Selective Induction of Tumor Cell Apoptosis by a Novel P450-mediated Reactive Oxygen Species (ROS) Inducer Methyl 3-(4-Nitrophenyl) Propiolate

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Background: Generating ROS has become a novel anti-cancer approach. NPP preferentially induces tumor cell apoptosis through P450-catalyzed ROS production. Cell susceptibility to ROS-induced death is influenced by cellular redox status, p53 mutation, STAT3 activation, and location of ROS production. Our study not only discovered a novel drug candidate but also shed new light on the understanding of ROS generation and function.

Induction of tumor cell apoptosis has been recognized as a valid anticancer strategy. However, therapeutic selectivity between tumor and normal cells has always been a challenge. Here, we report a novel anti-cancer compound methyl 3-(4-nitrophenyl) propiolate (NPP) preferentially induces apoptosis in tumor cells through P450-catalyzed reactive oxygen species (ROS) production. A compound sensitivity study on multiple cell lines shows that tumor cells with high basal ROS levels, low antioxidant capacities, and p53 mutations are especially sensitive to NPP. Knockdown of p53 sensitized non-transformed cells to NPP-induced cell death. Additionally, by comparing NPP with other ROS inducers, we show that the susceptibility of tumor cells to the ROS-induced cell death is influenced by the mode, amount, duration, and perhaps location of ROS production. Our studies not only discovered a unique anticancer drug candidate but also shed new light on the understanding of ROS generation and function and the potential application of a ROS-promoting strategy in cancer treatment.

Induction of cell apoptosis has been a popular strategy for cancer therapy. Successful apoptosis-inducing anticancer drugs induce tumor cells to die by either directly turning on apoptotic pathways or turning off antiapoptotic pathways (1). However, selectively inducing cancer cells to die remains a big concern and challenge for cancer treatment. ROS play an important role in inducing apoptosis under both physiological and pathological conditions (2–5). Recently, inducing ROS generation has become a novel approach to treat cancer (6, 7). The exciting advantage of this strategy lies in its good selectivity. Cancer cells are usually under oxidative stress and, hence, have a relatively high basal level of ROS (8, 9). A small induction of ROS in tumor cells may push the level of ROS over the threshold of life and death to induce cell death, whereas normal cells can better tolerate the oxidative insults because of their lower basal level of ROS and stronger antioxidant capacities (10). Indeed, the natural compound β-phenylethyl isothiocyanate (PEITC), a novel ROS-inducing agent, exhibits superior anticancer selectivity and is currently under clinical investigation (11–13). Nonetheless, the ROS-generating anticancer strategy is still in its infancy. The manner of compounds to induce ROS are diverse, and the effects of the ROS inducers are not identical, but the detailed mechanism is unclear. More ROS-generating agents with different mechanisms are needed to fully understand their potential application in cancer treatment.

p53 is a critical regulator of apoptosis, and many cells undergo apoptosis through the p53 pathway (1, 14). Drugs that induce p53-dependent apoptosis, however, have limited effects on tumor cells because many of them escape from apoptosis because of their defective p53 functions and cause unwanted cell death in normal cells, generating toxicities (15). Therefore, novel strategies are needed to induce p53-defective cancer cells to die. Although previous studies indicate that promoting ROS formation might treat cancer cells selectively, it is unclear whether it can induce cell death in the p53-defective cancer cells.

We report here the identification of NPP as a preferential tumor cell death inducer. It induced apoptosis by cytochrome P450-catalyzed ROS formation. Our study uncovered a new ROS inducer with new mechanisms. Using NPP as a tool, we explored the susceptibility of tumor cells to ROS-induced cell death. Our results suggest that loss of p53 in tumor cells increases their sensitivity to the ROS inducer and that the cytotoxic effects of ROS inducers are influenced by the
mode, amount, duration, and perhaps location of ROS production.

