Nimboide Sensitizes Human Colon Cancer Cells to TRAIL through Reactive Oxygen Species- and ERK-dependent Up-regulation of Death Receptors, p53, and Bax

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TNF-related apoptosis-inducing ligand (TRAIL) shows promise as a cancer treatment, but acquired tumor resistance to TRAIL is a roadblock. Here we investigated whether nimboide, a limonoid, could sensitize human colon cancer cells to TRAIL. As indicated by assays that measure esterase activity, sub-G1 fractions, mitochondrial activity, and activation of caspases, nimboide potentiated the effect of TRAIL. This limonoid also enhanced expression of death receptors (DRs) DR5 and DR4 in cancer cells. Gene silencing of the receptors, we found that activation of ERK and p38 MAPK was required for sensitization to TRAIL but not for DR up-regulation. Overall, our results indicate that nimboide can sensitize colon cancer cells to TRAIL-induced apoptosis through three distinct mechanisms: reactive oxygen species- and ERK-mediated up-regulation of DR5 and DR4, down-regulation of cell survival proteins, and up-regulation of p53 and Bax.

Because acquired resistance to apoptosis is one of the hallmarks of tumor cells (1), therapeutic agents that can selectively kill tumor cells and overcome tumor resistance are critically needed. Since its discovery in 1995, TNF-related apoptosis-inducing ligand (TRAIL)(2) has emerged as a promising candidate for anticancer therapy because of its remarkable ability to induce apoptosis in a variety of cancer cells (2, 3). TRAIL has been shown to interact with five different proteins, namely death receptor (DR) 4, DR5, decoy receptor 1, decoy receptor 2, and osteoprotegerin, but it transduces an apoptotic signal by binding to DR4 and DR5 (4, 5). These receptors recruit the Fas-associated death domain and caspase-8, but also activate NF-κB (3, 6), whereas DR3 recruits the Fas-associated death domain and procaspase-8 in a death-inducing signaling complex. The autocatalytic processing of procaspase-8 in the death-inducing signaling complex leads to activation of caspase-3 or caspase-7 (7), which cleave the Bid to truncated Bid and activate NF-κB (3, 6), respectively. Like other members of the TNF receptor superfamily, DR4 not only contains the death domain, but also activates NF-κB (3, 6), thereby potentiating cell survival (8, 9). TRAIL resistance is a roadblock. Numerous types of cancer cells are insensitive to TRAIL-induced apoptosis (8). Two of the possible mechanisms by which cancer cells develop resistance to TRAIL are dysfunction of the signaling pathways of TRAIL-induced apoptosis and elevation of survival signals. Signaling pathway dysfunction includes altered expression of the DRs, the Fas-associated death domain, or caspase-8 (9). Elevation of survival signals consists of overexpression of anti-apoptotic Bcl-2 family proteins (10), Mcl-1 (11), survivin, and cellular FLICE-like inhibitory protein (cFLIP, a caspase-8 inhibitory protein) (12) (Table 1). In addition, high levels of expression of X-linked inhibitor of apoptosis protein (XIAP) have been observed in many tumor cell lines, and XIAP may lead to TRAIL resistance by directly inhibiting caspase-3, caspase-7, and caspase-9 (14). It
is also possible that cancer cells may simultaneously exhibit multiple mechanisms of TRAIL resistance (15).

It has been proposed that an agent that could enhance the expression of pro-apoptotic proteins and/or inhibit the expression of cell survival proteins might sensitize tumor cells to TRAIL-induced apoptosis. We hypothesized that nimboide is one such agent. Nimboide was first derived from the leaves and flowers of neem (Azadirachta indica), a tree in the mahogany family. In Persian, neem is known as azad dirakht, which means “free tree,” and in Swahili, it is called muarubaini, which means “the tree that cures 40 different diseases” (16). Natural products have played a significant role in the discovery of anticancer drugs; >60% of anticancer drugs are of natural origin (17).

