Potentiation of Nitric Oxide-induced Apoptosis of MDA-MB-468 Cells by Farnesyltransferase Inhibitor: Implications in Breast Cancer

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ABSTRACT

High amounts of nitric oxide (NO) produced by activated macrophages or NO donors are required to induce cytotoxicity and apoptosis in pathogens and tumor cells. High concentrations of NO may lead to nonspecific toxicity thereby limiting the use of NO donors in the treatment of cancer. In this study, we tested the possibility of potentiating the apoptotic action of NO in a human breast cancer cell line, MDA-MB-468, by combining it with a farnesyltransferase inhibitor (FTI), which has been shown to induce apoptosis in some other cancer cell lines with minimal toxicity to normal cells. DETA-NONOate, a long acting NO donor which has a half-life of 20 h at 37°C, was used in this study, DETA-NONOate (1 mM), which releases NO in the range produced by activated macrophages, induced apoptosis after 36 h in MDA-MB-468 cells via cytochrome c release and caspase-9 and -3 activation. FTI (25 μM) potentiated the action of lower concentrations of DETA-NONOate (25–100 μM) by inducing apoptosis in these cells within 24 h by increasing cytochrome c release and caspase-9 and -3 activation. This effect was observed preferentially in the cancer cell lines studied with no apoptosis induction in normal breast epithelial cells. This novel combination of FTI and NO may emerge as a promising approach for the treatment of breast cancer.

INTRODUCTION

NO, a highly reactive free radical gas, has emerged as an important mediator in many physiological and pathological processes (1, 2). Among the physiological processes, NO has an important role in the regulation of vascular tone, platelet aggregation, neurotransmission, learning, and memory, whereas its involvement in pathological processes include induction of cytotoxicity leading to apoptotic or necrotic death (3). Beneficial or deleterious effects of NO on a cell depends on the concentrations of NO, duration of exposure, and the surrounding microenvironment. Low amounts of NO (nm range) as produced by endothelial NO synthase regulate many physiological processes, whereas large amounts of NO (μM range) as produced by activated macrophages are required for inducing cytotoxicity to the cells (4). NO produced by macrophages plays an important role in modulating the host defense mechanism against tumor cells in vitro as well as in vivo (5–8). Several in vitro studies have also demonstrated that NO donors are cytotoxic to tumor cells leading to apoptosis (9).

These NO donor molecules target not only the tumor cells but also the normal cells to induce apoptosis, and their use for the treatment of cancer is thereby limited (10). Because higher amounts of NO are required for their cytotoxic actions compared with its physiological effects on other systems like the cardiovascular system, administration of high amounts of NO could lead to unwanted side effects, which would preclude their use alone as a single chemotherapeutic agent. Hence, we wanted to test the possibility of potentiating the apoptotic action of NO by combining it with other drugs which, in the long term, may lead to the development of combination therapy with NO donors for the treatment of some forms of cancer.

FTIs block the growth of tumors with minimal toxicity to normal cells (11–13). These compounds inhibit the protein farnesyltransferase, an enzyme that catalyzes the farnesylation of a number of proteins including members of the Ras superfamily of small G proteins (12, 14). A variety of animal studies has demonstrated the ability of FTIs to block or even regress growth of tumor cells (11–13). Their effectiveness in treating certain malignancies is currently being assessed in various clinical trials (15, 16). However, in some preclinical studies, although there was near ablation of residual tumors, there was proliferation of a residual tumor after withdrawal of these agents (17). Thus, FTI treatment may require long-term administration, and this increases the possibility of side effects and the development of resistance to these agents. Combination of FTIs with other drugs may therefore be more beneficial than FTIs alone.

The proliferation of malignant cells is regulated by a variety of intracellular signaling pathways, and these pathways cross-talk with each other. Combination therapies using two or more drugs are therefore expected to be more effective for cancer therapy compared with single-drug therapy. In this regard, the combination of FTIs with a variety of commonly used anticancer agents on human tumors has been tested (18–23). In all these studies, FTIs alone had less cytostatic or cytotoxic effect. Antimicrotubule agents that prevent tubulin depolymerization such as taxanes and vincristine have been shown to synergize with FTIs to affect cancer cell lines as well as tumor xenografts (19–21). The combination of chemotherapeutic agents such as fluorouracil with a minimally effective concentration of FTIs was demonstrated to produce enhanced antiproliferative activity against cultured human breast cancer cells, as well as established tumor xenografts (19, 21, 22). The combination of FTIs with a PI 3-kinase inhibitor or a Cdk inhibitor led to the enhancement of apoptosis of a variety of human cancer cells (18, 23).