EXPERIMENTAL PROCEDURES

Chemical Synthesis of Compound NPP—NPP (molecular weight, 312.28 Da) was synthesized via a straightforward six-step synthetic route, starting from the commercially available 3-chloro-2-methyl aniline and employing Neimentowski synthesis as the key step. The final product was characterized by 1H-nuclear magnetic resonance, mass spectrometry, and elemental analyses. All other chemicals were purchased from Sigma.

Cell Lines and Reagents—HepG2/STAT3 cells, a gift from Prof. Xinyuan Fu (National University of Singapore, Singapore), were HepG2 cells stably transfected with a STAT3-responsive firefly luciferase reporter plasmid. All other cell lines were obtained from the ATCC. HepG2 cells were cultured in α-minimal essential medium (Invitrogen) with 10% FBS (Life Technologies). All other cell cultures were cultured in DMEM (Invitrogen) with 10% FBS. The sources of chemicals and antibodies were as follows. Hoechst and PI were from Sigma-Aldrich. Secondary HRP-conjugated antibodies were from Jackson ImmunoResearch Laboratories. Mouse anti-α-tubulin and mouse anti-β-actin antibodies were from Santa Cruz Biotechnology, Inc. Mouse anti-cytochrome c antibodies were from BD Biosciences. Mouse anti-phospho-STAT3, mouse anti-caspase 3 antibodies, and rabbit anti-poly(ADP-ribose) polymerase antibodies were from Cell Signaling Technology, Inc.

Determination of Cellular ROS—Accumulation of intracellular ROS was detected with the probe DCFH2-DA as described previously (16). In brief, after drug treatment, cells were labeled with 10 μM DCFH2-DA (2′,7′-dichlorofluorescin diacetate) for 20 min at 37 °C in a humidified atmosphere at 5% CO2. The labeled cells were washed and collected. To quantify ROS, the fluorescence intensity (FL-1 channel) was measured by flow cytometry (FACS Calibur, BD Biosciences).

Cell Viability Assay—About 5000 cells/well were seeded into 96-well plates. Twenty-four hours later, cells were treated with vehicle control or various concentrations of NPP, PEITC, menadione, or taxol for 72 h. After various treatments, 20 μl of MTT solution (5 mg/ml, Sigma Aldrich) was added to each well and incubated at 37 °C for 3 h. The supernatant was aspirated, and the MTT-formazan crystals were dissolved in 150 μl of dimethyl sulfoxide. The absorbance was measured by a microplate reader (FACS Calibur, BD Biosciences).

Immunoblotting Analysis—Whole cell lysates were prepared in 1× Laemmli sample buffer (Sigma) to extract total proteins. Equivalent amounts of total cellular protein were electrophoresed on an 8% SDS-PAGE gel and transferred onto nitrocellulose membranes (Millipore). Membranes were blocked in 5% nonfat milk in TBS containing 0.1% Tween 20 (TBST) for 1 h at room temperature and then incubated with primary antibodies in 5% BSA in TBST at 4 °C overnight. Membranes were then washed with TBST and incubated with HRP-conjugated secondary antibody in 5% BSA in TBST for 1 h at room temperature. Immune complexes were detected by enhanced chemiluminescence (Pierce).

RNAi and Transfection—TP53 siRNA-1 5′-GACUC-CAGUGGAUACUCGdTdT-3′, TP53 siRNA-2 5′-CUAC-UUCCUAGAAAACACGdTdT-3′, and a random sequence control siRNA were purchased from Genepharma (Shanghai, China). Synthetic siRNAs were transfected into LO2 cells using Lipofectamine 2000 (Invitrogen).