Nimboide, a tetrnortriterpenoid that consists of a classic limonoid skeleton with an αβ-unsaturated ketone system and a δ-lactone ring (18), has been shown to exhibit numerous types of biological activity, including antimalarial (19) and anticancer (20–23) activity. Nimboide was found to exhibit anticancer activity in a wide variety of tumor cells, including neuroblastoma, osteosarcoma, choriocarcinoma (24), leukemia (23), and melanoma (22) cells, and in macrophages. In animal tumor models, nimboide (at 10–100 mg/kg) was shown to exhibit chemopreventive activity against 7,12-dimethylbenz[a]anthracene-induced oral carcinogenesis (25). The αβ-unsaturated ketone structural element of nimboide has been linked to its anticancer activity (26).

Although the mechanism of the anticancer effect of nimboide is not fully understood, it has been shown that Nimboide at G0/G1, cell cycle arrest (22), to modulate the Notch pathway, and to increase the generation of reactive oxygen species (ROS) in cancer cells (24). ROS induce signal transduction pathways that lead to cell survival, progression, or death. Important downstream proteins of ROS-induced signaling are the MAPKs (27) and the PI3K/Akt pathway, and ERK. These factors led us to hypothesize that nimboide potentiates TRAIL-induced apoptosis by modulating signal transduction pathways that regulate apoptosis. Therefore, the objective of this study was to determine whether nimboide can modulate the sensitivity of colon cancer cells to TRAIL-induced apoptosis and, if so, through what mechanism(s).

Our results indicate that nimboide can sensitize tumor cells to TRAIL-induced apoptosis, and up-regulation of pro-apoptotic proteins. Our results indicate that nimboide can modulate the sensitivity of colon cancer cells to TRAIL-induced apoptosis. We hypothesized that nimboide is one such agent. Nimboide was first derived from the leaves and flowers of neem (Azadirachta indica), a tree in the mahogany family. In Persian, neem is known as azad dirakht, which means “free tree,” and in Swahili, it is called muarubaini, which means “the tree that cures 40 different diseases” (16). Nimboide is one such agent. Nimboide was first derived from the leaves and flowers of neem (Azadirachta indica), a tree in the mahogany family. In Persian, neem is known as azad dirakht, which means “free tree,” and in Swahili, it is called muarubaini, which means “the tree that cures 40 different diseases” (16).

**EXPERIMENTAL PROCEDURES**

**Materials**—Nimboide was isolated from A. indica leaves as described previously (28). A 50 mM solution of this triterpenoid was prepared in dimethyl sulfoxide and then diluted as needed in cell culture medium. Soluble recombinant human TRAIL was purchased from PeproTech. Penicillin, streptomycin, DMEM, RPMI 1640, FBS, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), and the kit for the live/dead assay were purchased from Invitrogen. Antibodies against DR4, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bcl-xl, clAP-1, clAP-2, Bid, Bcl-2-associated X protein (Bax), p53, ERK1, ERK2, p-ERK1/2, caspase-3, caspase-8, caspase-9, cytochrome c, p38, JNK1, and p-JNK (Thr183/185) were obtained from Santa Cruz Biotechnology. Anti-DR5 was purchased from ProSci, Inc. Anti-XIAP was purchased from BD Biosciences. Antibodies against cleaved caspase-9 and p-p38 (Thr180/Tyr182) were obtained from Cell Signaling Technology. Mouse monoclonal anti-β-actin, N-acetylcyesteine (NAC), GSH, cycloheximide, actinomycin D, H2O2, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Antibodies against survivin and phycoerythrin-conjugated DR5 and DR4 were obtained from R&D Systems. siRNAs for DR5, DR4, ERK1, and ERK2 were purchased from Qiagen.

