Thioredoxin Reductase as a Potential Molecular Target for Anticancer Agents That Induce Oxidative Stress

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ABSTRACT

Redox-sensitive signaling factors regulate multiple cellular processes, including proliferation, cell cycle, and prosurvival signaling cascades, suggesting their potential as molecular targets for anticancer agents. It is logical to set constraints that a molecular target should meet at least one of the following criteria: (1) inhibition of prosurvival signaling pathways; (2) inhibition of cell cycle progression; or (3) enhancement of the cytotoxic effects of anticancer agents. Therefore, we hypothesized that thioredoxin reductase 1 (TR), a component of several redox-regulated pathways, might represent a potential molecular target candidate in response to agents that induce oxidative stress. To address this issue, permanent cell lines overexpressing either the wild-type (pCXN2-myc-TR-wt) or a Cys-Ser mutant (pCXN2-myc-mTR) TR gene were used, as were parental HeLa cells treated with 1-methyl-1-propyl-2-imidazolyl disulfide (IV-2), a pharmacologic inhibitor of TR. Cells were exposed to the oxidative stressors, H2O2 and ionizing radiation (IR), and analyzed for changes in signal transduction, cell cycle, and cytotoxicity. Analysis of HeLa cells overexpressing the pCXN2-myc-TR-wt gene showed increased basal activity of nuclear factor kB (NFkB) and activator protein (AP-1), whereas HeLa cells expressing a pCXN2-myc-mTR gene and HeLa cells treated with IV-2 were unable to induce NFkB or AP-1 activity following H2O2 or IR exposure. Fluorescence-activated cell sorting analysis showed a marked accumulation of pCXN2-myc-mTR cells in the late G1 phase, whereas pCXN2-myc-TR-wt cells showed a decreased G1(subpopulation). Chemical inhibition of TR with IV-2 also completely inhibited cellular proliferation at concentrations between 10 and 25 μmol/L, resulting in a G1 phase cell cycle arrest consistent with the results from cells expressing the pCXN2-myc-mTR gene. Following exposure to H2O2 and IR, pCXN2-myc-mTR- and IV-2–treated cells were significantly more sensitive to oxidative stress-induced cytotoxicity as measured by clonogenic survival assays. Finally, IV-2–treated cells showed increased tumor cell death when treated with H2O2 and IR. These results identify TR as a potential target to enhance the cytotoxic effects of agents that induce oxidative stress, including IR.

INTRODUCTION

The cytotoxicity of agents that induce oxidative stress, including H2O2 and ionizing radiation (IR), originates from intracellular damage caused by reactive oxygen intermediates (ROIs). Ideally, a metabolically active cell should strike a balance between ROI production and the cellular antioxidant defense system, resulting in a reduced cellular environment (1–3). Although relatively small amounts of ROIs are easily tolerated by cells (4, 5). However, levels of ROI production that exceed endogenous cellular antioxidant capacity can create a condition referred to as "oxidative stress," in which the resulting lipid peroxidation and DNA damage can lead to cell death (6–9). It is believed that ROI levels that result in oxidative stress and accumulation of oxidative damage are produced after exposure to H2O2, IR, chemotherapeutic agents, and hyperthermia (10–14).

Thioredoxin reductase 1 (TR), thioredoxin (TRX), and NADPH comprise a highly conserved, ubiquitous system (15) that plays an important role in the redox regulation of multiple intracellular processes, including DNA synthesis, transcriptional regulation, cell growth, and resistance to cytotoxic agents that induce oxidative stress and apoptosis (16–18). TR is a member of a recently identified class of signaling factors that use critical cysteine motif(s) to act as redox-sensitive "sulfhydryl switches." These switches reversibly modulate specific signal transduction cascades, which regulate downstream proteins with similar redox-sensitive sites (19–21). TR is a homodimeric selenocysteine-containing protein that catalyzes the NADPH-dependent reduction of TRX and numerous other oxidized cellular proteins (15, 19, 20). Following oxidative stress, TR initiates a signaling cascade in response to free radicals in the cytoplasm and then activates transcription factors in the nucleus that regulate downstream genes, which appear to protect the cell from the oxidative stress induced by free radicals (22–25). Collectively, these observations appear to suggest a possible role for TR in the cellular defense against oxidative damage.