Real-time Quantitative PCR Assay—The mRNA abundances of antioxidant genes were determined by quantitative real-time PCR assays. The ΔΔCt method of relative quantification and SYBR Green chemistry were used, and β-actin was used as an endogenous control for normalization. PCR primer sets were designed using Primer Premier 5, and the sequences were as follows: TP53, 5′-AGAATCTCCGCAAGAAAGG-3′ (forward) and 5′-CAAGCAAGGGTCTAAGAC-3′ (reverse); CDKN1A, 5′-ACTTTTGATTACGACGGGAACA-3′ (forward) and 5′-CTGGCTATGCTCTATTGG-3′ (reverse); SESN2, 5′-AAGACCCACCGAAGATGT-3′ (forward) and 5′-AGGAGTCAGGTCATGTAGCG-3′ (reverse); SOD1, 5′-CTGGTTTGCTGCTTAG-3′ (forward) and 5′-CTTCTGTGCAGGACT-3′ (reverse) and 5′-CGTTTGCTGCTTAG-3′ (forward).

Luciferase Assay—HepG2/STAT3 cells (1.5 × 105 cells/well) were seeded into 24-well cell culture microplates (Corning), allowed to grow for 24 h, and then treated with reagents for 2 h followed by stimulation with 10 ng/ml IL-6 for 5 h. Equal numbers of cells were collected, and the luciferase activity was measured by a luminometer using a luciferase assay system (Promega). All luciferase assay experiments were performed at least three times to minimize the differences caused by cell numbers.

Assessment of Apoptosis—NPP-induced apoptosis was determined by an annexin V-FITC apoptosis detection kit (KeyGen). Briefly, MDA-MB-468 cells were harvested after exposure to NPP for 24 h. The cells were washed twice with cold PBS and then resuspended in 500 μl of binding buffer at a concentration of 1 × 106/ml. Cells were then stained with annexin V-FITC and PI and analyzed with a FACScan flow cytometer (BD Biosciences). Viable cells were negative for both PI and annexin V. Apoptotic cells were positive for annexin V and negative for PI, whereas late apoptotic cells and necrotic cells displayed both annexin V and PI labeling.

Detection of Cytochrome c Release—The method of subcellular fractionation was modified on the basis of the method described previously (17). Cells were resuspended in a homogenization buffer (250 mM sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM PMSF (pH 7.5)). Homogenization was carried out with a Sigma Wheaton homogenizer until the trypan blue was taken up by > 90% of the cells. The organelle pellet containing mitochondria was collected by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatant cytosol was collected. Samples of cytosol and mitochondria...
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were resolved by SDS-PAGE. Immunoblotting for cytochrome c was performed with a specific antibody.

Total Antioxidant Capacity Assay—The antioxidant capacities of cells were measured according to the method described previously on the basis of cell quenching capacity toward the 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation (18). Trolox was used as reference antioxidant. Total antioxidant capacity was determined using a total antioxidant capacity kit (Beyotime) according to the instruction of the manufacturer.

TP53 Mutation Information—Most of the TP53 information was obtained from the World Health Organization International Agency for Research on Cancer TP53 database (Version R16, November 2012) (19), except for the information on Wi-38, LO2, and Chang, which were obtained from external references (20–22).

Statistical Analysis—Data are graphically represented as mean ± S.D. All experiments were replicated at least three times. Treatment means were compared using one-way analysis of variance, whereas other data were analyzed using unpaired Student’s t test.

RESULTS

NPP Preferentially Induced Apoptosis in Tumor Cells—We identified NPP, a methyl 3-(4-nitrophenyl) propiolate (Fig. 1A), as a selective inducer of cancer death from a large-scale compounds screening. To investigate its molecular mechanisms and therapeutic potential, we analyzed the effects of the compound on both tumor cells and normal cells. Interestingly, NPP demonstrated a striking selectivity against tumor cells, including leukemia and breast, liver, lung, prostate, colon, skin and cervical cancer cells compared with normal cells. 8 μM NPP inhibited more than 80% of the cell growth in transformed cell lines Hs578T and MDA-MB-468, whereas it had very little growth inhibition on the two less transformed cell lines Wi-38 and LO2 (Fig. 1B) and another two normal cell lines, HMEC and Chang (Fig. 1D). On the other hand, the general cytotoxic anticancer reagents taxol and cisplatin showed much less cell selectivity between the tumor and normal cells (Fig. 1C and data not shown).

