Toxicology and Applied Pharmacology 265 (2012) 342–350

Contents lists available at SciVerse ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Inhibition of glutathione synthesis eliminates the adaptive response of ascitic hepatoma 22 cells to nedaplatin that targets thioredoxin reductase

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ARTICLE INFO

Article history:
Received 29 May 2012
Revised 1 September 2012
Accepted 3 September 2012
Available online 12 September 2012

Keywords:
Buthionine sulfoximine
Glutathione
Nedaplatin
Thioredoxin reductase

ABSTRACT

Thioredoxin reductase (TrxR) is a target for cancer therapy and the anticancer mechanism of cisplatin involves TrxR inhibition. We hypothesize that the anticancer drug nedaplatin (NDP), an analogue of cisplatin and a second-generation platinum complex, also targets TrxR. Furthermore, we investigate whether the therapeutic efficacy of NDP can be enhanced by simultaneous modulation of 1) TrxR, *via* NDP, and 2) glutathione (GSH), *via* the GSH synthesis inhibitor buthionine sulfoximine (BSO). Mice bearing ascitic hepatoma 22 (H22) cells were treated with NDP alone or NDP plus BSO. TrxR activity of H22 cells was inhibited by NDP in a dose-dependent manner. A high correlation between the inhibition of TrxR activity at 6 h and the inhibition of ascitic fluid volume at 72 h was established ($r = 0.978$, $p < 0.01$). As an adaptive response, the viable ascitic cancer cells after NDP treatment displayed an enlarged cell phenotype, assembled with several-fold more antioxidant enzymes and GSH-predominant non-protein free thiols. This adaptive response was largely eliminated when BSO was co-administered with NDP, leading to the decimation of the H22 cell population without enhancing renal toxicity, since at this dose, NDP did not inhibit renal TrxR activity. In conclusion, the pharmacological effect of NDP involves TrxR inhibition, and the adaptive response of NDP-treated ascitic H22 cells can be efficiently counteracted by BSO. Simultaneous modulation of TrxR and GSH on ascitic H22 cells using NDP plus BSO greatly enhances therapeutic efficacy as compared with the single modulation of TrxR using NDP alone.

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Introduction

The thioredoxin system, comprising selenocysteine-containing thioredoxin reductase (TrxR), thioredoxin (Trx) and nicotinamide-adenine dinucleotide phosphate (NADPH), participates in a broad range of cellular functions, such as the redox control of apoptosis and proliferation-associated signaling and regulatory proteins (Arnér, 2009). TrxR is over-expressed in many cancer cells, where it incites pro-survival effects and enhances tumor development and resistance to therapeutic modalities (Gromer et al., 2004; Nguyen et al., 2006). TrxR1 knockdown Lewis lung carcinoma cells showed a dramatic reduction in tumor progression and metastasis (Yoo et al., 2006). Several commonly used anticancer drugs including cyclophosphamide, ifosfamide, cisplatin and oxaliplatin (Wang et al., 2007, 2008; Witte et al., 2005; Zhang et al., 2008) inactivate TrxR activity, thus adding to the cytotoxic potential of these agents. The selenocysteine-dependent TrxR enzyme has emerged as an important molecular target for anticancer drug development (Cai et al., 2012b; Pennington et al., 2007; Tonissen and Di Trapani, 2009).

Glutathione (GSH), a predominant nonprotein free thiol (NPFT) molecule found in cells at millimolar concentrations, plays a crucial role in cell defense mechanisms by acting as an antioxidant or conjugating with toxic electrophiles (Davis et al., 2001; Lu et al., 2007). The sensitivity of cancer cell lines to chemotherapy is inversely correlated with their GSH content (Andringa et al., 2006; Dai et al., 1999; Meurette et al., 2005; Wu et al., 2004). A positive correlation between elevation of intracellular GSH levels and resistance to platinum or alkylating agents has been established (Biroccio et al., 2004; Dai et al., 1999; Troyano et al., 2001). In cultured MCF-7 cells, GSH depletion by buthionine