**Cell Lines**—The human cell lines HCT-116 and HT-29 (colon adenocarcinoma), KBM-5 (chronic myeloid leukemia), U266 (multiple myeloma), A293 (embryonic kidney carcinoma), AsPC-1 (pancreatic adenocarcinoma), MDA-MB-231 (breast adenocarcinoma), MCF-7 (breast cancer cells), and H1299 (lung adenocarcinoma) were obtained from the American Type Culture Collection. MCF-10A (human nontumor breast cells) was supplied by Dr. Kapil Mehta of our Institute. HCT-116 variant cell lines in p53 and Bax were supplied by Dr. Philip Rutka (The Johns Hopkins University). The other cells were cultured in McCoy’s 5A medium (Gibco) supplemented with 10% FBS and penicillin/streptomycin. MCF-10A and MDA-MB-231 cells were cultured in Iscove’s modified Dulbecco’s medium with 15% FBS, 20 ng/ml EGF, 100 ng/ml human hydrocortisone, and penicillin/streptomycin. The other cells were cultured in Iscove’s modified Dulbecco’s medium with 15% FBS and penicillin/streptomycin; H460, A293, MCF-7, and MDA-MB-231 cells were cultured in DMEM, and the other cells lines were cultured in RPMI 1640 with 10% FBS and penicillin/streptomycin.

**Cytotoxicity Assay**—The effects of nimboide on the cytotoxic potential of TRAIL were assessed by measuring mitochondrial dehydrogenase activity using MTT as the substrate (29). The assay relies on the fact that mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of MTT, yielding purple MITT formazan, which is measured spectrophotometrically at 570 nm.

**Clonogenic Assay**—The assay tests every cell in a given population for its ability to undergo unlimited division and form colonies. Treated and untreated cells were allowed to form colonies for 14 days and then stained with 0.3% crystal violet solution (30).

**Live/Dead Assay**—To measure apoptosis, we used the live/dead assay, which assesses intracellular esterase activity and plasma membrane integrity. It is a two-color fluorescence assay that simultaneously determines numbers of live cells and dead cells. Intracellular esterases from live cells convert nonfluorescent, cell-permeable calcein acetoxymethyl ester to the intensely fluorescent calcein, which is retained within cells. This assay also examines dead cells that have damaged membranes; the ethidium homodimer-1 enters damaged cells, is fluorescent when bound to nucleic acids, and produces a bright red fluorescence. The assay was performed as described previously (31). Four different microscopic fields were selected to count the number of live and dead cells, and the
Nimbolide-induced TRAIL Sensitization

data are presented as the mean ± S.D. of three independent experiments.

Propidium Iodide Staining for DNA Fragmentation—DNA content was analyzed using propidium iodide staining as described previously (30). This method is based on the fact that cells undergoing apoptosis will lose part of their DNA (because of DNA fragmentation), and those cells may be detected as a sub-G₁ population after propidium iodide staining.

RNA Isolation and RT-PCR—Total RNA was extracted from control and treated cells using TRIzol reagent (Invitrogen). DR5 and DR4 transcripts were detected using the SuperScript One-Step RT-PCR kit (Invitrogen) (32). PCR products were run on a 2% agarose gel and then stained with ethidium bromide. Stained bands were visualized under UV light and photographed.

Transfection with siRNA—Cells were plated in 6-well plates and allowed to adhere for 24 h. On the day of transfection, 12 μl of HiPerFect transfection reagent (Qiagen) was added to 25 nmol/liter siRNA in a final volume of 100 μl of culture medium and added to the cells. After 48 h of transfection, cells were treated with nimbolide for 6 h and then exposed to TRAIL for 24 h (32).

Western Blot Analysis—To determine the levels of protein expression, we prepared protein extracts and separated them by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes, probed with relevant antibodies, and detected by an ECL reagent (GE Healthcare) (33). After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes, probed with relevant antibodies, and detected by an ECL reagent (GE Healthcare) (33).

Analysis of Cell-surface Expression of DR5 and DR4—Intracellular ROS generation was estimated by flow cytometry using the membrane-permeable dye DCFH-DA. DCFH-DA is converted by intracellular esterases to DCF₂, which is then oxidized by ROS to highly fluorescent DCF. The assay was carried out as described previously (32).

Measurement of Intracellular ROS—Intracellular ROS generation was estimated by flow cytometry using the membrane-permeable dye DCFH-DA. DCFH-DA is converted by intracellular esterases to DCF₂, which is then oxidized by ROS to highly fluorescent DCF. The assay was carried out as described previously (32).

Statistical Analysis—Experiments were repeated a minimum of three times, with consistent results. Data represented are given as the mean ± S.D. The statistical analysis was carried out using a two-tailed unpaired Student’s t test. A value of p < 0.05 was considered statistically significant.

