2-[(1-Methylpropyl)dithio]-1*H*-imidazole inhibits tubulin polymerization through cysteine oxidation

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Abstract

2-[(1-Methylpropyl)dithio]-1H-imidazole (IV-2) is a known inhibitor of the thioredoxin system. It causes the oxidation of cysteine residues from both thioredoxin reductase and thioredoxin, with only the latter leading to irreversible inhibition of protein function. Although IV-2 is considered to be the first specific inhibitor of thioredoxin to undergo evaluation in cancer patients (under the name PX-12), it is unclear whether the oxidative ability of IV-2 is limited to proteins of the thioredoxin family. The current study investigated the specificity of IV-2 by examining its interaction with tubulin, a protein in which cysteine oxidation causes loss of polymerization competence. The cellular effects of IV-2 were examined in MCF-7 breast cancer and endothelial cells (human umbilical vein endothelial cells). Immunocytochemistry revealed a loss of microtubule structure with Western blot analysis confirming that treated cells contained a higher proportion of unpolymerized tubulin. Cell-free tubulin polymerization assays showed a dose-dependent inhibition of tubulin polymerization and depolymerization of preformed microtubules, confirming a direct interaction between IV-2 and tubulin. Further investigation of the tubulin interaction, through analysis of sulfhydryl reactivity and disulfide bond formation, suggested that IV-2 acts through the oxidation of cysteines in tubulin. Biochemical assays indicated that the oxidative properties of IV-2 are not limited to thioredoxin and tubulin, as cysteine-dependent proteases were also inhibited. Breast cancer cells with thioredoxin silenced by short interfering RNA remained sensitive to IV-2, albeit at higher antiproliferative GI_{50} values than in cells with normal thioredoxin function. These findings show that modulation of targets other than thioredoxin contribute to the effects of IV-2 on proliferating cells. [Mol Cancer Ther 2008;7(1):143–51]

Introduction

2-[(1-Methylpropyl)dithio]-1H-imidazole (IV-2), also known as PX-12, is an inhibitor of the thioredoxin/thioredoxin reductase system (1). This compound is the first specific thioredoxin-1 inhibitor to undergo clinical studies in cancer patients, having already completed phase I clinical trials (2), and having recently begun phase II trials in patients with advanced pancreatic carcinoma. The mechanism of action of IV-2, in terms of its inhibition of the thioredoxin system, has been well characterized. IV-2 contains a reactive disulfide bond and causes the inhibition of thioredoxin and thioredoxin reductase through the oxidation of cysteine residues in the catalytic sites of these enzymes. However, as these inhibitions are reversible, IV-2's main effects have been attributed to its ability to oxidize the noncatalytic site Cys^{73} of thioredoxin, giving a form of thioredoxin that cannot be reduced by thioredoxin reductase, resulting in an irreversible loss of function (1).

In vitro studies have shown that IV-2 causes growth inhibition in cancer cells, with a mean GI_{50} value of 6.5 μmol/L when tested in the NCI-60 cell line panel (3). Additional *in vitro* studies have shown that IV-2 causes irreversible arrest in the G_2-M phase of the cell cycle, accompanied by increased Cdk1 activity, decreased Cdk1 phosphorylation, and hyperphosphorylation of Bcl-2 (4). *In vivo*, IV-2 has been shown to affect angiogenesis, decreasing the levels of hypoxia-inducible factor 1α and vascular endothelial growth factor in MCF-7 xenographs (5), and causing a rapid decrease in tumor blood vessel permeability within 2 h of administration (6).

Although the effects of IV-2 have traditionally been attributed to thioredoxin inhibition, some evidence suggests that there may be other targets. Hyperphosphorylation of Bcl-2 and a rapid increase in vascular permeability could be attributed to downstream effects of thioredoxin inhibition; however, this profile is similar to that of microtubule-perturbing agents (7, 8).

The tubulin dimer contains 20 cysteine residues, 12 of which are on the α subunit and 8 of which are on the β subunit (9), and cysteine oxidation in tubulin causes loss of polymerization competence (10). Tubulin-interacting agents, such as paclitaxel, the *Vinca* alkaloids, and

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colchicine have gained great success in cancer chemotherapy (11), and recently, tubulin-oxidizing agents such as diallyl trisulfide have been shown to be effective cytotoxic agents (12).

In the course of our studies on the role of the thioredoxin system in angiogenesis and tumor vascularization, we have examined a number of relevant agents in terms of mode of action and specificity. Here, we report our findings with one such agent, IV-2, which, we found, also affects tubulin polymerization.

Materials and Methods

Tissue Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords using the collagenase perfusion technique (13), detailed in an earlier publication (14). Cells were used between passages 2 and 6 and were grown in a 1:1 mix of Ham's F12 medium (Sigma-Aldrich) and Medium 199 (Sigma-Aldrich), sterile water (Baxter) enriched with 20% iron-supplemented donor calf serum (PAA Laboratories), 1% HEPES solution (Sigma-Aldrich), 1% sodium bicarbonate (Sigma-Aldrich), 1% penicillin-streptomycin (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), 15,000 units of heparin (CP Pharmaceuticals Limited), 20 ng/mL of basic fibroblast growth factor, and 5 ng/mL of epidermal growth factor (Peprotech). For all experiments, HUVEC were grown on culture surfaces coated with 0.2% gelatin (Sigma-Aldrich). MCF-7 cells were obtained from American Type Culture Collection (Teddington), used between passages 15 and 25 and grown in MEM, enriched with 10% iron-supplemented donor calf serum (PAA Laboratories), 1% penicillin-streptomycin (Sigma-Aldrich), and 1% L-glutamine (Sigma-Aldrich).

IV-2 Synthesis

IV-2 was synthesized from 2-butanethiol and 2-mercaptoimidazole according to a previously published procedure (15).