We then chose one of the NPP-sensitive cell lines, MDA-MB-468, as our model to investigate the mechanisms of NPP. NPP inhibited growth of the MDA-MB-468 cells with an EC50 of around 2 μM (Fig. 1D). Further studies revealed that NPP dose-dependently induced cell apoptosis, as indicated by chromosomal condensation and annexin V-positive/PI-negative staining (Fig. 1, E and F). We then analyzed several key apoptosis-related events in response to the NPP treatment, which included the cleavages of nuclear enzyme poly(ADP-ribose) polymerase and caspase 3, and cytochrome c release. We observed both cleavages of poly(ADP-ribose) polymerase and caspase-3 following NPP treatment (Fig. 1, G and H). We also observed the release of cytochrome c into the cytosol after NPP treatment (Fig. 1I). These results demonstrated that NPP induced apoptosis through the intrinsic mitochondrial pathway.

The Effects of NPP Are Mediated by ROS—ROS have been suggested to specifically inhibit tumor cell growth according to the ROS threshold theory (10). We therefore investigated ROS levels after NPP treatment using a fluorescent probe, DCFH2-DA (2’,7’-dichlorofluorescin diacetate). Indeed, ROS levels, as indicated by fluorescence intensity, significantly increased in response to NPP treatment in a dose-dependent fashion in MDA-MB-468 cells (Fig. 2A). We also found that NPP increased ROS levels in all cell lines we studied, including transformed Hepa, SKBr3, HepG2, H460, SKOV3, MDA-MB-231, MDA-MB-453, and A549 cells as well as less transformed HMEC and Wi-38 cells (data not shown), indicating ROS induction as a general effect of NPP. The induction of ROS by NPP was rapid, occurred quickly in less than 10 min, reached its maximum level at 30 min, and began to decrease in less than 60 min after the treatment, suggesting that the ROS induction was the primary effect of NPP (Fig. 2B).

Because GSH is the major antioxidant reagent in cells to counterbalance ROS and maintain redox balance, we investigated the involvement of GSH in the NPP-mediated ROS formation. We observed a NPP dose-dependent decrease in the intracellular level of GSH after NPP treatment in MDA-MB-468 cells (Fig. 2, C and D). We found similar effects in another transformed cell line, MCF-7, and one less transformed cell line, Wi-38 (data not shown), suggesting GSH decrease as a general effect of NPP. A comparison between the time courses of ROS production and GSH deduction suggested that NPP-induced oxidative stress was the cause of the GSH deduction (Fig. 2, B and D).

To clarify the cause-effect relationship between ROS generation and cell death, we examined the effects of free radical scavenger GSH and N-acetyl-L-cysteine (Nac) on NPP-treated MDA-MB-468 cells. The addition of GSH effectively attenuated the NPP-induced intracellular accumulation of ROS (Fig. 2E). Both GSH and Nac abrogated NPP-induced cell death (Fig. 2F). Taken together, our results demonstrated that oxidative stress was the primary event upon NPP treatment and that it subsequently caused tumor cell apoptosis.

Cytochrome P450s Mediated NPP-induced ROS Formation—We next investigated the mechanisms by which NPP induced ROS formation. Mitochondria are the major source of intracellular ROS under physiological conditions (3, 23). Disruption of mitochondrial electron transport flow has been reported to result in generation of ROS. To determine whether NPP induced ROS generation through interfering with mitochondrial function, we examined the effects of NPP on ROS levels in both normal A549 cells and A549 Rh0 cells whose mitochondrial function was disabled by depletion of mitochondria DNA. However, we found that NPP also induced ROS formation in A549 Rh0 cells, similar to the parental normal A549 cells (Fig. 3A). There were no significant differences in the NPP-induced growth inhibition between A549 and A549 Rh0 cells (Fig. 3B). These data suggested that the NPP-induced ROS did not result from mitochondria.

