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Disruption of Mitochondrial Thioredoxin System as a cell death Mechanism of Cationic Triphenylmethanes

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Abstract

Alteration of mitochondrial structure and function is a hallmark of cancer cells different from normal cells and thus targeting mitochondria emerges as an novel approach for cancer therapy. Mitochondrial thioredoxin2 (Trx2) system is critical for cell viability, but the role in cancer biology is not well understood. Recently some cationic triphenylmethanes such as brilliant green (BG) and gentian violet showed antitumor and antiangiogenic activity by unknown mechanisms. Here we demonstrate that BG killed cells at nanomolar concentrations and targeted mitochondrial Trx2, which was oxidized and degraded. HeLa cells were more sensitive to BG than fibroblasts. In HeLa cells, Trx2 down-regulation by siRNA resulted in increased sensitivity to BG, whereas for fibroblasts, the same treatments had no effect. BG was visualized to accumulate in mitochondria and cause a rapid and dramatic decrease of mitochondrial Trx2 protein. With a redox Western blot method, we found that treatment with BG caused oxidation of both Trx1 and Trx2, followed by release of cytochrome c and apoptosis inducing factor from mitochondria into cytosol. Moreover, this treatment resulted in an elevation of mRNA level of Lon protease, a protein quality control enzyme in mitochondrial matrix, suggesting that the oxidized Trx2 may be degraded by Lon protease.

Keywords

Thioredoxin; anticancer target; mitochondria; triphenylmethane; brilliant green; Lon protease

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Introduction

Cationic triphenylmethane dyes including gentian violet, brilliant green and malachite green have a long history of use as antifungal and antibacterial agents because of their toxic effects [1–3]. Recently some triphenylmethanes have shown potent anticancer and antiangiogenic activities in mice and humans [4,5]. However, the anticancer mechanism of triphenylmethane dyes is not fully understood. The Trx systems, composed of NADPH, TrxRs, and Trxs are the major thiol-dependent reduction system in cytosol and mitochondria, respectively. Trx system proteins are critical for maintaining DNA synthesis and the cellular redox balance [6]. Trx1 is an electron donor for ribonucleotide reductase (RNR), which converts ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs) [7]. Dithiol Trx1 also reduces methionine sulfoxide reductases (MSRs), and thioredoxin peroxidases (peroxiredoxins, Prxs), and is involved in the repair of oxidatively damaged proteins, or signaling via hydrogen peroxide [8,9]. Compared to normal cells, cancer cells exhibit the striking properties of changes of cellular redox states [10]. Trx1 and TrxR1 have been found over-expressed in many cancer cells and TrxR1 is proposed as a cancer therapeutic target [11–13]. However, the roles of mitochondrial Trx2 system in cancer cells are less clear.

Trx2 possesses the conserved thioredoxin active site WCGPC and catalyzes the disulfide reduction in mitochondria together with TrxR2 and NADPH [14]. Trx2 provides electrons to Prx3 to scavenge H_2O_2 in mitochondria, a major source of cellular ROS. Trx2 is a key regulator in mitochondria-dependent apoptosis by binding to apoptosis signal-regulating kinase 1 (ASK1) and thioredoxin interacting protein [15–17]. In addition, Trx2 plays an important role in the regulation of mitochondrial permeability transition [18–20]. Trx2^{+/-} mice with a 50% decrease of Trx2 protein expression have an impaired mitochondrial function and increased oxidative stress, and homozygosity for Trx2 results in embryonic lethality [21,22].

Despite the fact that triphenylmethanes have been used by humans for over a century, there is little known about the mechanism of activity of these compounds [4,23]. However, it is known that the biological action of some triphenylmethanes such as gentian violet is dependent on the oxidation-reduction potential [24] and may involve free radical formation [25]. Recently, triphenylmethane dyes were demonstrated to have potent inhibitory activity against NADPH oxidases Nox2 and Nox4 upon acute exposure [4,26]. In an effort to explore the effects of chronic brilliant green exposure, SVR angiosarcoma cells were cultured in constantly increasing concentrations of brilliant green, and gene array was performed on resistant cells. TrxR1 was highly upregulated and this was confirmed with RT-PCR, where a 25-fold induction was noted (Arbiser JL. et al., preliminary results). Given this observation, we decided to investigate the effects of these commonly used compounds on the Trx systems.