Growth Assay

The proliferation status of cells was assessed after drug treatment. Cells (3×10^5) were plated in 25 cm^2 tissue culture flasks. Cells were allowed to attach overnight, and then exposed to drugs or vehicle alone for 72 h under normoxic conditions. Cell numbers were assessed by counting on a hemacytometer. All points were in duplicate and experiments were repeated at least twice. GI_{50} values were calculated as the concentration of drug necessary to cause a 50% reduction in cell growth as compared with vehicle-treated control cultures.

Clonogenic Survival Assay

Cell survival assays were used to measure reproductive integrity. These assays were done according the protocol of Liebmann et al. (16), as detailed in an earlier publication (14). Briefly, cells were treated with drugs for 72 h, as in the growth assays above. Cells were then washed, trypinized, counted and replated in drug-free medium to access reproductive integrity following drug exposure. Plates were incubated for 3 weeks for MCF-7 cells and 2 weeks for HUVEC. Colonies were fixed with methanol, stained with 1% crystal violet (Sigma-Aldrich), and counted. All survival points were in triplicate and experiments were repeated at least twice. LD_{50} values were calculated as the concentration of drug necessary to cause a 50% reduction in colony formation as compared with untreated controls.

Insulin Reduction Assay

Thioredoxin reductase/thioredoxin-dependent insulin reduction assays were conducted according to the protocol modified from Kunkel et al. (17), as used previously (18). Insulin-reducing activity was measured in an incubation with a final volume of 100 μL containing 100 mmol/L of Hepes buffer (pH 7.2), 5 mmol/L of EDTA, 1 mmol/L of NADPH, 0.4 μmol/L of thioredoxin reductase (Sigma-Aldrich), 0.32 μmol/L of thioredoxin (Merck Chemicals, Ltd.), 10 mg/mL of bovine insulin, and the indicated concentration of drug. Reactions were incubated at 37°C for 30 min and stopped by the addition of 150 μL of 6 mol/L guanidine HCl, 50 mmol/L of Tris (pH 8.0), and 10 mmol/L of DTNB [5,5′-dithio-bis(2-nitrobenzoic acid); Ellman's reagent]. Absorbance was read at 405 nm and experiments were repeated at least twice.

Immunocytochemistry

Cells were seeded at 1×10^4 cells per well in Permanox chamber slides (Fisher Scientific) in complete medium. They were allowed to attach overnight before being replaced with fresh medium containing the indicated concentration of drug before further incubation at 37°C under normoxic conditions. After 16 h of drug exposure, the medium was removed, cells were washed with PBS and fixed in methanol for 20 min at −20°C. Nonspecific binding was blocked with blocking buffer (5% bovine serum albumin, 0.1% Triton X-100) for 30 min at room temperature. Cells were incubated with a mouse anti-human β-tubulin (diluted 1:100 in blocking buffer; Chemicon Europe, Ltd.) for 1 h, washed thrice in PBS, and incubated with goat anti-mouse IgG/FITC (diluted 1:100 in blocking buffer; Dako U.K., Ltd.) for 1 h. Cells were again washed thrice with PBS, and then incubated with 0.2 μg/mL of propidium iodide (Sigma-Aldrich) for 2 min. Coverslips were mounted using fluorescence mounting medium (Dako U.K.). Experiments were repeated twice on both MCF-7 and endothelial cells.

Western Blotting for Soluble and Insoluble Tubulin Fractions

Cells were seeded in six-well plates at 3×10^5 cells per well in complete medium and allowed to attach overnight. This was replaced with fresh medium containing the indicated concentration of drug and incubated at 37°C under normoxic conditions. After 16 h of drug exposure, the medium was removed, cells washed with PBS, and soluble and insoluble tubulin fractions were collected as described previously (19, 20). To collect the soluble tubulin fraction, 500 μL of prewarmed lysis buffer [80 mmol/L Pipes-KOH (pH 6.8), 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.2% Triton X-100, 10% glycerol, 0.1% protease inhibitor cocktail (Sigma-Aldrich)] was gently added to the cells, without mixing, and incubated for 3 min at 30°C. Lysis

buffer was gently removed, and mixed with 500 μL of 2× Laemmli's sample buffer [125 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 10% SDS, 130 mmol/L DTT]. Samples were immediately heated to 95°C for 3 min. To collect the insoluble tubulin fraction, 1 mL of 1× Laemmli's sample buffer was added to each well, collected, and heated to 95°C for 3 min. Samples were either frozen at −20°C for up to 1 month or used immediately. Samples were run on an SDS-10% polyacrylamide gel (21), with 25 μL sample per lane, and transferred to a nitrocellulose membrane by a semidry transfer at 150 mA for 2 h. Blots were probed with mouse anti-human β-tubulin diluted 1:2,000 (Chemicon Europe) and horseradish peroxidase rabbit anti-mouse secondary antibody diluted 1:5,000 (Dako U.K.). Bands were analyzed with an enhanced chemiluminescence protocol (Amersham Plc) and visualized on radiographic film (Amersham).

Tubulin Polymerization Assays

A tubulin polymerization assay kit (Cytoskeleton, Inc.) was used according to the manufacturer's instructions. Briefly, the assay plate was prewarmed to 37°C prior to use; 5 μL of a 10× stock solution of drug was added to each well, followed by 45 μL of a tubulin reaction mix. Polymerization was followed by fluorescence enhancement due to the incorporation of a fluorescence reporter into microtubules as polymerization occurred. Readings were taken at an excitation wavelength of 350 nm and emission at 440 nm for 60 min at 1-min intervals. All data points were in triplicate and experiments were repeated at least twice.

For tubulin depolymerization assays, the above protocol was slightly modified. Forty-five microliters of the tubulin reaction mix was added to each well and readings were taken for 30 min, until the tubulin polymerization reached a steady-state equilibrium. Five microliters of the prewarmed 10× drug was then added to wells and fluorescence readings were resumed for a further 30 min. All data points were in triplicate.