Quinones has been reported to induce ROS through a semiquinone intermediate in the presence of NADPH-cytochrome P450 reductase and cofactor NADPH (24–26). During our analyses of the structure-activity relationship of NPP, we found that the propargyl ester group or its analogs are the key structures responsible for the NPP activity3. Alkynyl groups in

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these compounds are chemically active and could be reduced. Cytochrome P450s are the most important oxidoreductases for metabolizing exogenous reagents. We therefore examined the roles of P450s in NPP-induced ROS production. We first examined the effects of two general cytochrome P450 inhibitors, SKF-525A and 1-aminobenzotriazole (27–29) on NPP-induced
ROS generation. The ROS accumulation induced by NPP was blocked dose-dependently by SKF-525A (Fig. 3C). Both SKF-525A and 1-aminobenzotriazole inhibited NPP-induced cell death, suggesting that P450 is involved in NPP-induced ROS production (Fig. 3D).

Cytochrome P450 is a large and diverse family of enzymes (30, 31). We found that two specific CYP3A4 inhibitors, oleandomycin triacetate (TAO) and chloramphenicol (CHL) (29, 32), dose-dependently blocked NPP-induced ROS formation, whereas the CYP1A-specific inhibitor α-naphthoflavone (ANF) (33) did not (Fig. 3E). These data suggest that CYP3A4 is one of the key P450 members that are responsible for NPP-induced ROS.

Intracellular Redox Status and p53 Mutation Determined the Cell Selectivity of NPP—We observed a strong correlation between the cell sensitivity to NPP, the basal cellular ROS level, and the total antioxidant capacity of the cell (Figs. 1B and 4, A and B). Cells with higher basal ROS levels and lower antioxidant capacities, especially in the case of the leukemia cells, were more sensitive to the NPP-induced cell death. Conversely, cells with lower basal ROS levels and higher antioxidant capacities, as in the case of the less transformed cells, were less sensitive.

We selected three cell lines with distinct sensitivities to NPP, HL-60 representing the high, MCF-7 the moderate, and Wi-38 the low, and analyzed their gene expression microarray data to obtain their characteristic expression profiles. We found that the expression of 41 of 85 reported antioxidant genes, including the well known SOD, GPX, and SESN genes and the antioxidant-related genes CDKN1A and TP53, were lower in the HL-60 cells but higher in the Wi-38 cells (supplemental Fig. S1). Quantitative real-time PCR results confirmed the microarray data (Fig. 4C).
Interestingly, we also found that the p53 status was strongly correlated to the sensitivity of the cell to NPP. As shown in Fig. 4D, most of the NPP-sensitive cell lines harbored loss-of-function mutations in p53, whereas all NPP-resistant cell lines had the wild-type p53. Previous studies have shown that p53 regulates the transcription of many antioxidant genes, such as SESNs and GPXs, and that loss of p53 function increases ROS levels in normal cells (4, 34). We further investigated the roles of p53 in the effect of NPP. Knockdown of p53 by siRNA increased the cytotoxic effects of NPP in the non-transformed LO2 cells, which were originally insensitive to NPP (Fig. 4, E and F). The half inhibition concentration of NPP decreased substantially after p53 knockdown.

One exception for the correlation of p53 status and NPP sensitivity was the relative resistance of Hep3B (p53 null) to NPP, which was due to high expression of NPP-inactivating enzyme carboxylesterase in hepatocytes (supplemental Fig. S2, A–C). Treatment of carboxylesterase inhibitor bis-(p-nitrophenyl) phosphate (35) significantly sensitized the Hep3B cells, whereas it had scarcely synergistic effects on non-hepatic cells (supplemental Fig. S2, D–K).
These results clearly demonstrate that the cellular redox status, especially the p53 mutation status, determines the cell responsiveness to NPP. Tumor cells that have impaired antioxidant capacity and mutant p53 will be particularly vulnerable to the treatment of NPP and other ROS inducers.

