lines (Fig. 2). Currently, it is known that DR5 can be regulated in a p53-independent manner (43, 48), but the underlying mechanisms remain largely unclear.

CHOP has been demonstrated recently to regulate DR5 expression through the CHOP-binding site in the DR5 gene (22, 23), revealing a novel p53-independent regulation of DR5 expression. In our study, LNF appeared to increase DR5 expression at the transcriptional level because it increased DR5 mRNA levels and the activity of the DR5 promoter (Fig. 2). The deletion and mutation analyses of the DR5 5′-flanking region revealed that the region containing the CHOP-binding site is essential for LNF-mediated DR5 transactivation (Fig. 4). Indeed, LNF induced a time-dependent CHOP expression accompanied by the up-regulation of DR5 expression. Blockage of LNF-mediated CHOP expression by the CHOP siRNA accordingly inhibited DR5 up-regulation (Fig. 4). We conclude that LNF induces DR5 expression through a CHOP-dependent mechanism.

It is known that CHOP is an ER stress-regulated protein (21). It was reported that another FTI called R115777 induces ER stress in myeloma cells because it increased the levels of both CHOP and particularly BiP/GRP78 after a prolonged treatment (i.e., 72 h) (49). In our system, LNF at a concentration that induces DR5 expression (e.g., 5 μM) increased the levels of CHOP, but not BiP/GRP78 (Fig. 4), arguing that LNF induces ER stress in our system. Nonetheless, whether
CHOP elevation by LNF is due to ER stress or other mechanisms needs to be studied further.

In addition to LNF, R115777 also increased DR5 expression.4 The LNF analog SCH66337, which was much weaker than LNF in inhibiting protein farnesylation, also showed weaker activity than LNF in inducing DR5 expression and enhancing TRAIL-induced apoptosis (supplemental Fig. S3). Therefore, future studies should address whether there is a relationship between inhibition of protein farnesylation and induction of CHOP and DR5.

Although R115777 was reported to induce apoptosis in the presence of a caspase-8 inhibitor in myeloma cells (49), this FTI did enhance death receptor-induced apoptosis mediated by Fas ligand or TRAIL in myeloma cells (50) and lung cancer cells.4 These findings are in agreement with our current finding that LNF enhances TRAIL-induced apoptosis in human lung cancer cells. In our study, we have clearly shown that LNF induces caspase-8-dependent apoptosis through both pharmacological (inhibitor) and molecular (siRNA) approaches (Fig. 1). Because both LNF and R115777 induced DR5 expression in myeloma cells,4 further studies are needed to address whether the death receptor-mediated apoptotic pathway is also involved in FTI-induced apoptosis in myeloma cells as well as in other types of cancer cells.

Our findings that FTIs increase DR5 expression and enhance TRAIL-induced apoptosis have clinically meaningful implications. It is known that TRAIL functions as the DR5 ligand and rapidly induces apoptosis in a wide variety of transformed cells, but is not cytotoxic in normal cells in vitro and in vivo (18, 19). Therefore, TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and a promising new candidate for cancer treatment. In addition, agonistic anti-DR5 antibodies can also induce DR5 trimerization, which triggers the extrinsic apoptotic pathway, thus having great cancer therapeutic potential (51). In fact, the agonistic anti-DR5 antibody is already being tested in phase I clinical trials. Therefore, LNF, as well as other FTIs that increase cell-surface DR5 expression, can be used in combination with TRAIL or an agonistic anti-DR5 antibody to achieve an enhanced effect on apoptosis induction in human cancer cells. In summary, our study has demonstrated, for the first time, that an FTI (e.g. LNF) induces CHOP-dependent DR5 expression at a clinically achievable concentration range, contributing to FTI-induced apoptosis.

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