Determination of Intermolecular and Intramolecular Disulfide Bonds by Electrophoresis

Disulfide bond formation in IV-2–treated tubulin was analyzed using the protocol described by Britto et al. (22). Briefly, 10 μmol/L of tubulin was reacted with varying concentrations of IV-2 or DTNB (as a positive control) in Mes buffer [0.1 mol/L Mes, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA (pH 6.9)] for 1 h at 25°C. Four micrograms of each sample was then separated by SDS-PAGE on NuPAGE 7% Tris-acetate mini-gels (Invitrogen Ltd.) under nonreducing conditions at 150 mA for 1 h. Bands were visualized using Coomassie blue staining.

Reaction of Tubulin-SH with IV-2 and DTNB

Cysteine oxidation of IV-2–treated tubulin was examined by titrating pretreated tubulin with DTNB. For the tubulin standard curve, 500 μmol/L of DTNB was reacted with varying tubulin concentrations (+4 mol/L of urea for some samples) in Mes buffer for 1 h at 25°C. For IV-2 prereactions, 2 μmol/L of tubulin was reacted with IV-2 in Mes buffer at 25°C for 1 h. Reactions were then dialyzed using 3,500 molecular weight cutoff Slide-A-Lyzer Dialysis Cassettes (Perbio Science U.K., Ltd.) against Mes buffer twice for 2 h at room temperature, followed by overnight dialysis at 4°C. These samples were then reacted with DTNB (final concentrations: 500 μmol/L DTNB, 1 μmol/L tubulin) for 1 h at 25°C. SH modifications of tubulin with DTNB were measured by following the solution turbidity at 412 nm.

Papain and Ficin Proteolysis Assays

In order to examine IV-2–mediated inhibition of cysteine proteases, papain and ficin activity assays were carried out according to a protocol modified from Guttmann et al. (23). All assays were carried out at 25°C with 9 units/mL of papain (Sigma-Aldrich) or 1 unit/mL of ficin (Sigma-Aldrich) in a buffer containing 50 mmol/L of Hepes (pH 7.5), 1 mmol/L of $CaCl_2$, and the indicated concentrations of IV-2 or H_2O_2. To initiate the reaction, the substrate Suc-Leu-Leu-Val-Tyr-AMC (Bachem U.K., Ltd.) was added at a final concentration of 0.117 mg/mL. Reactions were carried out in a 96-well plate and reaction kinetics were followed on a Fluostar Optima (BMG Labtech) with excitation at 380 nm and emission at 460 nm. All experiments were repeated at least twice.

Thioredoxin RNAi

MCF-7 cells (2×10^5) were seeded into each well of a six-well plate and left overnight. Short interfering RNA (siRNA) sequences (Applied Biosystems) were prediluted in nuclease-free water to a working solution of 2 μmol/L and then further diluted in RPMI 1640 (Sigma-Aldrich) to obtain the final concentrations desired in a final volume of 200 μL (tube 1). Two microliters of the DharmaFECT lipid transfection reagent (Fisher Scientific) were added to 198 μL of RPMI 1640 and incubated, alongside tube 1, for 5 min at room temperature, then combined with tube 1 and incubated for an additional 20 min. Reaction mixtures were then added to 1.6 mL of MCF-7 medium without penicillin-streptomycin. Medium was aspirated from wells and replaced with the appropriate 2 mL of transfection solution. Cells were transfected with either 25 nmol/L of thioredoxin siRNA or mock-transfected with a scrambled siRNA to control for nonspecific siRNA-related effects. Transfection medium was removed after 24 h, replaced by fresh medium and incubated for an additional 48 h. Transfected cells were then plated for growth curve assays, as above. Western blot analyses of thioredoxin and β-actin (as a loading control) were used to confirm that thioredoxin expression was suppressed for the duration of the experiment.

Results

IV-2 Is Cytotoxic to MCF-7 Breast Cancer Cells and HUVECs and Causes Inhibition of the Thioredoxin System

MCF-7 cells and HUVEC were exposed to IV-2 for 72 h under normoxic conditions and evaluated for changes in proliferation and clonogenicity. Growth assays show that both cell types are equally sensitive to IV-2, with a GI_{50}

dose of ~5 to 10 μmol/L. Clonogenic assays suggest that the effects of IV-2 are predominantly cytotoxic, with an LD_{50} of ~15 μmol/L in both cell types. Insulin reduction assays confirm that IV-2 is capable of inhibiting the thioredoxin system with an IC_{50} of ~20 μmol/L. This value is slightly higher than previously reported (3); however, this is most likely attributable to variations in thioredoxin and thioredoxin reductase enzyme activity levels, not to differences in drug potency.

Cells Treated with IV-2 Show Loss of Microtubule Organization

In order to assess the effects of IV-2 on microtubule integrity, we examined the microtubule organization in both MCF-7 cells and HUVEC treated for 16 h with IV-2 as compared to treatments with known microtubule-perturbing agents. Immunocytochemical analysis of cells treated with vehicle alone reveals a clear and organized microtubule distribution (Fig. 1A.1 and B.1). At 10 μmol/L concentrations of IV-2, HUVECs show a decrease in intact microtubules, with the majority of tubulin in the cells appearing dispersed and unpolymerized (Fig. 1B.2), whereas MCF-7 cells seem to be less sensitive to the drug, displaying a normal microtubule organization at this dose (Fig. 1A.2). At 50 μmol/L of IV-2, both MCF-7 and HUVEC exhibit the same pattern of diffuse tubulin staining, with a distinct lack of microtubules (Fig. 1A.3 and B.3). Colcemid and vinblastine, at concentrations of 1 μmol/L, show a similar pattern of microtubule disruption in both cell types, whereas paclitaxel shows the expected pattern of condensed microtubule organization (Fig. 1A.4–6 and B.4–6).