RESULTS

The objective of this study was to determine whether and how nimbolide can modulate the sensitivity of colon cancer cells to TRAIL-induced apoptosis. Because of availability of several variants of HCT-116, we used this cell line for most of the studies, although other cell types were also employed to determine the specificity of the effect of nimbolide. Apoptosis and growth inhibitory effects of TRAIL and nimbolide were examined by multiple methods that reflected different stages of apoptosis.

Nimbolide Sensitizes Human Colon Cancer Cells to TRAIL-induced Apoptosis—We first determined whether nimbolide can enhance TRAIL-induced apoptosis in human colon cancer cells. Intracellular esterase activity measured by the live/dead assay indicated that nimbolide treatment increased TRAIL-induced apoptosis in HCT-116 and HT-29 cells in a dose-dependent manner. The percentage of apoptotic cells was increased from 5 to 60% in HCT-116 cells and from 4 to 50% in HT-29 cells (Fig. 1A) by treatment with 5 μM nimbolide.

When the sub-G₁ fraction was examined using propidium iodide staining in HCT-116 cells, we found that apoptosis was induced at 7.4% by TRAIL, at 9.2% by nimbolide, and at 45.5% by TRAIL plus nimbolide (Fig. 1B), suggesting a synergistic interaction between the two agents. We also examined the effect of nimbolide on TRAIL-induced cytotoxicity by the MTT assay, which measures mitochondrial activity. Nimbolide and TRAIL alone were minimally cytotoxic to HCT-116 and HT-29 cells; however, pretreatment of cells with nimbolide enhanced the cytotoxic effect of TRAIL in a dose-dependent manner in both cell lines (Fig. 1C).

Activation of PARP is a hallmark of apoptosis. We next examined the effect of nimbolide on caspase activation and PARP cleavage in colon cancer cell lines. The extrinsic pathway involves death receptor ligation, whereas the intrinsic pathway involves mitochondrial dysfunction and subsequent caspase activation and subsequent cleavage of PARP (34). Overall, these results indicate that nimbolide can enhance the apoptotic potential of human colon cancer cells through both extrinsic and intrinsic pathways.

We also examined the effect of nimbolide on TRAIL-induced suppression of long-term colony formation. Although nimbolide and TRAIL alone were minimally effective, nimbolide significantly enhanced the effect of TRAIL on suppression of the colony-forming ability of tumor cells (Fig. 1E). The number of colonies decreased from 954 in control medium to 913 with nimbolide and to 824 with TRAIL. When cells were pretreated with nimbolide before TRAIL treatment, the number of colonies decreased to 193.

Nimbolide Up-regulates the Expression of TRAIL Receptors—To explore the mechanism involved in the enhancement of TRAIL-induced apoptosis, we examined the effect of nimbolide on DRs. The limonoid up-regulated DR5 and DR4 in HCT-116 and HT-29 cells in a dose- and time-dependent manner (Fig. 2A and B). Treatment of cells with 5 μM nimbolide for 12 h was found optimal for up-regulating DRs without affecting cell viability.

We also examined whether nimbolide can enhance the cell-surface expression of DRs. Nimbolide increased cell-surface expression of DR5 and DR4 in HCT-116 cells (Fig. 2C).

We examined whether up-regulation of DRs by nimbolide occurs at the transcriptional or post-transcriptional level. Our results indicated that nimbolide enhanced DR5 and DR4 transcripts in HCT-116 cells in a dose-dependent manner (Fig. 2D). The transcriptional (actinomycin D) and translational (cycloheximide) inhibitors were found to block nimbolide-induced DR5 and DR4 expression (Fig. 2E). These results in-
Nimbolide Enhances Protein Stability of DR5 and DR4—To further confirm whether the effect of nimbolide on DR5 and DR4 expression is due to posttranscriptional regulation, we examined the protein stability. After 6 h of administration to the HCT-116 cells, nimbolide was washed from the medium, and cycloheximide was given from 30 min to 6 h in the presence or absence of nimbolide. Results indicated that DR5 and DR4 proteins were maintained in the group of cells treated in combination with nimbolide and cycloheximide, whereas they were gradually dropped in the group of cells treated with cycloheximide alone (Fig. 2F). Taken together, these results indicate that nimbolide increases protein stability of DR5 and DR4.
Up-regulation of DR5 and DR4 by Nimbolide Is Not Cell Type-specific—We examined whether the limonoid-induced up-regulation of DR5 and DR4 is specific to colon cancer cells or also occurs in other cancer cell types. Nimbolide up-regulated DR5 and DR4 in chronic myeloid leukemia (KBM-5), multiple myeloma (U266), embryonic kidney carcinoma (A293), pancreatic adenocarcinoma (AsPC-1), and breast adenocarcinoma (MDA-MB-231) cells (Fig. 2G). These results suggest that the up-regulation of DR5 and DR4 by nimbolide is not cell type-specific.