A class of proto-oncogenes referred to as immediate early response genes is activated as a consequence of a wide variety of environmental agents that induce oxidative stress (7, 26, 27). These genes encode nuclear transcription factors, including the activator protein (AP-1) complex and nuclear factor kB (NFkB), which play central roles in the transmission of intracellular information through multiple cellular signaling pathways (28–31). One possible role for the induction of these transcription factors is to modulate the expression of specific target genes involved in a protective or reparative cellular response to the damaging effects of oxidative stress induced by exogenous cytotoxic agents (32–35). As such, knowledge of these signaling pathways provides fundamental insight into how tumor cells respond to cytotoxic agents.

The activities of NFkB and AP-1 also are affected following exposure to chemicals, drugs, or other exogenous agents that appear to alter the cellular reduction/oxidation (redox) status, including H2O2 (1, 36, 37). From these observations, it has been suggested that changes in cellular redox status, which are communicated via a series of cellular redox-sensitive signaling circuits using metal- and thiol-containing proteins, serve as common mechanisms linking environmental stressors to adaptive cellular responses (7, 18, 36). These transcription factors have been speculated to provide a prosurvival or antiapoptotic function in tumor cells (20, 34, 37). As such, these transcription factors are ideal paradigms to study the mechanism and possible physiologic significance of early response genes in the cellular response to changes in intracellular redox status induced by environmental stress.

Because TR appears to regulate prosurvival signaling factors in

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Note: D. K. Smart and K. L. Ortiz contributed equally to this manuscript.

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response to oxidative stress, we hypothesized that TR might represent a potential candidate for a molecular target in response to oxidizing agents. It is our position that an ideal molecular target should result in at least one of the following events: (1) inhibition of prosurvival signaling pathways; (2) inhibition of cell cycle progression; and/or (3) enhancement of the cytotoxic effects of anticancer agents. Genetic or chemical inhibition of TR inhibited two prosurvival transcription factors, NFκB and AP-1, induced G1-phase growth delay, and sensitized tumor cells to the cytotoxic effects of H2O2 and IR. These results identify TR as a potential target to enhance the cytotoxicity of agents that induce oxidative stress, including IR.

MATERIALS AND METHODS

Cell Culture, IR and H2O2 Conditions, and 1-Methyl-1-Propyl-2-Imidazolyl Disulfide Synthesis. Parental HeLa cells (human cervical carcinoma) were cultured in MEM (a modification) supplemented with 10% heat-inactivated (56°C, 30 minutes) calf serum, penicillin (100 μg/mL), and streptomycin (100 units/mL). HeLa cell lines stably overexpressing TR (25) were cultured in MEM-α containing 5% fetal bovine serum, penicillin, streptomycin, and G418 (300 μg/mL). All of the cell lines were maintained in a humidified 37°C incubator with 5% CO2 and air. The TR wild-type (pCXN2-myc-TR-wt) and Cy5-Ser mutant (pCXN2-myc-mTR) expression plasmids have been described previously (22). All of the TR-overexpressing cell lines used in the study are pools of at least 15 clones; cells tested negative for Mycoplasma contamination before experimentation.

Cells were exposed to IR in an X-RAD 3200 (Precision X-Ray, Madison, CT) operated at 300 kV and 10 mA; 2-mm Al filtration was used to deliver IR at a dose rate of 1.8 Gy/min (14). Hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) stock solutions were made in sterile PBS, and their molar concentrations were determined by a spectrophotometric method described previously (6). Doses of H2O2 were delivered directly to the growth medium, and cells were returned to the incubator for designated periods following exposure to either IR or H2O2.

The unsymmetrical 1-methyl-1-propyl-2-imidazolyl disulfide (IV-2) was prepared as reported previously (38), and all of the reagents were obtained from Sigma-Aldrich. Briefly, 1-methyl-1-propanethiol (11.6 g, 129.9 mmol) was dissolved in aqueous EtOH (H2O, 40 mL; EtOH, 130 mL) with thiourea (7.70 g, 101.2 mmol). The solution was cooled in an ice bath, and concentrated HCl (11 mL) was added, followed by dropwise addition of 30% H2O2 (12.4 g). After 3 hours, the solvent was removed, and the residue taken up in minimal EtOH, diluted with diethyl ether, and refrigerated for 18 hours. The precipitated 1-methyl-1-propanethioisothioureia intermediate was collected, dried in vacuo, and used directly thereafter. 2-Mercaptomidazole (0.5 g, 5.05 mmol) and the intermediate isothioureia (1.20 g, 5.95 mmol) were dissolved in methanol (12 mL), and aqueous 0.4 M sodium bicarbonate (20 mL) was added dropwise while stirring. After 1 hour, the solution was refrigerated, and the solid product was collected on a Hirsch funnel (0.78 g, 82% yield).

