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Original Contribution

Enhanced targeting of mitochondrial peroxide defense by the combined use of thiosemicarbazones and inhibitors of thioredoxin reductase

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ABSTRACT

Peroxiredoxin-3 (Prx3) accounts for about 90% of mitochondrial peroxidase activity, and its marked upregulation in many cancers is important for cell survival. Prx3 oxidation can critically alter peroxide signaling and defense and can be a seminal event in promoting cell death. Here it is shown that this mechanism can be exploited pharmacologically by combinations of clinically available drugs that compromise Prx3 function in different ways. Clinically relevant levels of the thiosemicarbazone iron chelators triapine (Tp) and 2,2′-Dipyridyl-*N*,*N*-dimethylsemicarbazone (Dp44mT) promote selective oxidation of mitochondrial Prx3, but not cytosolic Prx1, in multiple human lung and ovarian cancer lines. Decreased cell survival closely correlates with Prx3 oxidation. However, Prx3 oxidation is not merely an indicator of cell death as cytotoxic concentrations of cisplatin do not cause Prx3 oxidation. The siRNA-mediated suppression of either Prx3 or thioredoxin-2, which supports Prx3, enhances Tp's cytotoxicity. Tp-mediated Prx3 oxidation is driven by enhanced peroxide generation, but not by nitric oxide. Many tumors overexpress thioredoxin reductase (TrxR) which supports Prx activity. Direct inhibitors of TrxR (e.g. auranofin, cisplatin) markedly enhanced Tp's cytotoxicity, and auranofin enhanced Prx3 oxidation by low dose Tp. Together, these results support an important role for Prx3 oxidation in the cytotoxicity of Tp, and demonstrate that TrxR inhibitors can significantly enhance Tp's cytotoxicity. Thiosemicarbazone-based regimens could prove effective for targeting Prx3 in a variety of cancers.

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1. Introduction

Peroxiredoxins (Prxs) are a ubiquitous family of highly abundant peroxidases that have important roles in peroxide defense and redox signaling [1–3]. In contrast to other peroxidases, cysteine (Cys) is essential to their active site [3]. Most Prx isoforms show high affinity and reactivity with hydrogen peroxide (H_2O_2) [3–6]. Prx isoforms are differentially localized to the cytosol (Prx1, Prx2, Prx6), endoplasmic reticulum (Prx4), or mitochondria (Prx3, Prx5). Prx5 has also been detected in peroxisomes, the cytosol, and the nucleus.

Mitochondria are a major site for cellular reactive oxygen species (ROS) generation including H_2O_2 [7]. Cancer cells typically generate elevated levels of ROS, and they are often significantly more susceptible than normal cells to agents that promote additional ROS [8–12]. To enhance defense against ROS, many cancers (incl. lung, ovarian, cervical, endometrial, liver, breast, and prostate) overexpress Prx3 relative to normal tissues [13–23]. Prx3 accounts for ~90% of total mitochondrial peroxidase activity [3]. Elevated levels of Prx3 are associated with enhanced cell/tumor proliferation [13,19,21,22] and with enhanced cell survival to several insults including H_2O_2, *t*-buytlhydroperoxide, hypoxia, platinum antitumor drugs, staurosporine, tumor necrosis factor-α, and antiandrogen drugs (e.g. bicalutamide) [15,17,18,24,25]. Conversely, the siRNA-mediated suppression of Prx3 results in elevated cellular H_2O_2 and renders cancer cells more sensitive to H_2O_2, and to agents that promote mitochondrial ROS (e.g. staurosporine and tumor necrosis factor-α) [17,18,25].

Prx3 oxidation compromises mitochondrial peroxide defense

Abbreviations: ANF, auranofin; cDDP, cisplatin (*cis*-diamminedichloroplatinum (II)); Cys, cysteine; DMSO, dimethylsulfoxide; DMEM, Dulbecco's Modified Eagle's Medium; Dp44mT, 2,2′-Dipyridyl-*N*,*N*-dimethylsemicarbazone; FBS, fetal bovine serum; Fe, iron; GPx, glutathione peroxidase; HBSS, Hank's balanced salt solution; L-NAME, L-N^G-Nitroarginine methyl ester hydrochloride; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; Prxs, peroxiredoxins; Prx1, peroxiredoxin-1 (cytosolic); Prx3, peroxiredoxin-3 (mitochondrial); Prx4, peroxiredoxin-4; Prx5, peroxiredoxin-5; ROS, reactive oxygen species; SeCys, selenocysteine; Tp, triapine; Trx1, thioredoxin-1; Trx2, thioredoxin-2; TrxR1, thioredoxin reductase-1 (cytosolic); TrxR2, thioredoxin reductase-2 (mitochondrial)