Up-regulation of DRs by Nimbolide Is Essential for TRAIL-induced Apoptosis—To determine whether the up-regulation of DR5 and DR4 by nimbolide is essential to sensitize tumor cells to TRAIL, we silenced DR5 and DR4 by specific siRNA. Transfection of cells with DR5 and DR4 siRNAs substantially reduced nimbolide-induced expression of DR5 and DR4, respectively. Control siRNA, however, had no effect on nimbolide-induced expression of DR5 and DR4 (Fig. 3A). DR5 siRNA had no effect on nimbolide-induced DR4 expression. Similarly, DR4 siRNA had no effect on nimbolide-induced DR5 up-regulation.

We next examined whether silencing of DR5 or DR4 by siRNA could also alter the sensitizing effects of nimbolide on TRAIL-induced apoptosis. The effect of nimbolide on TRAIL-induced apoptosis was substantially decreased in cells transfected with either DR5 or DR4 siRNA (Fig. 3B). DR5 silencing had a more drastic effect on TRAIL-induced apoptosis than DR4 silencing.
Nimbolide Down-regulates the Expression of Cell Survival Proteins—The anti-apoptotic proteins, such as Bcl-2, Bcl-xL, cIAP, I-FLICE, survivin, and XIAP, are linked to TRAIL resistance in cancer cells. We examined whether nimbolide can modulate the expression of these proteins. Our results revealed that nimbolide down-regulated the expression of Bcl-2, Bcl-xL, cIAP-1, cIAP-2, I-FLICE, survivin, and XIAP. The effect of nimbolide was most prominent at 5 and 10 μM (Fig. 3, top). Overall, these results suggest that nimbolide can down-regulate cell survival proteins.

Nimbolide Up-regulates the Expression of Pro-apoptotic Proteins—We also examined whether nimbolide can modulate the expression of pro-apoptotic proteins. Nimbolide up-regulated the expression of Bax and cytochrome c in a dose-dependent manner. Levels of Bid, a pro-apoptotic Bcl-2 family protein that is cleaved by caspase-8 to truncated Bid was also decreased in a dose-dependent manner (Fig. 3C, bottom). These results suggest that nimbolide can up-regulate pro-apoptotic proteins.

Up-regulation of DR5 and DR4 by Nimbolide Is ROS-dependent—We first investigated whether the limonoid can induce ROS generation. In the cells treated with nimbolide, ROS generation was 10-fold higher than that in untreated cells. This increase in ROS generation was abrogated when the cells were pretreated with NAC (Fig. 4A).

We investigated whether nimbolide-induced expression of DR5 and DR4 is ROS-dependent. When HCT-116 cells were pretreated with the ROS scavenger NAC, a dose-dependent decrease in nimbolide-induced expression of DR5 and DR4 was observed (Fig. 4B, left). GSH, another ROS scavenger, also decreased nimbolide-induced expression of DR5 and DR4 in a dose-dependent manner (Fig. 4B, right). These results suggest that ROS generation is essential for the limonoid-induced up-regulation of DR5 and DR4.