The Biological Effects of NPP Are Distinct from Other ROS Inducers—To further understand the cytotoxic effects of ROS inducers on tumor cells, we compared NPP with the known ROS inducers menadione (36, 37) and PEITC (13, 38, 39) for efficacy and selectivity on inducing tumor cell death. We
observed that NPP exhibited a more potent induction of cell death and better selectivity toward tumor cells than PEITC or menadione (Figs. 1B and 5A, B). The superiority of NPP did not seem to be due to the amount and duration of ROS produced because the PEITC-induced ROS level was higher and lasted longer (Fig. 5C).

Interestingly, we observed that NPP inhibited tyrosine phosphorylations of STAT3 and decreased JAK/STAT signaling. As shown in Fig. 5, D and E, NPP inhibited both cytokine-stimulated STAT3 phosphorylation as well as constitutive STAT3 phosphorylation. The free radical scavengers GSH and Nac blocked the inhibitory effects of NPP on STAT3 phosphorylation, suggesting that the inhibition of JAK/STAT signaling by NPP is dependent on ROS generation. However, the other ROS inducer, PEITC, had similar effects but to a much lesser extent (Fig. 5F). A luciferase reporter assay also showed that NPP inhibited STAT3 signaling (Fig. 5G). Because the HepG2 cells, in which the luciferase reporter systems were established, express a high level of the NPP-inactivating enzyme carboxylesterase (supplemental Fig. S2, A and B), it diminished the inhibitory effect of NPP toward STAT3 signaling. Combination treatment of NPP and specific carboxylesterase inhibitor bis-(p-nitrophenyl) phosphate synergistically inhibited the cytotoxic effect of NPP toward STAT3 signaling. Combination treatment of cell apoptosis. Indeed, we observed that NPP induced more death in MDA-MB-468 and SKBr3 cells, which had constitutively activated STAT3, than in MCF-7 and Hs578T cells, which had normal STAT3 (Fig. 5I). PEITC, on the other hand, showed little preference over the STAT3-activated tumor cells (Fig. 5J). Our data suggest that the biological effects of ROS inducers are distinct and cellular content-dependent.

DISCUSSION

Although induction of apoptosis has long been considered as an anticancer strategy, the goal of treating tumor cells selectively is far from being achieved. Here we report the identification of a methyl 3-(4-nitrophenyl) propiolate, NPP, as a novel selective tumor cell apoptosis inducer. We present evidence to show that NPP induces tumor cell death through ROS-mediated mechanisms. By analyzing a set of cellular parameters, including basal ROS levels, total antioxidant capacities, expressions of antioxidant enzymes, and p53 mutation status, we demonstrate a strong correlation between cellular redox status and cell responsiveness to NPP. Thus, using NPP as a tool, we provide solid evidence to support the ROS threshold theory.

Furthermore, p53 is a key apoptosis mediator that is mutated in most cancer cells. Apoptosis-inducing anticancer strategies face the problems of toxicity for normal cells and drug resistance for tumor cells because of the p53 status (15). Interestingly, NPP preferably kills cells with p53 loss-of-function mutations. Knockdown of p53 increased NPP-induced cytotoxicity, suggesting that NPP-induced apoptosis is not mediated by p53. Instead, loss of p53 may increase the basal level of intracellular ROS and render p53-deficient tumor cells more sensitive to NPP. These data add strong evidences to support ROS-promoting strategy as a potential anti-cancer strategy.

Our data show that the NPP-induced ROS is catalyzed by the cytochrome P450 enzymes, and we further identified CYP3A4 as one of the key enzymes for the ROS production. CYP3A4 might catalyze NPP to produce ROS through a free radical intermediate, similar to the mechanism of quinones (25). We hypothesized that the propargyl form of NPP converted to an allenic intermediate in the presence of P450, and then the allenic NPP coupled to the oxygen molecule to produce a superoxide anion that has a very short half-life and could be quickly transformed into H$_2$O$_2$, another form of ROS molecules with a relatively long half-life, by dismutation of superoxide. H$_2$O could be further transformed into a hydroxyl radical by a transitional metal (Fig. 6). Enzyme-catalyzed free radical formation may have the advantage that ROS are produced moderately and continuously instead of a burst of stress (41), avoiding overproduction of ROS and unwanted cytotoxicity in normal cells.