Characterization and purity determination of IV-2 were achieved by application of a combination of standard analytical techniques, including 300 MHz proton NMR (Gemini System; Varian, Palo Alto, CA), chemical ionization mass spectrometry (Mat Finnegan 4600; Finnigan Corporation, San Jose, CA), and elemental combustion analysis (Atlantic Microlabs, Norcross, GA). Data from the three techniques are presented in detail (vide infra): [1H NMR (CDCl3)] 6 7.133 (s, 2H), 2.957 (sex, 1H, J = 6.6), 1.731 (sept, 1H, J = 7.2), 1.542 (sept, 1H, J = 7.2), 1.302 (d, [3H], J = 6.9), 0.909 (t, [3H], J = 6.9); [mass spectrometry (ClN)] 189 (M+1); ref. 38]; [combustion analysis for carbon, hydrogen, and nitrogen (corresponding to C13H22N2S3); calculated C = 44.62, H = 6.43, n = 14.87; found: C = 44.44; H = 6.47; n = 15.02]. The analyses detected no other species present than the described product (i.e., the proton NMR consistently matched the previously reported synthesis with no other signals detected; the mass spectrum registered no higher molecular weight species present and a consistent fragmentation pattern for the product; and the combustion analysis indicated expected elemental ratios). The characterization data are fully consistent with the previously reported synthesis and indicate acceptable and unequivocal purity (>98%) as required by American Chemical Society standards. Immediately before cell treatment, IV-2 was dissolved in 10-mmol/L stock solutions in 100% EtOH and added to the growth media to achieve specific molar concentrations.

Preparation of Whole Cell and Subcellular Extracts. Whole cell, nuclear, and cytoplasmic extracts were prepared using a modification of the previously described method (39). No reducing agents were added to the buffers used to make extracts. Protein concentrations were determined using the Bradford assay (Bio-Rad Labs, Hercules, CA) and a Beckman DU-640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Following preparation and protein analysis, samples were stored at −80°C and thawed on ice immediately before use.

Electrophoretic Mobility Shift Assay. Protein samples were prepared, stored, and quantified by methods described previously (40); no reducing agents were added to the electrophoretic mobility shift assays (EMSA). EMSAs were performed as described previously using a 32P-radiolabeled oligonucleotide corresponding to the consensus NFκB or AP-1 DNA binding site (24). Nuclear extracts (10 μg) were incubated with poly(dI-dC) for 10 minutes on ice, followed by the addition of radiolabeled oligonucleotide (100,000 cpm of radiolabeled probe per reaction), and incubated at 25°C for 20 minutes. Samples were electrophoresed on a 6% nondenaturing polyacrylamide gel, dried, exposed to a phosphorimager screen, and analyzed using a TYPHOON 860 Phosphorimager (Amersham Biosciences, Piscataway, NJ) with ImageQuant software (Amersham Biosciences).

Plasmids, Cotransfections, and Luciferase Assays. HeLa cells were plated at 2 × 105 cells per 100-mm plate, serum starved (0.5% FCS) for 8 hours, and transfected via calcium phosphate precipitation for overexpression of NFκB or AP-1 (24). Either 2 μg of p45-xκB- tk-LUC (containing four copies of the NFκB DNA binding site) or p7X-AP-1-Itk-LUC (containing seven copies of the AP-1 DNA binding site) upstream of the luciferase reporter gene in ptk-LUC (Stratagene, Inc, La Jolla, CA) were added to each plate. As an internal control, 1 μg of the β-galactosidase expression plasmid (pcMV-β-Gal) was also used for each plate. Thirty-six hours after transfection, cells were exposed to H2O2 and harvested after 8 hours. Luciferase activity was determined using a luminometer (Zylux Corp, Maryville, TN), and the relative-fold induction of luciferase activity was calculated by normalizing to β-Gal activity.

SDS-PAGE and Immunoblot Analysis. Following preparation of whole cell extracts, equal protein amounts (10 to 20 μg/sample) were mixed with Laemmli lysis buffer, boiled for 5 minutes, and loaded into denaturing SDS-polyacrylamide gels for electrophoresis as described previously (39, 40). Membranes were probed with antibodies against NFκB, inhibitor of NFκB (IκB), c-Fos, c-Jun, Rb, p16 (Santa Cruz Biotechnology, Santa Cruz, CA), TR1, TR2 (Upstate Biotechnology, Lake Placid, NY), and actin (Chemicon, Temecula, CA) and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Bands were analyzed using an enhanced chemiluminescence protocol (Amersham Biosciences) and visualized on radiographic film (Eastman-Kodak, Rochester, NY). Figures are representative of the outcome in at least two independent experiments.