The mitochondria have emerged as a mediator for transducing apoptotic stimuli in situations of nonreceptor-stimulated apoptosis (24). We have reported previously that cytochrome c is released from the mitochondria in FTI-induced apoptosis (18, 25). There are also reports that NO changes mitochondrial membrane potential and induces cytochrome c release and caspase activation (26, 27). On this basis, we tested whether NO could act synergistically with FTIs to induce apoptosis in tumor cell lines. We investigated this possibility using the human breast cancer cell line MDA-MB-468. We used DETA-NONOate as the NO donor because this compound has a long half-life (20 h at 37°C), and the rate of NO release can be adjusted by using a different concentration of this compound to mimic the amount of NO released by activated macrophages. The steady state of NO released from 1 mM DETA-NONOate in DMEM is 0.5 μM, and this rate of release of NO remains constant over a 24-h period (28). This steady-state release of NO is similar to that produced by 10⁶ macrophages/ml (29).
We show that the NO donor DETA-NONOate at 1 mM concentration induced apoptosis of MDA-MB-468 human breast cancer cells, whereas this apoptotic effect was not observed when 10 and 100 μM NO donor were used. However, when lower concentrations of the NO donor were used in combination with the FTIs (25 μM), apoptosis was induced although FTIs at this concentration alone did not lead to apoptosis. Importantly, this effect was observed preferentially in the cancer cell line MDA-MB-468, with no apoptosis induction observed with normal breast epithelial cells.

MATERIALS AND METHODS

Chemicals. The NO donor DETA-NONOate was purchased from Alexis Biochemicals (San Diego, CA). FITC SCH565282 (30) was provided by Dr. W. R. Bishop (Scheiring-Plough Research Institute). The fluorogenic substrate for caspase-3, Ac-DEVD-AMC, was obtained from PharMingen (San Diego, CA), and caspase-9 substrate, Ac-LEHD-AMC, was obtained from Alexis Biochemicals. Inhibitors of PI 3-kinase (LY294020; Ref. 31), Cdk (roscoivitine; Ref. 32) [2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropyl purine], were purchased from CalBiochem (San Diego, CA). Antibodies used were from the following suppliers: rabbit polyclonal anti-caspase-3 (65906E) and mouse monoclonal anti-cytochrome c (65981A) from PharMingen, rabbit polyclonal anti-caspase-9 (H-83) and goat polyclonal anti DFF-45 (K-17) from Santa Cruz Biotecnology (Santa Cruz, CA), and anti-porin H1 31 monoclonal (Ab 4) antibody from CalBiochem. Protease inhibitor cocktail was obtained from Boehringer Mannheim.

Cell Culture. MDA-MB-468, MCF-10A, and MCF-10AneoN cell lines were used in our study. MDA-MB-468 breast cancer cell line was obtained from American Type Culture Collection. MCF-10A, a spontaneously immortalized untransformed human mammary epithelial cell line, and MCF-10AneoN (MCF-10A transformed with H-ras oncogene), were obtained from Robert J. Pauley (Barbara Ann Karmanos Cancer Institute, Detroit, Michigan).

MDA-MB-468 cells were cultured in DMEM containing 10% nonessential amino acids, 2 mM t-glutamine, 1 μg/ml insulin, and 10% fetal bovine serum. MCF-10A and MCF-10AneoN cells were cultured in DMEM:Ham's F-12 (1:1) supplemented with 5% equine serum, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 100 ng/ml cholera enterotoxin, and 0.5 μg/ml hydrocortisone.

Measurement of Cell Viability. Cells (2 × 10⁶) were seeded in 100-mm plates and allowed to grow. The cells were harvested at different time points after various treatments, and viability was determined on a hemocytometer by the trypan blue exclusion method.