Materials and Methods

Chemicals and enzymes

Human wild type Trx1 and mutant Trx1 (C61S/C72S), goat anti-Trx1, anti-rat TrxR1 antibodies were from IMCO Ltd (Stockholm, Sweden; www.imcocorp.se). Anti-Trx2, RNR R1, RNR R2, VDAC, Lamin B and GAPDH antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Prx3 antibody was from Abcam (Cambridge, MA, USA). Methyl-brilliant green and proton sponge dye were synthesized as described in supplementary methods. All the other chemicals were analytical grade.

Cell culture

HeLa cells from ATCC were cultured with 1 g/L glucose Dulbecco's modified Eagle's medium (GIBCO) and normal primary human foreskin fibroblast cells (kindly provided by Dr. Laura Papp, Queensland Institute of Medical Research, Australia) were grown in RPMI 1640 medium. The cells were plated at a density of 1×10^6 in 100 mm petri dishes and treated with different concentrations of triphenylmethanes. Following the treatment, cell lysates were prepared for Western blotting and TrxR activity assay as described previously [13]. MTT assay, trypan blue exclusion, and neutral red uptake assay were used to measure the cell viability in 96 or 6 well plates [13,27].

Transfection of siRNA

The transfection of siRNA was performed according to the Dharmacon transfection protocol. In brief, HeLa or fibroblast cells were split into a 6 or 96 well plate and grown overnight. siRNA transfection reagent (Dharmacon Inc. Lafayette, CO, USA), AllStars negative Control siRNA, Hs_TXN_1 HP siRNAs, Hs_TXNRD1_1, Hs_TXN2_1, Hs_TXNRD2_1 (Qiagen Inc, Valencia, CA) were diluted in serum and antibiotic free medium and left at room temperature for 5 min. Then siRNA transfection reagent and siRNAs were mixed and incubated for 20 min. The complete medium was added into the mixture to prepare the culture medium with 50 nM siRNA and added to 96 or 6 well plates. After 48 hours, the medium was changed to complete medium with or without 0.5 μ M brilliant green. After another 24 hour treatment, cells were harvested to prepare cell lysates for Western blotting or used for cell viability measurement by MTT or trypan blue methods.

Western Blotting and Quantitative real-time PCR analysis

The cell lysates obtained after the treatment with triphenylmethanes were applied for Western blotting. The samples with 25 μ g of proteins were separated on a 12% Bis-Tris gel with MES running buffer. TrxR1 and Trx2 were detected by rabbit anti-TrxR1 (1:1000 dilutions), anti-Trx2 (1:500) (23). Trx1, RNR R1, RNR R2, and GAPDH were detected with goat anti-Trx1 (1:1000 dilutions), anti-RNR R1 (1:500), anti-RNR R2 (1:500), anti-HO-1 (1:200), rabbit anti-GAPDH (1:200) antibodies respectively, followed by the detection of Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Shelton, CT, USA).

Quantitative real-time PCR analysis

Total RNA was extracted from BG-treated or siRNA transfected HeLa cells by RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Purified RNA was treated with RNase-free DNaseI to ensure complete degradation of potential genomic DNA contamination, followed by the addition of 25 mM EDTA and heat denaturation of the enzyme. Different RNA samples were reversely transcribed to cDNA with random hexamer primers using a SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Real-time PCR primers were designed with RealTimeDesign software (<http://www.bioserchtech.com>) and were made by Thermo.

The real-time PCR was carried out on an ABI PRISM 7900 System (Applied Biosystems) according to the manufacturer's instructions in a 384-well microtiter plate with a 12.5 μ L reaction volume containing 6.25 μ L SYBR Green PCR Master Mix (Applied Biosystems), 2.5 pmol of each primer, 12.5 ng of cDNA templates. The sequence of the primers was described in Supplementary data. Amplification of housekeeping gene actin was used as a control to normalize the expression level of gene of interest. Negative controls without template were performed in each run. Dissociation analysis was performed at the end of each run to confirm the specificity of the reaction. The quantitative real-time PCR results were analyzed with a $\Delta\Delta$ CT method.