Abbreviations: ASK1, apoptosis signal-regulated kinase-1; BSA, bovine serum albumin; BSO, buthionine sulfoximine; BUN, urea nitrogen; CDNB, 1-chloro-2,4-dinitrobenzene; Cr, creatinine; DTNB, 5,5′-dithiobis (2-nitrobenzotic acid); GPx, glutathione peroxidase; GR, glutathione reductase; GRAN#, granulocytes; GSH, reduced glutathione; GST, glutathione S-transferase; H22, ascitic hepatoma 22 cells; HGB, hemoglobin; i.p., intraperitoneally; LYM#, lymphocytes; NADPH, nicotinamide-adenine dinucleotide phosphate; NDP, nedaplatin; NPFT, nonprotein free thiol; NSCLC, non-small-cell lung carcinoma; SCLC, small cell lung carcinoma; PLT, platelets; RBC, red blood cells; RNR, ribonucleotide reductase; SecTRAPs, selenium compromised TrxR-derived apoptotic proteins; SOD, superoxide dismutase; Trx, thioredoxin; TrxR, thioredoxin reductase; WBC, white blood cells.

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0041-008X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2012.09.001

sulfoximine (BSO), an inhibitor of GSH synthesis, markedly potentiates the cytotoxicity of arsenic trioxide, a TrxR inhibitor. Accordingly, the simultaneous modulation of TrxR and GSH is considered as a powerful strategy for cancer therapy (Lu et al., 2007).

Cis-diammine-glycolate-O,O′-platinum II (nedaplatin, referred to as NDP) is a second-generation platinum complex developed and approved in Japan (Ogawa, 1996; Sasaki et al., 1991). As an analogue of cisplatin, NDP has a novel ring structure in which glycolate is bound to platinum by a bidentate ligand (Alberto et al., 2009). NDP is ten-fold more water soluble than cisplatin (Vermorken, 2001). NDP causes significantly less nausea, vomiting and nephrotoxicity than cisplatin and thus can be given without hydration (Desoize and Madoulet, 2002; Kawai et al., 2005; Ogawa, 1996; Sasaki et al., 1991; Vermorken, 2001). The dose-limiting toxicity of NDP is myelosuppression, in particular thrombocytopenia (Desoize and Madoulet, 2002; Kawai et al., 2005; Ogawa, 1996; Sasaki et al., 1991; Vermorken, 2001). The anticancer mechanism of NDP involves the formation of reactive platinum complexes that bind to nucleophilic groups in DNA, leading to intrastrand and interstrand DNA cross-links and cellular apoptosis (Alberto et al., 2009). Preclinical and clinical studies have demonstrated that nedaplatin has anticancer activities equivalent to that of cisplatin (Desoize and Madoulet, 2002; Ogawa, 1996; Sasaki et al., 1991; Vermorken, 2001). Since its approval in 1995, NDP has been used in the treatment of testicular tumors, small cell lung carcinoma (SCLC), non-small-cell lung carcinoma (NSCLC), esophageal cancer, bladder cancer, ovarian and cervical cancers, and head and neck tumors (Desoize and Madoulet, 2002; Kawai et al., 2005; Koshiyama et al., 2005; Monk et al., 1998; Ogawa, 1996; Vermorken, 2001).

We have reported previously that cisplatin inactivated TrxR activity in ascitic hepatoma 22 (H22) cells in mice (Zhang et al., 2008). The current work investigated whether NDP targets TrxR of cancer cells *in vivo*. We found that the TrxR activity of ascitic H22 cells in mice could be inhibited by NDP in dose-dependent fashion, and the inhibition of TrxR activity was highly correlated with the inhibition of ascitic fluid volume. As an adaptive response, the viable ascitic H22 cells after NDP treatment displayed an enlarged cell phenotype, equipped with 2 to 5-fold more of various antioxidant enzymes and GSH-predominant NPFT. This adaptive response could be almost fully counteracted by BSO, greatly enhancing the cytotoxicity of NDP without generating renal toxicity, since at the dose used, NDP did not inhibit renal TrxR activity.

Materials and methods

Chemicals and drugs. BSO, NADPH, HEPES, insulin, 5,5′-dithiobis (2-nitrobenzotic acid) (DTNB), Trx (*E. coli*), guanidine hydrochloride, reduced GSH, bovine serum albumin (BSA), 1-chloro-2,4-dinitrobenzene (CDNB), RNase A and propidium iodide were all purchased from Sigma (St. Louis, MO, USA). NDP was purchased from Nanjing Tung Chit Pharmaceutical Co., Ltd., PR China. Other chemicals were of the highest grade available.

Animals. Healthy male Kunming mice (body weight 20–22 g) and their diet were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., PR China. The mice were housed in plastic cages in a room with controlled temperature (22 ± 1 °C) and humidity ($50 \pm 10\%$) and 12 h light/dark cycle. The mice were allowed to obtain food and water *ad libitum*.