Caspase Assay. Cells were lysed in buffer [10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mM DTT] for 30 min at 4°C. The lysate (3 μg) was used for caspase-3 assay using the substrate Ac-DEVD-AMC (33). Active caspase-3 cleaves the substrate Ac-DEVD-AMC after the aspartic acid residue and before the AMC group. The released AMC becomes fluorescent, and the fluorescence was quantified using a fluorometer (VersaFluor, Bio-Rad) with excitation at 380 nm and emission at 440 nm. Caspase-9 was similarly assayed using 10 μg of the cell lysate and a fluorogenic substrate Ac-LEHD-AMC.

Western Analysis. Cells were lysed in buffer containing 50 mM HEPES (pH 7.5), 1 mM DTT, 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride at 4°C for 30 min. Lysates (3 μg) were resolved electrophoretically on 10% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad) using a tank blot procedure (Bio-Rad Mini Protein 11). The membranes were incubated in 1:1000 dilution of anti-caspase-3, caspase-9, or DFF-45 antibody. The washed filter was incubated with 1:1000 dilution of horseradish peroxidase-linked Fab) fragment secondary antibody (Amersham Corp., Piscataway, NJ) for 1 h. Immunoreactive bands were visualized by enhanced chemiluminescence detection system (Amersham Corp.).

Detection of Cytochrome c Release into Cytosol. Cytochrome c release into cytosol was detected as described previously with minor modifications (34). Briefly, 1 × 10⁰ cells were harvested and washed with PBS. The cells were suspended in Buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, 1 × protease inhibitor cocktail] and homogenized by a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 1,000 g for 10 min at 4°C. The supernatant was further centrifuged at 10,000 g for 20 min. The supernatant was saved as a cytosolic fraction while the precipitate was dissolved in Buffer A containing 0.5% (v/v) NP40 and saved as the mitochondrial fraction. The mitochondrial and cytosolic fractions were analyzed by Western blot with an anti-cytochrome c monoclonal antibody, 7H8.2C12, or with an anti-porin HL31 monoclonal antibody, Ab4.

RESULTS

DETA-NONOate Induced Apoptosis in MDA-MB-468 Breast Cancer Cell Line. NO has the ability to exert cytotoxic effects on tumor cells. We investigated this effect of NO by using human breast cancer cell lines. MDA-MB-468 cells were treated with 1 mM DETA-NONOate, which releases NO within the range that is produced by activated macrophages (29). We observed that 10 and 100 μM of DETA-NONOate did not significantly affect cell proliferation, whereas at the 1 mM concentration, it induced an initial cytostasis (Fig. 1A). After the cytostasis, apoptotic cell death was detected by terminal deoxynucleotidyl transferase-mediated nick end labeling assay (data not shown).

To further investigate the mechanism by which 1 mM DETA-NONOate induced apoptosis in MDA-MB-468 cells, we examined caspase activation because caspases transduce cell death signals. Caspase-3 is an effector caspase whose activation commits the cells to apoptosis by cleavage of important intracellular substrates and induces apoptotic cell death including DNA fragmentation. Caspase-3 activation was examined in these cells after treatment with 1 mM DETA-NONOate using Ac-DEVD-AMC as a substrate. As shown in Fig. 1B, caspase-3 activation increased by 18-fold after 36 h of exposure to DETA-NONOate. We next examined caspase-9 activation, which functions upstream of caspase-3 using fluorogenic substrate Ac-LEHD-AMC. Activation of caspase-9 has been implicated in NO-mediated apoptosis in different cell lines (26). An increase of caspase-9 activation was detected after 36 h of DETA-NONOate treatment (Fig. 1B). We also assessed caspase-3 and -7 activation by examining their cleavage by Western analysis, as their activation is regulated by proteolytic cleavage. Pro-caspase-3 appears as a band of 32 kDa. After 36 h of exposure to DETA-NONOate, a 17 kDa band that corresponds to cleaved activated caspase-3 was observed (Fig. 1C). Also, pro-caspase-9 detected as a band of 55 kDa was cleaved to produce a 45-kDa band, which was evident after 36 h of exposure to DETA-NONOate.