Fluorescence staining

HeLa cells were cultured in chamber slides and treated with 1.0 μM BG at 37°C for 1 h. MitoTracker-Red (Invitrogen-Molecular Probes, Eugene, OR) was added to the culture medium 20 min before fixation with 4% paraformaldehyde. Digital images were taken by a Zeiss Axioplan 2 microscope and collected using an AxioCam HRm Camera and axiovision 4.2 software.

Redox Western Blotting of Trx1 and Trx2

The *in vivo* redox Western Blotting was performed based on an electrophoretic mobility shift assay with some modifications [28]. Briefly, after the treatment with BG, HeLa cells were lysed with a sample solution (Tris-HCl 50 mM, EDTA 1 mM, Urea 8 M, pH 8.3) containing 30 mM iodoacetic acid (IAA). After incubation at 37°C for 30 min, the proteins were precipitated and washed with ice-cold acetone/1 M HCl (98:2 v/v) three times. Then the precipitate was resuspended in the sample solution containing 3.5 mM DTT and then alkylated with 10 mM iodoacetamide (IAM). The proteins were separated by a PAGE in buffer containing 8 M urea and transferred with Invitrogen Transfer System. The bands of Trx1 and Trx2 were detected by Trx1 and Trx2 antibodies. Trx1 or Trx2 with more free thiols were more negatively charged by alkylation with IAA and migrated faster. HeLa cells were lysed with sample buffer containing 3.5 mM DTT to reduce all cysteine residues to sulfhydryl form. The Trx markers as fully reduced, oxidized were prepared by incubation with the DTT-reduced cell lysates with 30 mM IAA, 10 mM IAM. The DTT-reduced cell lysates were also incubated with 30 mM IAA-10 mM IAM, and 10 mM IAA-30 mM IAM. The equal amount of four fractions (alkylated with 30 mM IAA, 10 mM IAM, 30 mM IAA-10 mM IAM, and 10 mM IAA-30 mM IAM) were mixed to prepare the intermediate redox state form of Trxs.

Isolation of mitochondrial fractions

The preparation of nuclear extracts was as described in reference [29]. HeLa cells (5×10^6) treated with BG were washed and suspended in mitochondrial isolation buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes-NaOH, pH 7.5) containing protease inhibitor cocktails. Cells were disrupted by homogenizing with 20–25 strokes followed by centrifugation at $2000 \times g$ for 10 min. The supernatants were subsequently centrifuged at $13\,000 \times g$ for 10 min to yield the mitochondrial pellets. These supernatants were further centrifuged at $100\,000 \times g$ for 1 h to obtain the cytosolic fractions [30]. Mitochondrial pellets were resuspended and lysed in the cell lysis buffer and were loaded on SDS-PAGE for subsequent analysis. TrxR activities in cytosol and mitochondria were measured by a sensitive method using fluorescent labeled insulin as the substrate (Montano SJ, Lu J, and Holmgren A, manuscript in preparation), which is based in the insulin assay [31].

Results

Cell viability of HeLa and fibroblast cells against brilliant green treatment

Since brilliant green (structure is shown in Supplemental Fig. 1) exhibited an efficient inhibitory effect on the hemangioma growth in a mice model [4], in this study we compared the toxicity of BG to fibroblast and HeLa cells (Fig. 1A and B). Cell viability was measured by MTT assay. The cell viability did not exhibit obvious difference after the treatment for 24 h. But after the treatment for 48 and 72 hours, HeLa cells showed enhanced sensitivity to BG. (Fig. 1A). Upon the treatment of BG (above 0.06 μM), the cell viability for HeLa cells decreased to around 20% of the control, compared to about 60% for fibroblast cells (Fig. 1A and B). Besides MTT assay, we used trypan blue exclusion and neutral red uptake assays to

investigate the effects of triphenylmethane dyes on cell viability. All three methods yielded similar results (data not shown).