Thioredoxin Reductase Enzymatic Assay. TR activity was determined by a previously developed method (41) and performed as described (25). Briefly, cell extracts from log-phase cells were prepared by freeze-thaw lysis and quantified as described (40), and 20 μg of extract were incubated with bovine insulin, NADPH, and TRX in 0.2 mol/L HEPES (pH 7.6), for 20 minutes at 37°C. Reactions were terminated after the addition of 6 mol/L guanidine hydrochloride/0.4 mg/mL dithiothreitol (2-nitrobenzoic acid) prepared in 0.2 mol/L Tris (pH 8.0). In each case, a corresponding experimental sample without TRX was used to correct for the basal level of TR activity (because of endogenous TRX and NADPH). Reactions without cell extracts and reactions with pure TR in place of cell extracts also were used as negative and positive controls, respectively. Triplicate samples were measured for enzymatic activity by spectrophotometric absorbance at 412 nm.

Cellular Growth Assays and Fluorescence-Activated Cell Sorting Analysis. Cells were seeded at a density of 2 × 105 cells per 35-mm dish and returned to a humidified 37°C, 5% CO2 incubator for growth. Designated samples contained IV-2 at 10-, 25-, or 50-μmol/L concentrations in the media; EtOH vehicle was added to control samples. For the following days, three...
plates from each treatment condition were trypsinized and quantified via a Z2 counter (Beckman-Coulter) with fresh medium added every 3 days. Plating efficiencies were similar for all of the samples before treatment. Growth curves were plotted as the mean number of cells per dish as a function of time (hours).

For fluorescence-activated cell sorting (FACS) analysis, HeLa cells were seeded into 100-mm tissue culture dishes at a density of 1 × 10⁶ cells per dish and incubated at 37°C in the presence of 10, 25, or 50 μmol/L of IV-2 for 24 to 192 hours. After treatment with IV-2, cells were harvested by trypsinization, centrifuged at 1000 rpm for 5 minutes, washed twice sequentially in PBS, and fixed in 70% EtOH.

The fixed samples were stored at 4°C until analysis, then washed and centrifuged for two more cycles. The samples were incubated in RNase (0.2%) for 30 minutes at 4°C, labeled with propidium iodide, and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

**Clonogenic Cell Survival Assays.** For clonogenic cell survival assays, cells were seeded into 100-mm tissue culture dishes at a density of 2.0 × 10⁵ and grown to 75% confluence before experimental treatment. Doses of H₂O₂ were delivered directly to the growth medium, or designated doses of IR were administered as described previously. At 1 hour following exposure, cells were trypsinized and counted using a Z2 counter (Beckman-Coulter). Dilutions of the treated cells were prepared, and duplicate 60-mm tissue culture dishes were seeded with 200 to 20,000 cells each, depending on the severity of the challenge treatment. Colonies were allowed to form in an undisturbed, humidified, 37°C, 5% CO₂ environment for 7 to 10 days, fixed with 70% EtOH, stained with crystal violet, and counted under a dissection microscope. Only those plates containing 25 to 250 colonies were computed as statistically relevant, and only those colonies containing at least 50 cells were considered to be viable survivors. Surviving fractions from the treated test cultures were normalized to sham-treated controls and plotted as a function of dose on a log/linear plot.

**RESULTS**

**TR-Overexpressing Cell Lines Have Altered NFκB and AP-1 Activity in Response to H₂O₂.** We have shown previously that AP-1 DNA binding activity is induced following exposure to IR via a pathway involving redox regulation of TR, TRX, and Ref-1 (18, 22, 30). This was accomplished using permanent cell lines derived from HeLa cells that stably overexpress (1) a control expression vector (pCXN2-myc-X); (2) a wild-type TR-containing vector (pCXN2-myc-TR-wt); or (3) a TR mutant-containing vector (pCXN2-myc-mTR; refs. 25, 42, 43). Expression of the wild-type or Cys-Ser mutant genes has been shown previously to alter intracellular TR activity (25). However, this work was only done in cells treated with a single putative oxidative stressor, IR, and following inspection of only one redox-sensitive transcription factor. As such, we extended this investigation to include an additional agent that induces oxidative stress, H₂O₂.