In addition to the mode, amount, and duration of induced ROS production, our data suggest that the location of ROS production may be another important factor to determine the outcomes and selectivities of ROS inducers. The half-life of free radicals is so short that their diffusion distances are very limited (3). Thus, the effects of ROS inducers could be variable. As shown in Fig. 6, NPP induces ROS generation through cytochrome P450-mediated mechanisms. P450s are primarily membrane-associated proteins, located near the cell membrane, in the inner membrane of mitochondria, or in the endoplasmic reticulum (42, 43). Previous studies report that ROS induces endoplasmic reticulum stress, leading to cell death (44, 45). We, however, did not observe endoplasmic reticulum stress during NPP treatment. On the other hand, our data showed that ROS accumulated rapidly, suggesting that the

![FIGURE 4. Intracellular redox status and p53 mutation determined the cell selectivity of NPP. A, basal ROS levels of 12 cell lines were measured and normalized to the lowest one. Data are mean ± S.D. of three experiments. B, total antioxidant capacities of 12 cell lines were detected and quantified by protein level measurement using the Bradford method. Data are mean ± S.D. of three experiments. C, NPP-resistant cells expressed high levels of antioxidant-related genes, and NPP-sensitive cells expressed low levels. Detection of mRNA levels of antioxidant genes was performed by real-time quantitative PCR using the ∆∆CT method. Data are mean ± S.D. of three experiments. The relative mRNA level = 2^(-ΔΔCt), where Ct stands for Ct of the detected gene in the x cell line, Ct$_{H9252}$ stands for Ct of the housekeeping gene β-actin in the x cell line, Ct$_{H9252}$ stands for Ct of the detected gene in the calibrator cell line H9252, and Ct$_{H9252}$ stands for Ct of the housekeeping gene β-actin in the calibrator cell line H9252. D, correlation between p53 status and cell sensitivity to NPP. P53 mutation information, which was obtained from the International Agency for Research on Cancer TP53 database, was listed in the table so that cell sensitivity to NPP decreased from top to bottom. NA, data not available. The EC$_{50}$ of NPP on different cells was calculated from a 72-h MTT assay. E, knockdown of the TP53 gene in LO2 cells. LO2 cells were transfected with scramble siRNA or TP53 siRNAs for 48 h, and then mRNA were extracted for RT-PCR. The PCR products were separated on agarose gel. F, knockdown of TP53 increased cell sensitivity to NPP. LO2 cells were transfected with scramble siRNA or TP53 siRNAs. 72 h later, cell viabilities were measured by MTT assay. Data are mean ± S.D. of three experiments. The IC$_{50}$s were calculated. The knockdown and control data were compared using one-way analysis of variance. *p < 0.05; **p < 0.01.

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FIGURE 5. The biological effects of NPP are distinct from other ROS inducers. Growth inhibition of menadione (A) and PEITC (B) on the normal cell lines Wi-38 and LO2 and on the tumor cell lines Hs578T and MDA-MB-468. The cells were cultured with the indicated concentrations of the compounds for 72 h, and the cell viabilities were measured by an MTT assay. Data are mean ± S.D. of three independent experiments.

C, time course of ROS production induced by NPP or PEITC. MDA-MB-468 cells were treated with 10 μM NPP or 10 μM PEITC for the indicated time periods, and then the ROS levels were measured by flow cytometry. D, NPP inhibited the IL-6-induced STAT3 phosphorylation/activation through ROS. Hela cells were pretreated with the antioxidants GSH, Nac, or NPP for 40 min and then stimulated with 10 ng/ml IL-6 for 20 min. STAT3 phosphorylation was analyzed using Western blot analysis.