Western blot analysis of free versus polymerized tubulin confirms the immunocytochemistry results. In untreated HUVEC, there is a relatively even distribution of tubulin, with slightly more present in the unpolymerized fraction (Fig. 2). In cells treated with colcemid, a tubulin-disrupting agent, there is a distinct shift in the tubulin balance, with almost all tubulin present in the unpolymerized fraction. Cells treated with paclitaxel, a microtubule stabilizer, display the reverse proportion, with more

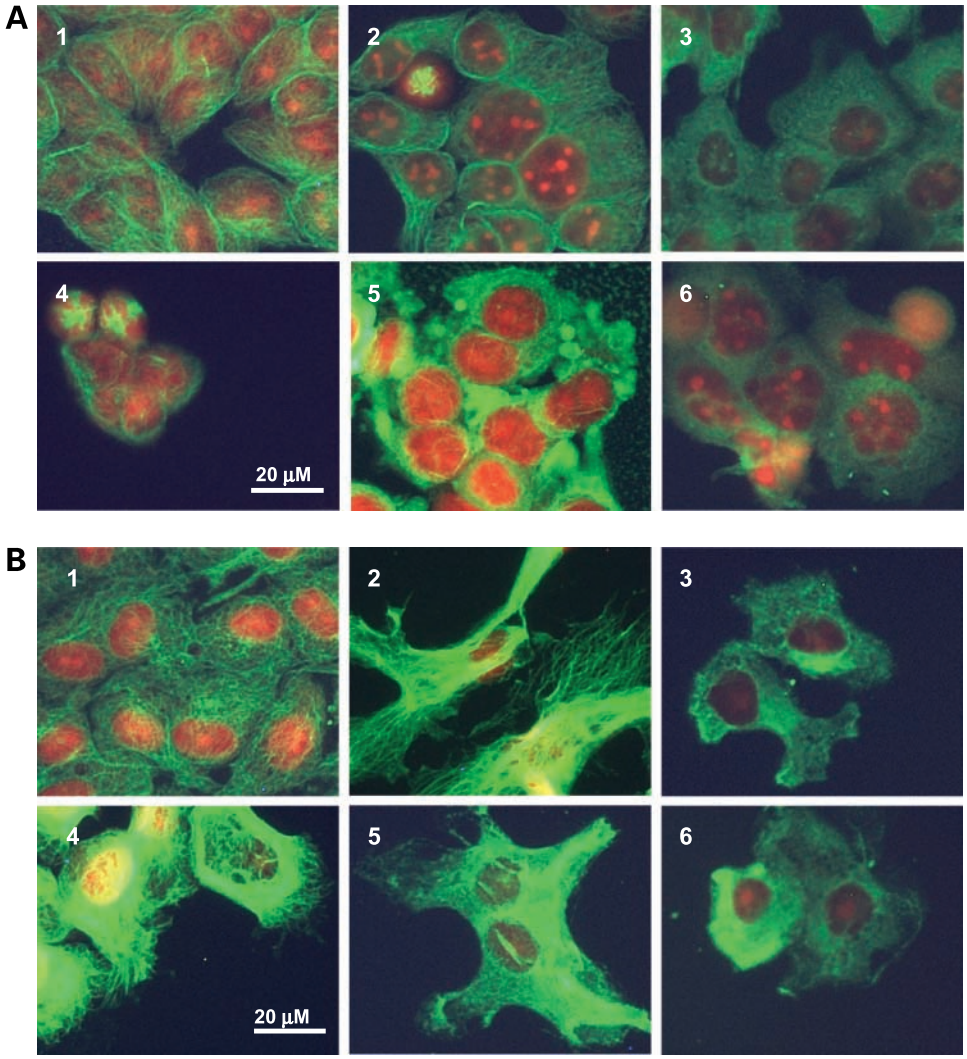

Figure 1. Effects of IV-2 on MCF-7 and HUVEC microtubule networks. MCF-7 cells (**A**) or HUVEC (**B**) were treated for 16 h with vehicle (0.5% DMSO; *1*), 10 μmol/L of IV-2 (*2*), 50 μmol/L of IV-2 (*3*), 1 μmol/L of taxol (*4*), 1 μmol/L of vinblastine (*5*), or 1 μmol/L of colcemid (*6*). Cells were fixed and stained with anti-human β-tubulin (*green*) and nuclei were counterstained with propidium iodine (*red*). Original magnification, ×400.

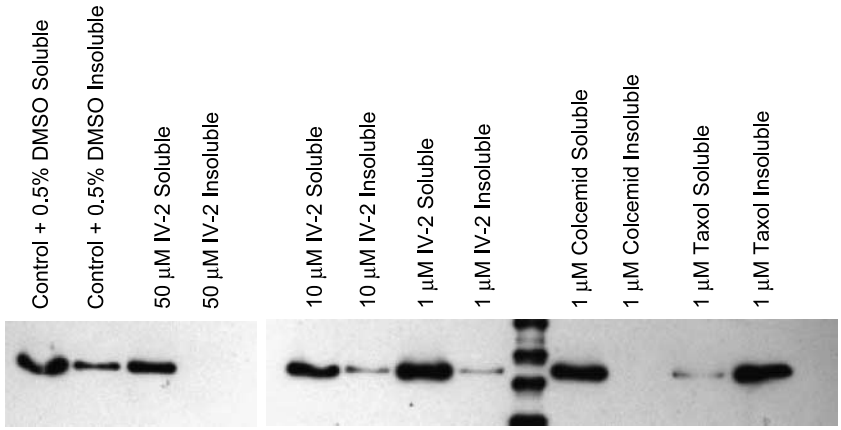

Figure 2. Western blot analysis of free versus polymerized tubulin in IV-2–treated HUVEC. HUVEC were treated with the indicated concentrations of drugs for 16 h. The fractions containing soluble and insoluble tubulin were collected and separated by SDS-PAGE. Tubulin was detected by Western blot analysis.

tubulin present in the polymerized fraction. IV-2–treated cells show a dose-dependent increase in unpolymerized tubulin, suggesting that IV-2 is preventing tubulin polymerization and/or destabilizing existing microtubules. The same pattern is seen in MCF-7 cells (data not shown).