EMSA performed on cell lines permanently overexpressing pCXN2-myc-X, pCXN2-myc-TR-wt, or pCXN2-myc-mTR showed that H₂O₂ induces NFκB DNA binding activity in vector controls (Fig. 1A, Lane 1 versus 4), whereas cells that overexpress the Cys-Ser mutant TR gene (pCXN2-myc-mTR) fail to induce DNA binding in response to H₂O₂ (Fig. 1A, Lane 3 versus 6). This lack of induction in the wild-type overexpressing cells is likely because NFκB activity is already up-regulated and/or maximally activated, and additional induction of activity is not possible. Luciferase assays performed following transient cotransfection experiments with p4x-κB-lκ-LUC also showed that inhibition of TR activity via overexpression of pCXN2-myc-mTR inhibits H₂O₂-induced NFκB-dependent gene expression (Fig. 1B). Similar results also were observed for AP-1 activity and gene expression in response to H₂O₂ in HeLa cells (Fig. 2A and B). The results of these experiments confirm and expand our previous results, suggesting that TR is involved in an intracellular signaling pathway that regulates the activation of redox-sensitive transcription factors (e.g., NFκB and AP-1) in response to agents that induce oxidative stress (e.g., H₂O₂).

**HeLa Cells Overexpressing a Wild-Type or TR-Mutant Gene Have Altered Cell Cycle Distribution and Doubling Times.** It is a well-established observation that redox pathways play critical roles in the regulation of multiple intracellular processes, including apoptosis and cell cycle progression (18, 22, 30, 36). In this regard, the factors connecting intracellular redox pathways to cell cycle regulation include TR, TRX, and glutathione systems and perhaps transcription, sensitivity to cytokines and growth factors, and components that regulate prosurvival pathways (18, 19, 36). Because genetically altered TR activity modulated the cellular response to NFκB and AP-1 in response to H₂O₂-induced oxidative stress, we determined whether cell lines that overexpress the wild-type or mutant TR gene would have altered cell cycle properties.

To address the possible effect of TR activity on cell growth and proliferation, HeLa cells that express the wild-type (pCXN2-myc-TR-
or mutant (pCXN2-myc-mTR) genes were initially analyzed for overall growth rate. Equal numbers of cells were plated, and for 5 days sequentially, the number of cells per plate for each cell line was determined, and average cell number per plate for each cell line was calculated. The results of these experiments show that overexpression of TR in HeLa cells (pCXN2-myc-TR-wt) caused accelerated growth compared with HeLa cells containing the empty vector (pCXN2-myc-X; Fig. 3A). In contrast, HeLa cells expressing the pCXN2-myc-mTR gene had a substantially decreased rate of proliferation (Fig. 3A), suggesting a role for TR in cell cycle regulation. To further address this observation, the three permanent cell lines were harvested, and their cell cycle profiles were assessed via FACS analysis. The data showed that overexpression of wild-type TR (pCXN2-myc-TR-wt) causes a marked decrease in the number of cells at the G1 phase of the cell cycle, whereas cells that express the mutant TR (pCXN2-myc-mTR) have a significant increase in the number of cells in the G1 phase of the cell cycle (Fig. 3B). Collectively, these experiments indicate a role for TR activity in regulation of HeLa cell progression through the G1 cell cycle checkpoint.

IV-2 Inhibits TR Activity in HeLa Cells. It has been shown previously that IV-2 inhibits TR activity at micromolar concentrations, selectively abrogates the TRX-dependent growth of tumor cells in culture, and has antitumor activity against MCF-7 and HL-60 tumors in vivo (38). This effect is independent of human glutathione reductase (44). It also has been shown that alkyl 2-imidazolyl disulfides interact directly with TRX via thioalkylation of critical cysteine residues or induced dimerization of the protein, leading to a loss of biological activity (44). As such, we hypothesized that TR might be a molecular target in cervical tumor cells, and IV-2 might be a potential agent through which to test this hypothesis. To address this idea, HeLa cells were treated with IV-2 (Fig. 4A), and the compound’s ability to inhibit TR activity was determined (41). The results of these experiments show a dose-dependent inhibition of constitutive TR activity in the concentration range of 5 to 100 μmol/L IV-2 (Fig. 4B) and provide proof-in-principle to use IV-2 to examine TR as a potential target.