E, NPP blocked constitutive STAT3 tyrosine phosphorylation. MDA-MB-468 cells were treated with 0.5 mM GSH and NPP simultaneously for 1 h. STAT3 phosphorylation was analyzed by Western blot analysis. F, NPP was more potent than PEITC on inhibiting STAT3 phosphorylation. HeLa cells were treated with the indicated concentrations of NPP or PEITC for 40 min and then stimulated with 10 ng/ml IL-6 for 20 min. STAT3 phosphorylation was analyzed by Western blot analysis. G, NPP inhibited the STAT3-responsive luciferase reporter gene activities. HepG2/STAT3 luciferase cells were treated with 0.5 mM GSH and NPP simultaneously for 1 h. STAT3 phosphorylation was analyzed using Western blot analysis.

H, the mRNA levels of the STAT-regulated prosurvival genes Bcl-2 and Bcl-xL were reduced by NPP. The mRNA levels were measured by real-time quantitative PCR using the ΔΔCt method. Data are mean ± S.D. of three independent experiments. I, the mRNA levels of the STAT-regulated prosurvival genes Bcl-2 and Bcl-xL were reduced by NPP. The mRNA levels were measured by real-time quantitative PCR using the ΔΔCt method. Data are mean ± S.D. of three independent experiments.
compound might be metabolized immediately by the plasma membrane-associated P450s after uptake into the cells. Thus, the ROS are produced near the plasma membrane, and the proteins nearby are prone to be affected. NPP inhibits STAT3 phosphorylation, possibly through upstream receptor-associated kinases such as JAKs. PEITC, on the other hand, produces ROS through depletion of the intracellular GSH level (46), which equally affects all cytosolic as well as membrane proteins. Therefore, NPP affects the JAK/STAT pathway more dramatically than PEITC does. Taken together, our data suggest an intriguing possibility that the better selectivity and potency of NPP may result from localized ROS generation. Further studies are needed to address this possibility.

How might NPP inhibit JAK/STAT signaling? It was recently reported that ROS play a central role as second messengers in many signal transduction pathways (3). The redox-sensitive sites of signaling proteins can be posttranslationally modified to serve as redox sensors. Cysteine residues are such sensors through the reversibility of the thiol/disulfide redox couple (47). We reported previously that 17-HJB, a natural compound with thiol-reactive epoxy groups, inhibits JAK/STAT signaling by covalently linking JAK2 through interaction with thiol groups of cysteines (48). Although the exact reactive cysteine residues in JAK2 remain to be elucidated, it was proposed that certain cysteine residues of the JAKs are exposed on the surface of the proteins, which can be easily accessed by the epoxy groups of 17-HJB. We therefore propose that the same cysteines of the JAK proteins might be attacked by the ROS generated by NPP.

In addition to JAKs, protein tyrosine phosphatases are frequently reported protein targets of free radicals. ROS inhibits protein tyrosine phosphatase activities by oxidizing the thiol groups of cysteines in the tyrosine phosphatase catalytic center (9, 47, 49). We observed that NPP induced phosphorylation of receptor tyrosine kinases such as the EGF receptor and PDGF receptor (data not shown), which might be through a membrane-proximal ROS production that inhibits protein tyrosine phosphatases. Although other anticancer therapeutics, such as ionizing radiation or genotoxic compounds, also induce ROS, their ROS induction levels are much lower compared with that of NPP, and the cytotoxic effects are mainly due to their damage effects on DNA, which are rather nonspecific (40, 50, 51). It is the particular P450-catalyzed ROS production in the membrane-proximal region that makes NPP a unique selective tumor cell death inducer.

As the understanding of ROS function under physiological condition accumulates and the threshold theory is being tested, ROS are no longer considered as simply tumorigenesis molecules. However, whether the ROS-promoting strategy is feasible in cancer treatment remains to be seen. We propose that NPP and other ROS inducers might be tools for such studies.

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