IV-2 Causes Loss of Tubulin Polymerization Competence

In order to assess the direct interaction between tubulin and IV-2, a cell-free tubulin polymerization assay was used. Tubulin polymerization was followed by the incorporation of a fluorescent marker into microtubules. Tubulin allowed to polymerize under control conditions showed a pattern of nucleation, growth, and eventual steady-state equilibrium (Fig. 3A). In the presence of paclitaxel, the nucleation period is vastly decreased, the growth rate faster, and the final polymer mass greater. In the case of microtubule-disrupting agents, colcemid and vinblastine, there is a decrease in the growth rate and a reduction in the final polymer mass. IV-2 causes a similar dose-dependent decrease in reaction rate and polymer mass, indicating a direct interaction between IV-2 and tubulin (Fig. 3B). The addition of IV-2 to microtubules that have already reached equilibrium causes a rapid, dose-dependent depolymerization of microtubules (Fig. 3C and D).

IV-2 Causes the Oxidation of Cysteine Residues in Tubulin

In order to examine the mechanism by which IV-2 inhibits tubulin polymerization, we used nonreducing SDS-PAGE to detect the formation of intermolecular and intramolecular tubulin disulfide bonds. Under control conditions, tubulin monomers appear on the gel as a single band of ~55 kDa. Oxidation of tubulin cysteines by IV-2 would be expected to cause the initial formation of reactive disulfide intermediates, available for further attack by another thiolate, and therefore, the subsequent formation of intermolecular and intramolecular disulfide bonds through disulfide exchange (Fig. 4A). Our results show that in the presence of IV-2, tubulin forms intermolecular bonds in a concentration-dependent manner, as indicated by the tubulin dimers and trimers (Fig. 4B). Additionally, tubulin forms intramolecular disulfide bonds, as indicated by the monomeric shift. A similar pattern is observed when tubulin is treated with the oxidizing agent DTNB.

As the formation of intermolecular and intramolecular bonds in tubulin reacted with tubulin indicate that cysteine oxidation is occurring, we examined the reaction of a known oxidizing agent, Dithio-bis(2-nitrobenzoic acid) (DTNB), with tubulin. The reaction of DTNB with tubulin causes the release of TNB, the concentration of which can be measured at an absorbance of 412 nm (Fig. 4C). When tubulin was reacted with DTNB, in the presence of urea, all 20 cysteine equivalents were available for DTNB oxidation, whereas in the absence of urea, only 19 cysteines were available (Fig. 4D). In the cases in which tubulin was prereacted with IV-2, only two cysteine equivalents were able to subsequently react with DTNB, suggesting that IV-2 is a strong oxidizing agent. In the case of colcemid, a microtubule-disrupting agent which directly binds tubulin, 16 cysteine equivalents reacted with DTNB, most likely due to a steric inhibition by the bound drug, as previously observed (24).

IV-2 Inhibits the Activity of the Cysteine Proteases Papain and Ficin

Although the main mechanism of action of IV-2 is attributed to irreversible oxidation of Cys^{73} on thioredoxin, IV-2 is also known to reversibly oxidize cysteine residues in the active sites of both thioredoxin and thioredoxin reductase. This information, combined with results showing that IV-2 causes the oxidation of tubulin cysteines, lead us to hypothesize that IV-2 might be a more promiscuous oxidizing agent than hitherto shown. We selected two cysteine proteases, papain and ficin, the functions of which are dependent on having reducing cysteines in the active site. The protease activity was measured by the cleavage of a substrate to produce a fluorescent product. Our results show that IV-2 is able to cause a dose-dependent inhibition of protease activity for both papain and ficin (Fig. 5A and B). In both cases, this inhibition is equal to or greater than the inhibition caused by the same concentration of hydrogen peroxide, a potent oxidizing agent. The cysteine protease inhibitor E64, the inhibition of which is not due to oxidation, was also tested in the system and resulted in the inhibition of protease activity (data not shown).

Thioredoxin siRNA Knockdown MCF-7 Cells Are Sensitive to IV-2

In order to assess the extent to which inhibition of the thioredoxin system is responsible for the cytotoxic effects of IV-2, siRNA was used to knock down the expression of thioredoxin in MCF-7 cells. These MCF-7 cells were treated with IV-2 as in the standard growth curve assays discussed previously. Thioredoxin knockdown cells were viable, but displayed a 50% to 60% decrease in the rate of proliferation. Western blot analysis showed that thioredoxin expression was below the level of detection for the duration of the drug treatment (Fig. 6A). The GI_{50} of IV-2 on the siRNA-treated cells was ~50 μmol/L, as compared with the GI_{50} of 5 to 10 μmol/L for normal MCF-7 cells (Fig. 6B). This 5- to 10-fold increase indicates that although the thioredoxin system is an important target of IV-2, other systems are also affected by the drug.

Discussion

IV-2 has made a successful progression through preclinical testing to its current phase II clinical trials. The phase I clinical trial on 38 patients with advanced solid tumors showed that PX-12 was well tolerated at doses up to 226 mg/m^2, and that high-grade side effects were uncommon. The best response achieved was stable disease in seven patients. The study showed a dose-dependent decrease in plasma levels of thioredoxin, presumably attributable to the removal of functionally inactivated protein from circulation. Intact PX-12 was undetectable in patient plasma at all drug doses, but the metabolite, 2-MI, was detectable within 15 min from the start of drug infusion (2, 25). Plasma levels of 2-MI reached ~80 μmol/L for the highest drug dosage (300 mg/m^2) and ~50 μmol/L at the reported maximum tolerated dose (226 mg/m^2). Furthermore, decreases in secreted thioredoxin and vascular endothelial growth factor levels in plasma from cancer patients treated with PX-12 have been reported (26).