Fig. 3. Permanent cell lines expressing the wild-type or a mutant TR gene have altered cell cycle profiles. A. Asynchronously growing HeLa cell lines that stably express vector alone (pCXN2-myc-X; Lane 1), wild-type TR (pCXN2-myc-TR-wt; Lane 2), or a mutant TR (pCXN2-myc-mTR; Lane 3) were exposed to 50 μmol/L of H2O2 (Lanes 4 through 6), harvested after 1 hour via subcellular fraction, and analyzed for AP-1 DNA binding activity via EMSA. Arrows indicate the AP-1 complex and free unbound AP-1 oligonucleotide. Sections of fluorograms from native gels obtained using a Typhoon Phosphorimager are shown. B. pCXN2-myc-X, pCXN2-myc-TR-wt, and pCXN2-myc-mTR cells were transfected with 2.0 μg of p7x-AP-1-tk-Luc, 1 μg of pCMV-β-Gal, and 12.0 μg of pUC. Sham-treated and H2O2-treated cells were harvested 1 hour following exposure, and analysis of reporter gene expression using luciferase and β-Gal activity was accomplished as described in Fig. 1. Error bars around data points represent 1 SD about the arithmetic mean, and statistical significance was established by Student’s t test (P < 0.05).
IV-2 Inhibits NFκB and AP-1 Activity in Response to H$_2$O$_2$ Exposure. Because IV-2 inhibits TR activity (Fig. 4B) and cell lines that overexpress the Cys-Ser mutant TR gene show impaired H$_2$O$_2$-induced NFκB activity (Fig. 1A and B), it seemed logical to also investigate whether IV-2 alters H$_2$O$_2$-induced NFκB activity. As such, parental HeLa cells were exposed to 20 or 50 $\mu$mol/L of H$_2$O$_2$ with or without pretreatment of 10 or 25 $\mu$mol/L of IV-2 and harvested via subcellular fractionation. EMSAs showed a fivefold increase in NFκB DNA binding activity following H$_2$O$_2$ exposure, and the effect was inhibited with pretreatment of IV-2 (Fig. 5A, Lanes 4 and 5 versus 6 and 7). However, IV-2 had no effect on unstimulated cells (Fig. 5A, Lanes 2 and 3). Transient cotransfection experiments in parental HeLa cells using p4x-κB-tk-LUC (Fig. 5B) confirmed that IV-2 inhibits NFκB DNA binding and gene expression in response to H$_2$O$_2$. Immunoreactive levels of NFκB also were increased in the nuclear fraction in response to H$_2$O$_2$, with a resulting decrease in response to treatment with IV-2 (Fig. 5C). In contrast, immunoreactive levels of IκB protein in the cytoplasmic fraction decreased in response to H$_2$O$_2$ treatment, and the effect was reversed with concurrent administration of IV-2 (Fig. 5C).

Analogous experiments to evaluate the response of AP-1 to IV-2 administration with or without H$_2$O$_2$ stimulation are presented in Fig. 6. These experiments also show that IV-2 inhibits H$_2$O$_2$-induced activation of AP-1–dependent DNA binding in EMSA (Fig. 6A) and gene expression using p7×-AP-1-tk-LUC (Fig. 6B), with corresponding changes in cytoplasmic c-Fos and c-Jun immunoreactive protein levels (Fig. 6C). The results of these experiments pharmacologically
and time-dependent manner (Fig. 7B) cells resulted in a G1-phase arrest that occurred in a concentration-and time (hours). FACS analysis showed that IV-2 treatment of HeLa cells treated with increasing doses of IV-2 at 24 and 48 hours follow-

### IV-2 inhibits Cellular Proliferation and Induces a G1-Phase Cell Cycle Arrest

To determine whether TR is a potential molecular target for \( H_2 O_2 \)-induced cytotoxicity, whereas pCXN2-myc-mTR (pCXN2-myc-mTR) -expressing cell lines and control cells (pCXN2-myc-X) were assayed for survival following exposure to IR (14, 32). When pCXN2-myc-X, pCXN2-myc-TR-wt, or pCXN2-myc-mTR cells were treated with 2, 4, 6, or 8 Gy of IR and assayed for clonogenic survival, the wild-type TR (pCXN2-myc-TR-wt) -expressing cells showed resistance to IR-induced cytotoxicity (Fig. 9A). In contrast, the cells expressing the mutant TR (pCXN2-myc-mTR) exhibited increased sensitivity to cell death when compared with control (pCXN2-myc-X) cells. Similar to the results with IR, HeLa cells that overexpress the wild-type TR (pCXN2-myc-TR-wt) were resistant to \( H_2 O_2 \)-induced cytotoxicity, whereas pCXN2-myc-mTR (pCXN2-
myc-mTR) cells were more sensitive to oxidative stress-induced death (Fig. 9B) as compared with controls.

To confirm these results in a similar model system and to determine whether IV-2 is a cytotoxic and/or a sensitizing agent in addition to being a cytostatic agent (Fig. 7A and B), HeLa cells were treated with either IV-2 alone or pretreated with IV-2 before exposure to IR or H2O2. When HeLa cells were treated with IV-2 alone for 24 hours at a concentration of 25 μmol/L, the cells showed 60% clonogenic survival compared with untreated controls. Likewise, cells treated with 50 μmol/L IV-2 showed 27% clonogenic survival, and cells treated with 100 μmol/L IV-2 showed 12% clonogenic survival compared with untreated controls. These data show that IV-2 induces a dose-dependent cytotoxic response.