We next examined whether scavenging of ROS could abolish the nimbolide-induced sensitization of cells to TRAIL. When cells were pretreated with NAC, the percentage of apoptotic cells induced by TRAIL plus nimbolide decreased from...
We also determined whether NAC pretreatment affects caspase activation and PARP cleavage induced by nimbolide and TRAIL. Our results showed that caspase activation and PARP cleavage in HCT-116 and HT-29 cells induced by nimbolide and TRAIL were completely suppressed when cells were pretreated with NAC (Fig. 4D). These results indicate that ROS play a critical role in sensitization of colon cancer cells to TRAIL-induced apoptosis.

Nimbolide-induced TRAIL Sensitization

58 to 18% (Fig. 4C). We also determined whether NAC pretreatment affects caspase activation and PARP cleavage induced by nimbolide and TRAIL. Our results showed that caspase activation and PARP cleavage in HCT-116 and HT-29 cells induced by nimbolide and TRAIL were completely suppressed when cells were pretreated with NAC (Fig. 4D). These results indicate that ROS play a critical role in sensitization of colon cancer cells to TRAIL-induced apoptosis.

Nimbolide Induces Sensitization Effects Selectively in Cancer Cells—Whether nimbolide could exert its effects in normal cells was investigated. The normal breast cells (MCF-10A) and breast cancer cells (MCF-7) were treated with nimbolide for 6 h followed by TRAIL for 24 h, and the number of apoptotic cells were examined. The results indicated that whereas nimbolide and TRAIL were minimally effective in inducing apoptosis in MCF-7 cells, the combination of both enhanced...
the number of apoptotic cells to 42%. Conversely, the combination of nimbolide and TRAIL was unable to evoke apoptosis in normal breast cancer cells (Fig. 5A). The mitochondrial dehydrogenase activity yielded similar results (Fig. 5B). We also investigated the potential of nimbolide to induce DR5 and DR4 in MCF-10A cells. Results showed a lack of DR5 and DR4 induction in MCF-10A cells by nimbolide, whereas a dose-dependent induction of these receptors was observed in MCF-7 cells (Fig. 5C). Overall, these results indicate that nimbolide exerts its effects selectively in the cancer cells.

**Hydrogen Peroxide Mimics the Events Observed after Nimbolide Treatment**—Because DCFH-DA determines mainly $H_2O_2$, we investigated whether the effects observed with nimbolide could be mimicked by exogenous administration of $H_2O_2$. Cells were treated with different concentrations of $H_2O_2$, and DR5 and DR4 proteins were analyzed by Western blot analysis. As shown in Fig. 5D, a dose-dependent increase in DR5 and DR4 expression was observed by $H_2O_2$ treatment. The ROS scavenger NAC completely suppressed $H_2O_2$-induced DR5 and DR4 expression. The role of $H_2O_2$ in sensitizing HCT-116 cells to TRAIL-induced apoptosis was also examined. As observed with nimbolide, pretreatment of cells with $H_2O_2$ was associated with an enhancement in TRAIL-induced apoptosis. The number of apoptotic cells was increased from 8 to 45% when cells were pretreated with $H_2O_2$ (Fig. 5E). We also examined the effect of NAC on $H_2O_2$-induced sensitization of HCT-116 cells to TRAIL. Results indicated that the number of apoptotic cells was significantly suppressed by NAC (Fig. 5E). Overall these results indicated that $H_2O_2$ mimics nimbolide-induced sensitization effects.

**Nimbolide-induced Up-regulation of DR5 and DR4 Is Mediated through MAPK Activation**—We investigated whether MAPKs play any role in nimbolide-induced up-regulation of DR5 and DR4. Pretreatment of cells with an ERK1/2 inhibitor...
Nimbolide-induced TRAIL Sensitization

(PD98059) suppressed the nimbolide-induced up-regulation of DR5 and DR4 in a dose-dependent manner (Fig. 6A, top). Similarly, when cells were pretreated with a p38 inhibitor (SB202190), a dose-dependent decrease in nimbolide-induced DR5 and DR4 up-regulation was observed (Fig. 6A, middle). A JNK inhibitor had no effect on nimbolide-induced up-regulation of DR5 or DR4 (Fig. 6A, bottom). Densitometry analysis from three independent experiments indicated that DR5 and DR4 expression was significantly inhibited by ERK and p38 MAPK inhibitors, whereas a JNK inhibitor had no effects (Fig. 6A).