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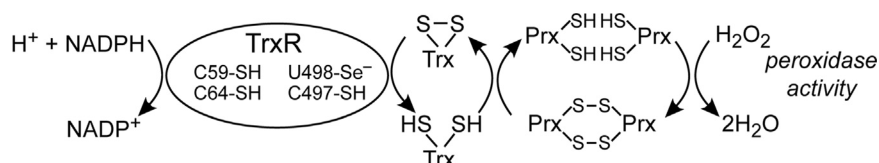

Fig. 1. TrxR and Trx reduce oxidized Prx, which is essential for Prx activity. Essential redox centers within TrxR include an FAD (not shown), a C59/C64 dithiol, and C497/U498 (U is selenocysteine, SeCys). There are distinct cytosolic (TrxR1, Trx1, Prx1, Prx2) and mitochondrial isoforms (TrxR2, Trx2, Prx3) of these proteins which are not in redox equilibrium with each other [86,87].

and can trigger cancer cell death [21,26,27], indicating that this mechanism could be developed as an antitumor strategy. While selective inhibitors of Prx3 activity are lacking, some chemicals have been shown to promote Prx3 oxidation. For example, phenethyl isothiocyanate causes the oxidation of Prx3, but not cytosolic Prx1 or Prx2, in Jurkat lymphoma cells, and only isothiocyanates that promote Prx3 oxidation induce cell death [26].

Many cancers also overexpress thioredoxin reductase (TrxR) [23,28–30] which provides reducing equivalents for Prxs (Fig. 1). TrxR inhibitors therefore have the potential to facilitate Prx oxidation by blocking reduction of oxidized Prx. By itself, auranofin (a gold-based inhibitor of TrxR) can cause Prx3 oxidation in A549 cells [27]. In other cells, the normal redox state of cytosolic Trx1 is maintained even when TrxR activity is inhibited by ~90% with aurothioglucose or with an siRNA to TrxR1 [31]. However, when combined with a pro-oxidant insult such as monomethylarsonous acid, TrxR inhibition or TrxR1 suppression can quickly lead to Trx1 oxidation [31]. Consistent with this idea, 2,4-dinitrochlorobenzene promotes Prx3 oxidation in Jurkat lymphoma cells [27], which likely reflects its dual ability to irreversibly inhibit TrxR and to be efficiently redox cycled to generate superoxide [27,32,33]. Although 2,4-dinitrochlorobenzene is a non-clinical agent, these findings suggest that simultaneously inhibiting TrxR and promoting mitochondrial ROS generation could be a highly effective approach to target Prx3 in cancer cells. While some TrxR inhibitors are clinically available (e.g. cisplatin (cDDP) and auranofin (ANF)), there is limited insight into existing antitumor drugs that specifically promote Prx3 oxidation.

In a preliminary study, we noted that the anti-tumor drug triapine (Tp) (3-aminopyridine-2-carboxaldehyde thiosemicarbazone) can promote Prx3 oxidation in lung cancer cells [34]. Tp (which has been included in multiple NCI-sponsored clinical trials) and other thiosemicarbazones such as Dp44mT (2,2′-Dipyridyl-N,N-dimethylsemicarbazone) are tridentate iron (Fe) chelators with pronounced activity against a variety of metastatic cancers [35–41]. While thiosemicarbazones have been associated with inhibition of ribonucleotide reductase in cells [35,41–43]. $Fe(Tp)_2$ and Fe $(Dp44mT)_2$ can also be redox-cycled to generate ROS including superoxide, H_2O_2, and hydroxyl radical [35,41,42,44–46]. There is growing evidence that this redox cycling is important for their antitumor effects [38,41,47]. The ability of catalase to protect human carcinoma cells from Tp [42] implies an important role for peroxide in particular. Furthermore, the high Fe content of many tumors [12,48–51] could promote enhanced ROS generation from the redox cycling of Fe-thiosemicarbazones [35]. This is consistent with the greater sensitivity of tumor cells to Tp relative to non-tumor cells [40,41,43].