However, when IV-2 treatment was combined with IR (Fig. 9C, open circle), the IV-2–treated cells were significantly more sensitive to IR-induced cytotoxicity than vehicle-treated HeLa cells (solid circle). Treatments of longer duration with IV-2 were not possible because cell cycle arrest was observed at these times (Fig. 7A and B).

When HeLa cells were pretreated with IV-2 before exposure to H2O2, a significant increase in H2O2-induced tumor cell death was observed (Fig. 9D), consistent with those from the genetically altered HeLa cell lines expressing the TR genes (Fig. 9B). Collectively, the results of these experiments suggest that the inhibition of TR can sensitize tumor cells to agents that induce oxidative stress and may function as either a major or minor mechanism of cell death.

DISCUSSION

With the ever-expanding discovery of the molecular components and metabolic mechanisms underlying the pathways involved in carcinogenesis and tumor cell resistance to anticancer agents, it now is possible to identify potential molecular targets that can be initially validated in vitro. TR has been shown to be overexpressed in several primary human malignancies, including breast cancer, thyroid, prostate, liver, colorectal carcinoma, and malignant melanoma (44, 45). In this regard, we investigated whether TR could serve as a potential molecular target. However, the identification and validation of cognate proteins as bona fide molecular drug targets using any of several potential techniques remains an underdeveloped field, with expectations far exceeding present capabilities (46, 47). We established a set of criteria for an ideal molecular target and determined which, if any, of these criteria were fulfilled by TR. Theoretically, an ideal molecular target should (1) be overexpressed in tumor cells; (2) enhance tumor proliferation; (3) exhibit prosurvival signaling attributes; (4) incite a prosurvival effect; and (5) enhance resistance to therapeutic modalities (e.g., IR and chemotherapy). Therefore, inhibition of the

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Fig. 7. IV-2 inhibits HeLa cell proliferation and induces a G1-phase cell cycle arrest.
A. HeLa cells were plated at 2 × 10^4 cells per plate and treated with 10, 25, or 50 μmol/L of IV-2. Cells were harvested at 1, 2, 3, 4, 5, 6, 7, and 8 days following initial treatment and counted as described. The number of HeLa cells per plate (three plates per condition) was determined and plotted as a function of days. B. HeLa cells were plated at 2 × 10^5 cells per plate and treated with 10, 25, or 50 μmol/L IV-2 or an identical volume of vehicle control and harvested at 24 or 48 hours. Cell cycle phase distribution was assessed by analysis of propidium iodide-labeled cells using flow cytometry.

Fig. 8. HeLa cells treated with IV-2 induce p16 cytoplasmic immunoreactive protein levels. Asynchronously growing HeLa cells were exposed to 25, 50, or 100 μmol/L of IV-2 for 24 hours and harvested via subcellular fractionation as described previously. Twenty micrograms of nuclear or cytoplasmic cellular protein were separated by SDS-PAGE, transferred onto nitrocellulose, and processed for immunoblot analysis. Immunoreactive protein levels for Rb, p16, TR1, and TR2 are shown. Equal protein loading was determined using a Bradford protein assay, and blots were reprobed with actin to ensure equal protein loading.
molecular target should reverse each of these effects and result in increased tumor cell response.

Exposure of eukaryotic cells to agents that induce oxidative stress, including IR, results in the immediate formation of short-lived free radicals. It has long been assumed that free radical damage to DNA is the primary target for IR-induced tumor cell death. However, it is increasingly clear that intracellular metabolic redox reactions can be affected by this initial IR-induced free radical insult and may remain perturbed for minutes, hours, or days. These processes include redox-sensitive signaling pathways, transcription factor activation, gene expression, and metabolic activities that govern the formation of intracellular oxidants and reductants. As such, it seems logical that these intracellular redox reactions may contribute to the activation of protective or damaging processes that could impact on the damaging effects of IR.

It is well established that cancer cells show altered metabolism when compared with normal, nontransformed cells (48, 49). The most common changes in metabolism involve glucose utilization and the loss of regulation between glycolytic metabolism and respiration when cancer cells exhibit increased rates of glycolysis and pentose phosphate cycle activity. It has been suggested that NADPH serves as the source of reducing equivalents for the glutathione-dependent decomposition of hydroperoxides via the peroxidase/glutathione reductase system and the TRX-dependent decomposition of hydroperoxides via the TRX peroxidase/TR system. This would account for why TR is up-regulated in tumor cells and supports the speculation that malignant cells may be more susceptible to the inhibition of these factors in regard to cytotoxicity, cytostasis, and radiosensitization than normal cells.