Previous studies have clearly established the ability of IV-2 to reversibly inhibit the catalytic sites of thioredoxin and thioredoxin reductase; and most importantly, to irreversibly inhibit thioredoxin through oxidation of Cys^{73} (1, 3). Early experiments suggested that IV-2 was a specific oxidizing agent due to its inability to inhibit glutathione reductase (1). Subsequent *in vitro* and *in vivo* studies did not directly examine the specificity of IV-2, but results hinted that perhaps the mechanism of action of IV-2 was more complex. One study using MCF-7 cells showed that IV-2 induces a G_2-M block and hyperphosphorylation of the antiapoptotic protein Bcl-2 (4), both of which are known

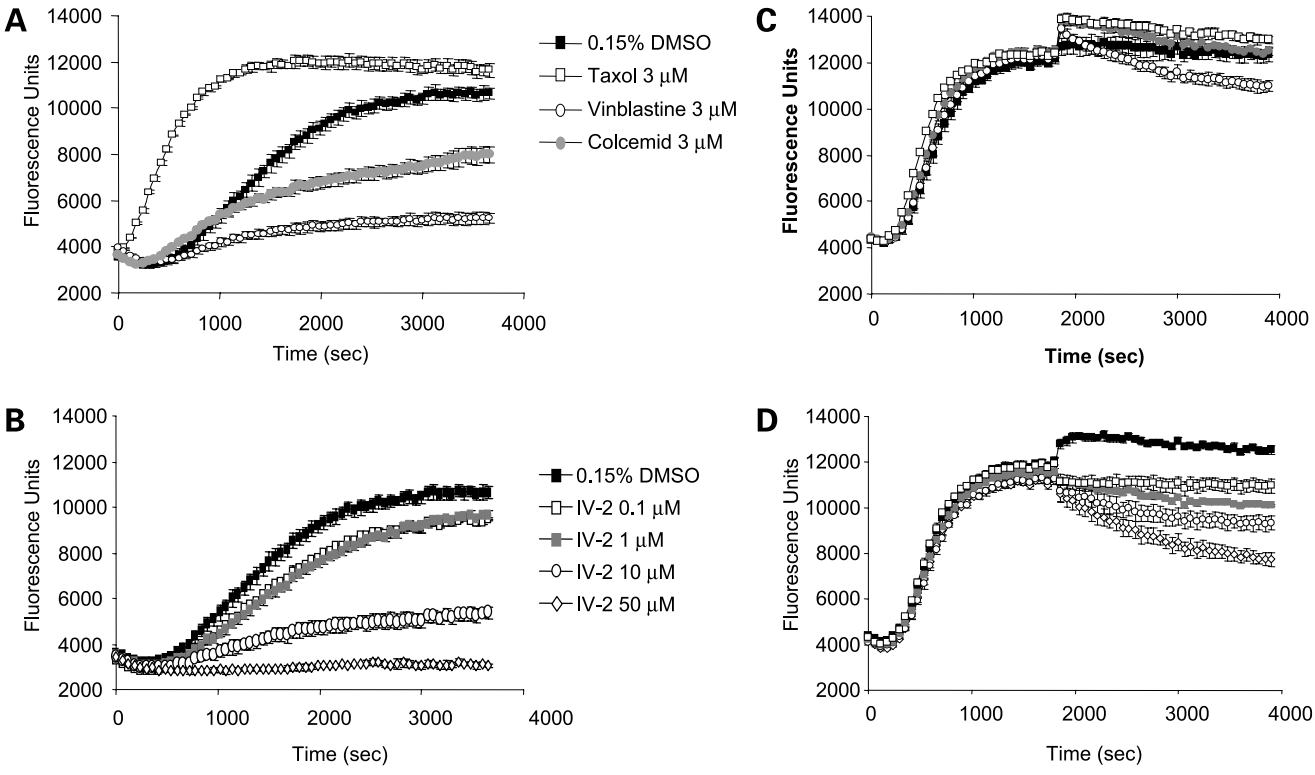

Figure 3. Inhibition of tubulin polymerization by IV-2. Polymerization of purified tubulin was followed by fluorescence enhancement due to incorporation of a fluorescence reporter into microtubules as polymerization occurred. Readings were taken at an excitation wavelength of 350 nm and emission at 440 nm for 60 min at 1-min intervals. Polymerization was followed in the presence of control drug (**A**) or IV-2 (**B**). Microtubules preformed in the absence of the drug were treated with control drugs (**C**) or IV-2 (**D**). Experiments were repeated independently; results of one representative experiment done in triplicate. *Points,* mean; *bars,* SE.