The primary goals of the studies reported here were to determine: (1) the specificity of thiosemicarbazones for mitochondrial vs. cytosolic Prxs; (2) the importance of Prx3 oxidation for Tp cytotoxicity; and (3) the potential for clinically available TrxR inhibitors to enhance Tp's cytotoxicity. We report that: (a) concentrations of Tp that are typical for human doses promote robust Prx3 oxidation in multiple cancer lines; (b) Tp does not promote the oxidation of cytosolic Prx1; (c) another thiosemicarbazone (Dp44mT) has similar Prx3-specific effects; (d) Prx3 oxidation is important for Tp cytotoxicity; (d) Prx3 protects cells from Tp; (f) clinically available TrxR inhibitors enhance the cytotoxicity of Tp; and (g) Tp-induced Prx3 oxidation is mediated by peroxide but not by nitric oxide.

2. Materials and methods

2.1. Reagents

Tp (kindly provided by Vion Pharmaceuticals) and Dp44mT (Calbiochem, La Jolla, CA) stock solutions were prepared in 95% dimethylsulfoxide (DMSO; cell culture grade) approximately 30 min prior to use. Stock solutions of ANF (Alexis Biochemicals) were prepared in 95% DMSO, whereas cDDP (Santa Cruz Biotechnology, Dallas, TX) was prepared 20–30 min before use in 0.9% saline and was filter-sterilized. The structures of these four compounds are shown in Fig. 2. L-NAME (L-N^G-Nitroarginine methyl ester hydrochloride) (Santa Cruz Biotech.) was dissolved in deionized water and filter-sterilized just before use. Bovine liver catalase was from Calbiochem (cat. no. 219001). Phenylmethylsulfonyl fluoride (PMSF) and Tris were obtained from Research Organics (Cleveland, OH). EDTA was obtained from Fisher Scientific (Hampton, NH). DMSO and all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

The following were purchased from Invitrogen (Carlsbad, CA): Hank's balanced salt solution (HBSS, cat. no. 14025-092), pre-cast electrophoresis gels (NuPAGE 10% Bis-Tris) and matching electrophoresis and loading buffers.

The following primary antibodies were obtained from Santa Cruz Biotechnology: Prx1 (sc-7381, goat polyclonal), Prx3 (sc-59661, mouse monoclonal 12B), Prx5 (sc-130337, mouse monoclonal), Prx4 (sc-23974, goat polyclonal), Prx2 (sc-23967, goat polyclonal), Trx2 (sc-50336, rabbit polyclonal), and GPx-1/2 (glutathione peroxidase-1/2, sc-133160, mouse monoclonal). The following primary antibodies were obtained from Abcam (Cambridge, MA): Prx3 (ab16751, mouse monoclonal 12B), GAPDH (ab9485, rabbit polyclonal), and Trx1 (ab16835, rabbit polyclonal). Secondary antibodies were anti-mouse IgG-HRP (Santa Cruz sc-2314), anti-rabbit IgG-HRP (Promega W401B or Santa Cruz sc-2313), and anti-goat IgG-HRP (Santa Cruz sc-2020).

2.2. Cell culture

All cells were grown at 37 °C in humidified air containing 6.3–6.5% CO_2. A549 cells (human alveolar carcinoma; ATCC CCL-185) were grown in pyruvate-free DMEM (Invitrogen 12430-047), supplemented with 10% fetal bovine serum (FBS) (Valley Biomedical, Winchester, VA), penicillin (100 U/ml), and streptomycin (100 μg/ml). The human lung cancer lines NCI-H23 (adenocarcinoma, ATCC CRL-5800) and NCI-H1703 (squamous cell cancer, ATCC CRL-5889) were grown in pyruvate-free RPMI (Invitrogen 22400-089), supplemented with 10% FBS, additional sodium bicarbonate (0.76 g/L), and amikacin plus cefoperazone (30 μg/ml each). Human ovarian cancer lines A2780 (adherent epithelial; cat. no. C0017002, AddexBio, San Diego, CA) and OVCAR-3 (adenocarcinoma; ATCC HTB-

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