TR is part of a family of signaling factors that use critical cysteine motif(s) as redox-sensitive “sulphydryl switches” to reversibly modulate specific prosurvival signaling cascades regulating downstream proteins with similar redox-sensitive sites. In this model, the redox status of these critical cysteine residue(s) is strongly influenced by its local protein microenvironment (1). Subtle changes in cellular redox potentially alter the ionization state of the cysteine sulfur molecule (–CH₂-SH to –CH₂-S–), resulting in profound changes in protein activity. The redox potential then could be passed along specific signal transduction cascades using proteins with redox-sensitive sites (24). It is possible that IR-induced changes in intracellular oxidation/reduction status may use TR as one target to activate prosurvival pathways to scavenge the cytotoxic free radicals formed following exposure.

To initially address the candidacy of TR as a molecular target, cell lines overexpressing wild-type or cysteine mutant forms of TR were used. The mutant form of TR lacked critical cysteine residues that presumably are involved with the passage of electrons from NADPH to TRX, effectively inhibiting the ability of TR to reduce TRX. Experiments with H₂O₂ confirm and extend our previous results and show that TR is an upstream signaling factor involved in the activation of NFκB and AP-1 activity in response to the oxidative stress induced by H₂O₂ (Figs. 2 and 3). These results were reaffirmed using IV-2, which inhibited H₂O₂-induced activation of NFκB and AP-1 activity in response to H₂O₂ (Figs. 5 and 6). IV-2 also inhibited IR-induced activation of NFκB and AP-1 activity (ref. 25; additional data not shown). The results of these experiments clearly show that genetic or chemical inhibition of TR prevents the activation of prosurvival signaling pathways in response to at least one cytotoxic agent that induces oxidative stress.

Cell proliferation growth curves showed HeLa cells overexpressing the wild-type TR gene exhibited a shorter doubling time, whereas cells expressing a dominant negative TR exhibited an extended doubling time (Fig. 4). FACS analysis results suggested that the difference in growth rates might be because of regulation of the G₁ phase of the cell
cycle. When asynchronously cycling HeLa cells were exposed to IR–2, a total inhibition of cellular proliferation was observed at concentrations between 10 and 25 μmol/L (Fig. 7). Similar to the results obtained with HeLa cells that overexpress a dominant negative TR gene (Fig. 4), asynchronously cycling HeLa cells exposed to IR–2 accumulated in the G1 phase of the cell cycle (Fig. 7). The results of these experiments not only show that IR–2 is cytostatic in HeLa cells in culture but also suggest a potential role for TR in the regulation of the transition through the G1 phase of cell cycle.

To address the role of IR–2 as a potential adjuvant cytotoxic agent, clonogenic cell survival experiments were designed and showed that pretreatment of HeLa cells with IR–2 before IR and H2O2 significantly increased the tumor cell cytotoxicity of these agents (Fig. 9). It should be noted that H2O2 is not an ideal model of anticancer agents that induce oxidative stress. However, when these results are combined with those observed for IR, a potential adjuvant role for IR–2 is clearly suggested. The results of these experiments identify IR–2 as a cytotoxic and sensitizing agent in vitro and combined with the aforementioned results strongly suggest that TR may be a useful molecular target.

If TR is upstream of and regulates TRX, it also would seem logical that TRX also may be a potential molecular target. Interestingly, it also has been shown that tumor cells overexpress TRX, suggesting a possible role in how tumor cells evade the cellular mechanism preventing uncontrolled and continued proliferation (16, 20). In addition and similar to TR, the chemical or genetic inhibition of TRX results in a cell cycle arrest and cytotoxicity that is enhanced when combined with several anticancer agents (16). Although this work did not specifically examine TRX, the results suggest that TR and TRX may be potential molecular targets for anticancer agents and sensitizing agents. Although the present work identifies TR as a potential anticancer target in vitro, additional work is required, including cell-targeted expression, in vivo monitoring of gene expression activity, and therapeutic trials that target particular malignancies. However, these results represent an initial effort to identify TR as a potential target to enhance the cytotoxicity of agents that induce oxidative stress, including IR.

REFERENCES
Thioredoxin Reductase as a Potential Molecular Target for Anticancer Agents That Induce Oxidative Stress

DeeDee K. Smart, Karen L. Ortiz, David Mattson, et al.