The reduction in nimbolide-induced up-regulation of DR5 and DR4 by inhibitors of MAPKs prompted us to investigate the ability of nimbolide to activate MAPKs. Our results indicated that the limonoid activated ERK in a time-dependent manner, activated p38 MAPK transiently, and was unable to induce JNK activation (Fig. 6B). These results suggest that MAPKs, in particular ERK and p38, may play a role in nimbolide-induced up-regulation of DR5 and DR4.

Nimbolide-induced MAPK Activation Is Mediated through ROS Generation—ROS have been reported to play a role in the induction of MAPK by various agents (30, 34). We investigated whether the limonoid-induced MAPK activation is dependent on ROS production. To determine this, we pretreated cells with NAC (at 20 mmol/liter) for 1 h before treatment with nimbolide. The NAC pretreatment abolished nimbolide-induced phosphorylation of ERK and p38 (36), which are also known to sensitize cancer cells to TRAIL-induced apoptosis through up-regulation of DR5 and DR4 through the ROS-dependent MAPK pathway, involving the high expression of pro-apoptotic proteins (Fig. 8).

We also investigated whether p53 and Bax play a role in nimbolide sensitization of tumor cells to TRAIL-induced apoptosis using the live/dead assay. Our results indicated that enhancement of apoptosis by nimbolide and TRAIL was significantly reduced in p53−/− and Bax−/− cells compared with the parent cells (Fig. 7E). These results indicate that p53 and Bax, however, play a role in nimbolide sensitization of tumor cells to TRAIL-induced apoptosis.

DISCUSSION

As is the case with most chemotherapeutic agents, cancer cells develop resistance to TRAIL through multiple mechanisms. The limitations of these mechanisms may have potential for enhancing the efficacy of TRAIL. We have provided evidence that nimbolide can enhance the sensitivity of cancer cells to TRAIL-induced apoptosis through three different mechanisms: up-regulation of pro-apoptotic proteins such as Bax, the ROS-dependent MAPK pathway, and down-regulation of cell survival proteins, and up-regulation of pro-apoptotic proteins (Fig. 8).

We found that this tetrannortriterpenoid up-regulated both DR5, DR3 and DR4, through which TRAIL interacts with tumor cells. This result is consistent with those previously reported for other triterpenoids such as celastrol (32) and butein (36), which are also known to sensitize cancer cells to TRAIL-induced apoptosis through up-regulation of these receptors. We also found that up-regulation of DRs by nimbolide was due to both increased transcription and protein stability. Furthermore, we found that the up-regulation of DR5 and DR4 by nimbolide was not restricted to colon cancer cells but also occurred in chronic myeloid leukemia, multiple myeloma, embryonic kidney, pancreatic cancer, and breast cancer cells. Our results also showed that up-regulation of DR5 and DR4 was critical as the silencing of either receptor significantly suppressed the effect of nimbolide on TRAIL-induced apoptosis. The suppression was more pronounced with silencing of DR5 than that of DR4. That DR5 is a most critical receptor is in agreement with results reported previously for baicalein (37).

We also investigated the molecular mechanism by which nimbolide up-regulated the DRs. We found that ERK is involved in up-regulation of both DR5 and DR4. An inhibitor of phosphatidylinositol 3-kinase (34) and zerumbone (30) have also been shown to induce these DRs through activation of ERK1/2. We found that silencing of ERK1 and ERK2 also suppressed the effect of the limonoid on TRAIL-induced apoptosis. We also examined how nimbolide activates ERK. We found that nimbolide-induced ROS, which led to ERK activa-
FIGURE 6. Up-regulation of DR5 and DR4 by nimboide is mediated through the ROS-dependent MAPK pathway. A, cells were pretreated with the indicated concentrations of PD98059, SB202190, and a JNK inhibitor for 1 h and then treated with 5 μM nimboide (NL) for 12 h. Whole-cell extracts were analyzed by Western blotting using anti-DR5 and anti-DR4 antibodies. B, nimboide-induced ERK activation is dependent on ROS. Cells were pretreated with NAC (at 20 mmol/liter) for 1 h and then exposed to 5 μM nimboide for the indicated times. Whole-cell extracts were analyzed by Western blotting using relevant antibodies. C, silencing of ERK1 and ERK2 abrogated nimboide-induced expression of DR5 and DR4. Cells were transfected with ERK1 siRNA, ERK2 siRNA, or control siRNA for 48 h and treated with 5 μM nimboide for 12 h. Whole-cell extracts were analyzed by Western blotting using anti-DR5 and anti-DR4 antibodies. D and E, ERK1 and ERK2-transfected cells were pretreated with 5 μM nimboide for 6 h and then incubated with 25 ng/ml TRAIL for 24 h. Apoptosis was measured by PARP cleavage or the live/dead assay. Values across each photomicrograph represent the mean ± S.D. of apoptotic cells (magnification ×100). One of three independent experiments is shown. Values below each blot in (A) represent percent inhibition compared to the NL + group.
Nimbolide-induced TRAIL Sensitization