NO stimulates cytochrome c release from the mitochondria, which leads to caspase-9 activation (27). To investigate whether cytochrome c is released by NO and to follow the time course of release of cytochrome c from the mitochondria into the cytosol, cytosolic and mitochondrial fractions were isolated after various time points after exposure to the NO donor. Western blot analysis was performed with the cytosolic fractions. We observed an increase of cytochrome c in the cytosol at 24 h with no further increase at later time points (Fig. 1C). To confirm that the cytosolic fractions had no mitochondrial contamination, the membrane was reprobed with porin antibody (data not shown). These results are in line with the idea that NO induced apoptosis of MDA-MB-468 cells by inducing cytochrome c release from mitochondria and activating caspase-9 and -3.

FTI Potentiates the Effect of DETA-NONOate to Induce Apoptosis in MDA-MB-468 Cells. The NO donor at 1 mM induced apoptosis, whereas at 10 and 100 μM, it did not affect either cell proliferation or was not able to induce apoptosis in these cells (Fig. 1A). To examine whether FTI in combination with these low concentrations of NO may potentiate apoptosis, MDA-MB-468 cells were treated with FTI SCH56582 (30) in combination with 1 mM DETA-NONOate. The combination of NO and FTI significantly increased the effect of NO on cell viability (data not shown). These results are in line with the idea that NO induced apoptosis of MDA-MB-468 cells by inducing cytochrome c release from mitochondria and activating caspase-9 and -3.
concentrations of the NO donor could induce apoptosis, we assessed caspase-3 activation at different concentrations of DETA-NONOate both in the presence and absence of the FTI (25 μM). As shown in

Fig. 2A, 25 μM FTI in combination with 25 μM DETA-NONOate induced a 3-fold increase in caspase-3 activity by 24 h. A total of 100 μM DETA-NONOate in combination with the FTI induced a 6-fold increase in caspase-3 activity. It is important to point out that FTI alone at 25 μM could not induce caspase-3 activity in these cells. Therefore, FTI potentiated the effect of the NO donor to induce apoptosis of MDA-MB-468 cells. We observed inhibition of farnesylation by this FTI during our mobility shift experiments using Hdj2 protein in SDS-PAGE (data not shown).

In addition to caspase-3, we observed an increase in caspase-9 activity in cells treated with a combination of FTI and 100 μM DETA-NONOate (Fig. 3B). Western analysis revealed cleaved caspase-3 (17 kDa) and cleaved caspase-9 (45 kDa) bands only in cells treated with DETA-NONOate + FTI, whereas cells receiving either DETA-NONOate or FTI had only the intact caspase-3 (32 kDa) and -9 (55 kDa; Fig. 2B). We further studied the role of cytochrome c release by isolating the cytosolic fractions and immunoblotting the cytosolic fractions for cytochrome c. We also observed an increase of cytochrome c release from the mitochondria into the cytosol in cells treated with both DETA-NONOate and FTI (Fig. 2C), suggesting that the release of cytochrome c by low concentrations of NO was potentiated by FTI.

To further confirm that a combination treatment with low concentrations of DETA-NONOate and FTI led to the apoptotic demise of the cells, we examined the cleavage of one of the caspase-3 substrates, DFF-45, after the cells were treated with the NO donor or FTI either alone or in combination. We found an increase of DFF-45 cleavage only in cells treated with a combination of DETA-NONOate and FTI (Fig. 2B). Absence of cleaved DFF-45 in cells treated with only the NO donor or the FTI also demonstrated the lack of caspase-3 activation in these groups of cells. To assess the percentage of viable cells after treatment with DETA-NONOate and FTI, trypan blue exclusion studies were done. With a combination of the NO donor and FTI, we observed that only 40% of the control cells were viable by 24 h (Fig. 2D), whereas the viability of cells in the other groups were similar. This decrease in viability of cells treated with combination of NO donor and FTI was attributable to apoptosis because the addition of zVAD fmk inhibited the death of these cells.

We have shown previously that FTI-induced apoptosis can be enhanced by combining with the PI 3-kinase inhibitor, LY294002 or with Cdk inhibitors (18). To examine whether these compounds can replace NO, we treated cells with the FTI (25 μM) + roscovitine (10 μM) or with a combination of FTI and LY294002 (50 μM) after which caspase-3 activation was measured. As seen in Fig. 2E, neither FTI + roscovitine nor FTI + LY294002 could induce caspase-3 activation in MDA-MB-468 cells. Therefore, among the combinations we tested, FTI in combination with NO was the only successful combination that induced caspase-3 activation and apoptosis in these cells.