Tumor cell inoculation. H22 murine carcinoma cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). In brief, viable cells in ascitic fluid were counted using trypan blue dye exclusion. The concentration of cells in ascitic fluid was adjusted to 100×10^6 cells/mL using saline, then 0.2 mL was intraperitoneally (i.p.) injected into the peritoneal cavity of mice; this procedure was carried out once weekly.

Animal treatments. All experiments involving mice were performed in strict compliance with the ethical guidelines issued by the Anhui Agricultural University.

In the first set of experiments, to investigate the dose effect of NDP on cellular antioxidant parameters and ascitic fluid volume, 48 mice were randomly divided into 4 groups of 12. Seventy-two hours after cell inoculation at a level of 20 million cells per mouse, group I was i.p. injected with saline as control, and groups II–IV were i.p. injected with NDP at the doses of 15, 25 and 35 mg/kg, respectively. Six mice in each group were euthanized by cervical dislocation at 6 h, and the rest were euthanized at 72 h after NDP treatment. Ascitic fluid was collected directly or with the aid of a known volume of ice cold saline if necessary and centrifuged to obtain H22 cells.

In the second set of experiments, to observe the effects of NDP on cell cycle, proliferation, morphology and cellular antioxidant inclusion, 24 mice were randomly divided into 2 groups of 12. Seventy-two hours after cell inoculation at a level of 20 million cells per mouse, group I was i.p. injected with saline as control, and group II was i.p. injected with NDP at a dose of 25 mg/kg. Six mice in each group were euthanized by cervical dislocation at 6 h, and the rest were euthanized at 72 h after NDP treatment. Ascitic fluid was collected directly or with the aid of a known volume of ice cold saline if necessary and centrifuged to obtain H22 cells.

In the third set of experiments, to inspect the potential toxicity of BSO and its effects on NPFT levels as well as H22 cell proliferation, twenty-four mice were randomly divided into 2 groups of 12. Seventy-two hours after cell inoculation at a level of 20 million cells per mouse, group I was i.p. injected with saline as control, and group II was i.p. injected with BSO at a dose of 500 mg/kg. Six mice in each group were euthanized by cervical dislocation at 6 h, and the rest were euthanized at 72 h after BSO treatment. Ascitic fluid samples were collected to obtain H22 cells, and renal tissues were dissected.

In the fourth set of experiments, to elucidate the effect of BSO on NDP cytotoxicity, 36 mice were randomly divided into 3 groups of 12. Seventy-two hours after cell inoculation at a level of 20 million cells per mouse, group I was i.p. injected with saline as control, groups II and III were i.p. injected with NDP at a dose of 25 mg/kg, and group III was simultaneously i.p. injected with BSO at a dose of 500 mg/kg. Six mice in each group were euthanized by cervical dislocation at 6 h, and the rest were euthanized at 72 h after NDP treatment. Ascitic fluid was collected directly or with the aid of a known volume of ice cold saline if necessary and centrifuged to obtain H22 cells.

In the fifth set of experiments, to address the impact of BSO on NDP toxicity, 42 mice were randomly divided into 3 groups of 14. Group I was i.p. injected with saline as control, groups II and III were i.p. injected with NDP at a dose of 25 mg/kg, and group III was simultaneously i.p. injected with BSO at a dose of 500 mg/kg. Six mice in each group were euthanized by cervical dislocation at 6 h after NDP treatment to dissect kidney tissues; the rest were euthanized at 72 h after NDP treatment to obtain blood samples and kidney tissues.

Sample preparation and biochemical parameters. H22 cells were homogenized in ice cold 0.15 M PBS (pH 7.2) with 1 mM $EDTANa_2$ by ultrasonication for 30 min in an ice bath. Kidney tissues were homogenized in a glass homogenizer with the above-mentioned PBS (1:9, w/v). Blood samples collected in EDTA-coated tubes were subjected to hematological evaluation including white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), platelets (PLT), lymphocytes (LYM#), and granulocytes (GRAN#) by using an auto analyzer. Blood samples collected in EDTA-coated tubes were centrifuged to obtain plasma for measuring urea nitrogen (BUN) and creatinine (Cr) levels by using commercial kits.

For the NPFT assay, immediately after homogenization, an aliquot of homogenate was taken out to mix with trichloroacetic acid (20%, w/v),

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