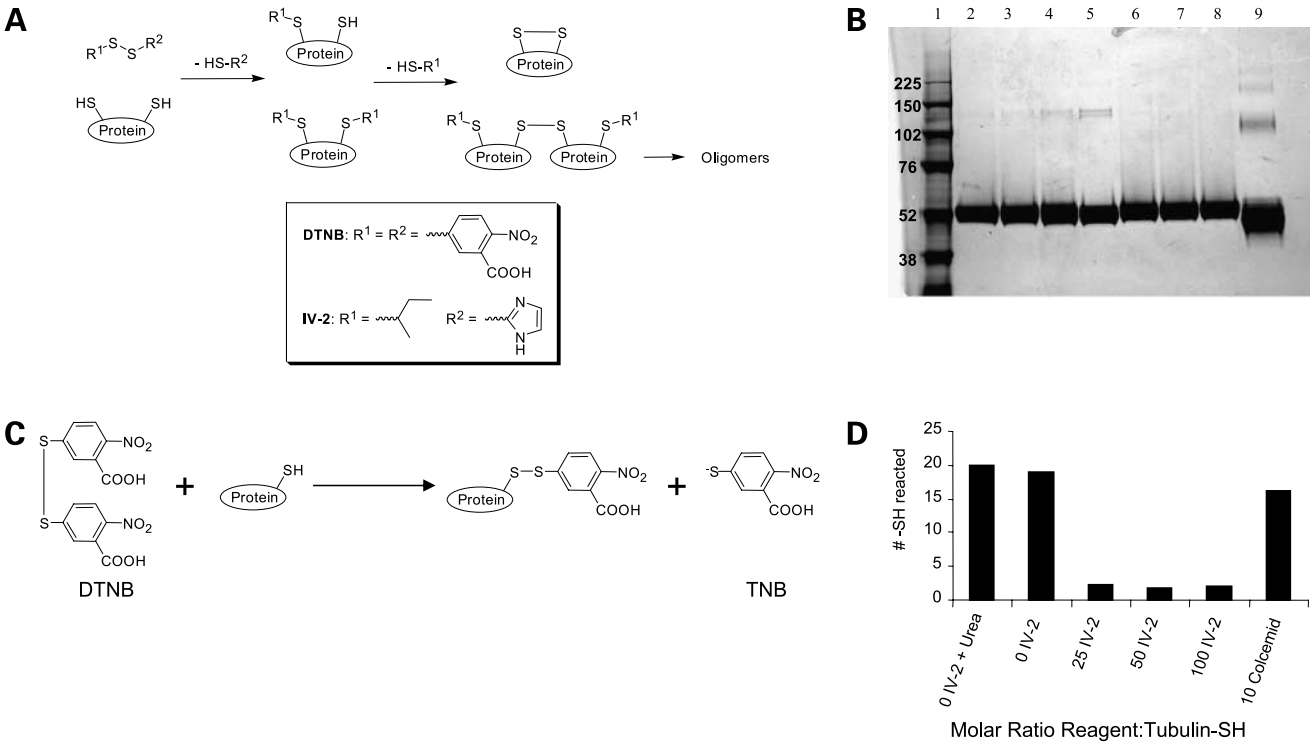

Figure 4. Effects of IV-2 on the oxidation of tubulin cysteines. **A,** proposed reaction of tubulin with IV-2 and DNTB resulting in the formation of intermolecular and intramolecular disulfide bonds. **B,** Coomassie blue staining of tubulin (10 μmol/L = 200 μmol/L SH) reacted with IV-2 at reagent/SH molar ratios of 0.2, 0.3, 0.5, and 20 in lanes 2 to 5, respectively. Lanes 6 to 8 contain controls with 0%, 0.004%, 0.4% DMSO as vehicle controls. Lane 9 contains tubulin treated with DTNB at a reagent/SH molar ratio of 0.5. All reactions were carried out in Mes buffer (pH 6.5) at 25°C for 60 min and separated under nonreducing conditions. Molecular weight standards are shown in kDa (*lane 1*). **C,** reaction of DTNB with tubulin cysteines causes the release of TNB the concentration of which can be measured at an absorbance of 412 nm. **D,** tubulin prereacted with IV-2 or colcemid in Mes buffer for 1 h at 25°C was dialyzed overnight and reacted with DTNB (final concentrations: 500 μmol/L DTNB, 1 μmol/L tubulin) for 1 h at 25°C. SH modifications of tubulin with DTNB were measured by A_{412} readings.

effects of microtubule-perturbing agents such as vinblastine, colcemid, or taxanes (8). Our own results have confirmed that IV-2 causes a strong G_2-M block in treated cells (data not shown). Additional *in vivo* studies have shown that IV-2 causes a rapid decrease in tumor vascular permeability within 2 h of administration (6); an effect similar to that seen with the vascular targeting agent combretestatin A-4 (7). Additionally, data coming from the most recent clinical testing provides compelling evidence to suggest that IV-2 has effects other than the oxidation of thioredoxin. The rapid metabolism of PX-12 observed in the phase I trial cannot be explained by the interaction of the drug with thioredoxin alone, and is most consistent with thiol-disulfide exchange with reduced cysteine residues present on plasma proteins.

In order to examine the specificity of IV-2, we first sought to confirm the *in vitro* activity reported previously. We initially examined the effects of IV-2 on MCF-7 breast cancer cells and HUVEC. The GI_{50} dose for MCF-7 and HUVEC treated with IV-2 for 72 h is between 5 and 10 μmol/L. This is consistent with the results from the screening of the compound against the NCI panel of 60 cancer cell lines, which showed a mean GI_{50} dose of 6.5 μmol/L (3). Clonogenic results indicate that the drug acts via a combination of cytotoxic and cytostatic mechanisms, with an LD_{50} of ~15 μmol/L in both cell lines. Additionally, insulin reduction assays confirmed that IV-2 inhibits the function of thioredoxin, in a cell-free system, with an IC_{50} of ~20 μmol/L. It should be noted that these *in vitro* potency values seem relevant to the clinically observed metabolite plasma level measurements. As PX-12 is safely administered and well tolerated at very high doses, midmicromolar physiologic IV-2 levels seem feasible.

By analyzing the tubulin distribution within IV-2–treated cells, our results indicate that the drug is acting as a microtubule-disrupting agent. Although this disruption could be a downstream effect of thioredoxin inhibition (27), additional assays looking at tubulin polymerization in a cell-free system confirmed our hypothesis that IV-2 is capable of causing thioredoxin-independent disruption of microtubules by both inhibiting tubulin polymerization and causing depolymerization of existing microtubules. As IV-2 has already been shown to function through the oxidation of cysteines on thioredoxin and thioredoxin reductase, it is not surprising that our results show that the inhibition of tubulin polymerization is also a result of IV-2–mediated oxidation of cysteine residues.

Many proteins contain cysteines which are critical for their function. Our results show that IV-2 causes an inhibition of the cysteine proteases, papain and ficin, confirming a broader range of drug activity than originally thought. The observed cellular disruption of microtubules is likely the combined result of oxidation of multiple proteins. Current results show that IV-2 could directly inhibit microtubules via the oxidation of tubulin, but in a cellular system, microtubule organization is additionally dependent on a number of microtubule-associated proteins. Oxidation of cysteines in microtubule-associated proteins, specifically microtubule-associated protein 2 and tau, has been shown to cause a reduction in their ability to promote microtubule assembly. Interestingly, the function of oxidized microtubule-associated proteins and tubulin can be restored through reduction by the thioredoxin system (27, 28). If IV-2 is causing the oxidation of thioredoxin, tubulin, and microtubule-associated proteins then the effects on microtubule organization in cells would be greatly enhanced.