DETA-NONOate with FTI Exhibits Apoptosis Induction Preferentially with Breast Cancer Cells. The above results establish that FTI in combination with the NO donor can efficiently induce apoptosis of MDA-MB-468 cells. Because FTI exhibits preferential effects on transformed cells (23, 25), we examined whether the combined treatment of FTI and NO is also preferential with cancer cells. To assess this aspect, effects of DETA-NONOate (100 μM) in combination with FTI (25 μM) on normal breast epithelial cells were examined. Two normal breast epithelial cell lines, MCF-10A and MCF-10A1neoN, were used. MCF-10A is a spontaneously immortalized untransformed human mammary epithelial cell line (35). MCF-10A1neoN is MCF-10A cells transfected with human H-ras proto-oncogene, which exhibits all of the characteristics of normal breast epithelial cells like MCF-10A cells except that they have higher...
These results support the idea that treatment with a combination of NO and FTI retains preference only for cancer cells.

**DISCUSSION**

In this study, we demonstrate that FTI in combination with NO is effective in inducing apoptosis of the breast cancer cell line MDA-MB-468. DETA-NONOate, the NO donor used in our study, at 1 mM induced apoptosis in these cells via cytochrome c release, followed by caspase-9 and caspase-3 activation. Low concentrations of the NO donor were not effective in inducing apoptosis. However, FTI in combination with low concentrations of DETA-NONOate induced apoptosis in these cells within 24 h. A similar potentiating effect of...
FTI in combination with NO was observed in two other human breast cancer cell lines: ZR 75–30 and T47D. This study represents the first report that FTI can potentiate the pro-apoptotic action of NO. We had reported previously that FTI in combination with either a Cdk inhibitor, roscovitine, or with a PI 3-kinase inhibitor, LY294002, could induce apoptosis of a number of human cell lines (18). This study adds another FTI combination to this growing list of combination treatments. The concentrations at which NO synergized with FTI were much below their cytotoxic levels and, therefore, their nonspecific actions. Therefore, the combination of NO with FTI is an effective means of overcoming the general toxic effects of NO. Another promising aspect of our finding is that this potentiation of NO by FTI induced apoptosis selectively in MDA-MB-468 breast cancer cells without affecting the normal breast epithelial cell lines MCF-10A and MCF-10A1neoN. This combination of a low concentration of NO with FTI eliminates a major concern of nonspecific actions associated with higher concentrations of NO and may emerge as a promising approach for the treatment of breast cancer.

There are very few reports on the therapeutic efficacy of NO in the treatment of cancer probably because the nonspecific effects of high concentrations of NO target both normal and tumor cells. Unfortunately, currently available NO delivery molecules do not target tumor cell preferentially. Before NO can be used as effective therapy in cancer, methods have to be devised for its specific targeting and selective delivery to the tumor. A study has exploited the overexpression of glucose transporter proteins and high levels of glucose transport characteristics of tumor cells to conjugate a NO donor to glucose. This glyco-NO conjugate was preferentially toxic to human ovarian carcinoma cells that overexpressed the particular transporter (37). A potential extension of our study is to use a combination of tamoxifen with a NO donor to target breast cancer tissues, which are estrogen receptor positive. It has also been reported that there is a significant infiltration of macrophages in some malignant breast tumors, and these macrophages because of inflammatory reactions are activated and express iNOS. It would therefore be interesting to assess whether FTIs would be more efficacious in treating this type of breast cancer where NO is produced by the tumor itself without affecting normal breast tissue.

We have shown that cytochrome c release by NO is potentiated by FTI. This suggests that both NO and FTI are acting on the mitochondria. Recently, mitochondria-associated mechanisms have been reported to play a key role in NO-mediated apoptosis in various cell lines including breast cancer cell lines (38–40). It has been reported that in human breast cancer cell lines, NO increases reactive oxygen species, triggers a drop in mitochondrial transmembrane potential, releases cytochrome c, and subsequently activates the caspase cascade (41). FTI when used alone has also been shown to induce cytochrome c release from the mitochondria of transformed cells and apoptosis (25). Our results suggest that both FTI and NO in combination target the mitochondria, facilitating the release of cytochrome c into the cytosol and initiating apoptosis. Additional studies are in progress to elucidate the precise target in the mitochondria where FTI and NO synergize leading to the release of cytochrome c.

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