We found that the limonoid also up-regulated p53 in colon cancer cells. Although several reports have shown that p53 up-regulation can lead to increased expression of TRAIL receptors (35), we found that nimbolide up-regulated the DRs through a p53-independent mechanism. We found that the receptors were up-regulated in p53-deleted cells, thus suggesting that the role of p53 in the up-regulation of DR5 and DR4 depends on the cell type and the nature of the stimulus. We found, however, that p53 was essential for nimbolide sensitization of tumor cells to TRAIL-induced apoptosis.

FIGURE 7. p53 and Bax are required for potentiation of TRAIL-induced apoptosis by nimbolide but are not essential for up-regulation of DR5 and DR4. A, cells were treated with the indicated concentrations of nimbolide (NL) for 12 h or with 5 μM nimbolide for the indicated times. Whole-cell extracts were analyzed by Western blotting using anti-p53 antibody. B and C, effect of p53 on nimbolide-induced expression of DR5 and DR4. Whole-cell extracts from control and nimbolide-treated WT, HCT-116<sup>−/−</sup>, and H1299 cells were analyzed by Western blotting using relevant antibodies. D, WT and Bax<sup>−/−</sup> HCT-116 cells were treated with the indicated concentrations of nimbolide for 12 h, and whole-cell extracts were analyzed by Western blotting using the indicated antibodies. E, cells were pretreated with nimbolide for 6 h and exposed to TRAIL for 24 h. Apoptosis was measured by the live/dead assay. Values across each photomicrograph represent the mean ± S.D. of apoptotic cells from three independent experiments (magnification ×100). One of three independent experiments is shown.

Retraction

July 8, 2016

This article has been retracted.
Nimbolide-induced TRAIL Sensitization

Although H$_2$O$_2$ was found to mimic the effects of nimbolide treatment, it cannot be employed to enhance the effect of TRAIL. The possible use of H$_2$O$_2$ in cancer therapy has been controversial over the years (44–47). However, a convincing amount of evidence has shown that H$_2$O$_2$-generating systems may be an efficient way of killing cancer cells. For example, the anticancer effect of various chemotherapeutic agents currently in the clinic, such as paclitaxel, cisplatin, and doxorubicin, is mediated, at least in part, by increasing intracellular levels of H$_2$O$_2$. Therefore, compounds such as nimbolide that trigger a significant increase in intracellular H$_2$O$_2$ could be an attractive strategy to potentiate the sensitivity of tumor cells to TRAIL. We found that these effects were tumor cell-specific as the limonoid was unable to induce DR5 and DR4 and sensitize normal breast cells to TRAIL. However, how nimbolide discriminates between normal and cancer cells remains to be elucidated.

In conclusion, our study provides strong evidence that nimbolide sensitizes tumor cells to TRAIL-induced apoptosis through three different mechanisms: ROS- and ERK-mediated up-regulation of DR5 and DR4, down-regulation of cell survival proteins, and up-regulation of pro-apoptotic proteins. Thus, our results suggest that the combination of nimbolide plus other effective anticaner agents in animal models, however, are crucial to the potential of nimbolide as a therapeutic agent to improve the clinical efficacy of TRAIL.

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