Microtubule disruption has become an important target for cancer therapy. Agents such as the *Vinca* alkaloids, colchicine, and most recently, the combretastatins have been shown to be effective antivascular agents (29). The microtubule-disrupting properties of IV-2 make it a promising vascular targeting agent. Our results show that HUVEC are more sensitive to the microtubule-disrupting effects of IV-2, with disorganization of microtubules

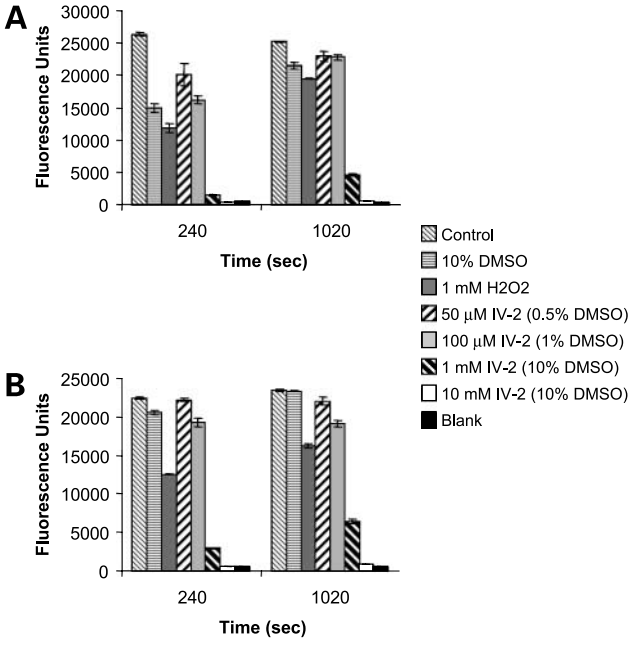

Figure 5. Effects of IV-2 on cysteine protease function. Papain (**A**) and ficin (**B**). Suc-Leu-Leu-Val-Tyr-AMC was digested at 25°C with 9.0 units/mL of papain (Sigma-Aldrich) or 1 unit/mL of ficin (Sigma-Aldrich) in a buffer containing 50 mmol/L of Hepes (pH 7.5), 1 mmol/L of $CaCl_2$, and the indicated concentrations of IV-2 or H_2O_2. Reaction kinetics were followed at an excitation of 380 nm and emission of 460 nm. *Columns,* mean; *bars,* SE (all data points are in duplicate).

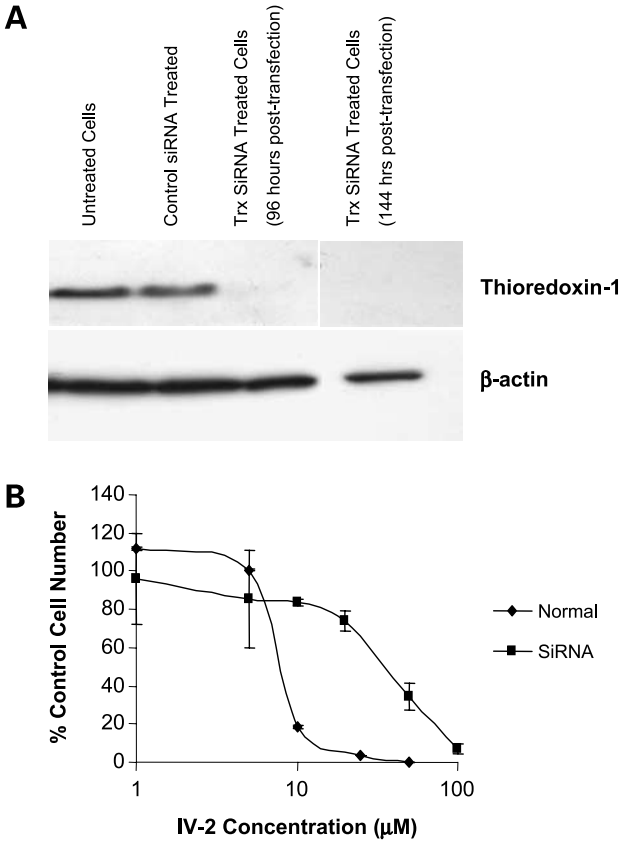

Figure 6. Effects of thioredoxin silencing on the proliferation of IV-2–treated cells. **A,** Western blot analysis of thioredoxin and β-actin (as a loading control) expression in untreated MCF-7 cells, cells transfected with 25 nmol/L of control siRNA, or with 25 nmol/L of thioredoxin siRNA: *96 h posttransfection,* time of drug addition; *144 h posttransfection,* the end point of the growth curve experiment. **B,** MCF-7 cells were transfected with thioredoxin siRNA and exposed to varying concentrations of IV-2, 96 h posttransfection, for 72 h. Cells were counted and proliferation values were expressed as a percentage of control cell numbers.

apparent at doses of 10 μmol/L within 16 h after drug exposure. A previous *in vivo* study using dynamic contrast-enhanced magnetic resonance imaging showed that IV-2 causes a rapid 63% decrease in tumor blood vessel permeability, accompanied by a decrease in plasma vascular endothelial growth factor levels, indicating the potential of IV-2 as an antivascular agent (6). Preliminary, unpublished, data from our studies investigating the antivascular effects of thioredoxin inhibitors show that IV-2 causes a 40% to 50% decrease in blood flow in dorsal xenograph tumors, with intravital microscopy showing vascular disruption.

The importance of these findings, in terms of the mechanism of action of IV-2, is further confirmed by the fact that cells which lack thioredoxin expression are still sensitive to IV-2, albeit with a greater GI_{50}. It seems that the oxidative abilities of IV-2 are not limited to the cysteines present on thioredoxin, thioredoxin reductase, or tubulin. Although the nonspecific activity of IV-2 does not change

the usefulness of the drug, as indicated by the early clinical trials data, current findings suggest that the drug may have a broader range of activity and applicability than originally contemplated (30).

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