Effects of Trx and TrxR knockdown on cell viability against BG treatment

Since Trx systems are critical for cell viability, we analyzed cytosolic and mitochondrial Trx systems in the fibroblast and HeLa cells. Both cells contained similar levels of Trx1, while fibroblast cells had lower level of TrxR1 and Trx2 compared to HeLa cells (Fig. 1C). Next, we examined the role of Trxs and TrxRs in protecting cell against BG treatment. We used a siRNA technique to deplete TrxRs or Trxs level in the cells and then compared BG induced cell toxicity. TrxR1, Trx1 and Trx2 siRNA led to a sharp decrease of TrxR1, Trx1, and Trx2 protein and mRNA levels in HeLa and fibroblast cells, respectively (Fig. 2). We also transfected HeLa and fibroblast cells with a TrxR2 siRNA (Hs_TXNRD2_1) to deplete mitochondrial TrxR2. However, TrxR2 mRNA level was not successfully reduced (data not shown). Trx1 knock-down resulted in a compensatory 3-fold increase of TrxR1 mRNA in fibroblasts, but not in HeLa cells. Trx2 knock-down caused a massive increase of mRNA level of TrxR2, Trx1, and TrxR1 in HeLa cell, but not in fibroblast cells (Fig. 2C and D).

The significance of Trx2 for HeLa cells was corroborated by cell viability assay. Trx2 knock down caused a decrease of cell viability for HeLa cells but not for fibroblast cells, while TrxR1 knock-down did not show significant effects on the cell toxicity for either HeLa or fibroblast cells (Fig. 3A and B). Moreover, in normal fibroblast cells down regulation of Trx1, TrxR1 or Trx2 did not enhance the cytotoxicity of BG (Fig. 3B). In HeLa cells, TrxR1 knockdown did not significantly affect BG induced toxicity, while the treatment with Trx2 siRNA caused a significant decrease of cell viability. This result not only suggests that BG-induced cell death is related to mitochondrial Trx system disruption, but also shows that the tumor cells are more dependent on mitochondrial Trx system than normal cells.

Cellular responses upon the treatment of triphenylmethane dyes

The treatment with BG for 24 h resulted in a trend of a marginal increase of TrxR1, and Trx1 protein level (Fig. 4A), but did not have significant effects of TrxR activity in the cell lysates (data not shown). Intriguingly, Trx2 protein level showed a sharp decrease upon treatment with low concentrations of BG (0.25–1.0 μ M). Upon treatment of 2.0 μ M BG, Trx2 amount just had a slight decrease compared to control (Fig. 4A), indicating that Trx2 degradation was attenuated upon the treatment with high concentration of BG. This may be of therapeutic importance, as it is more feasible to attain nanomolar concentrations rather than micromolar concentrations of triphenylmethanes *in vivo* [32]. The effects of BG on Trx systems in HeLa cells were also observed in fibroblast cells (Fig. 4B). Trx2 protein was nearly eliminated by treatment with 0.25 and 0.50 μ M BG.

RNR R1 protein level was not affected by the treatment of BG. However, RNR protein R2 which is present in S-phase [33–35] almost disappeared upon 1.0 μ M and 2.0 μ M BG treatments (Fig. 4A). We also detected the expression of the oxidative stress inducible protein heme oxygenase-1 (HO-1) upon treatment with BG. In the range of 0.25 to 1.0 μ M, the expression of HO-1 increased with higher BG; whereas at 2.0 μ M BG, the induction of HO-1 was blocked (Fig. 4A). The concentration dependent induction was also seen for another antioxidant enzyme NAD(P)H quinone oxidoreductase 1 (NQO1). Treatment with 1.0 μ M BG resulted in the elevation of NQO1 mRNA level, while there was no change upon the treatment of 2.0 μ M BG (Fig. 4C). These results suggest that BG induced up-regulation of cellular antioxidant enzymes and the outcome of cell death is concentration dependent. The strong toxicity for HeLa cells were also seen for other triphenylmethane dyes (Structures shown in supplemental Fig. 1). BG, methyl brilliant green (MBG), proton sponge dye (PS), gentian violet (GV), and carbazole blue (CB) exhibited the strongest cell

toxicity (supplemental Fig. 2). The positive charge of the compounds was critical for the cell toxicity, since other NADPH oxidase inhibitors, including fulvene-5, 4M5I and 4M5, which do not have positively charged nitrogens only had slight cellular toxicity. Treatment of HeLa cells by other triphenylmethane dyes such as GV and MBG also induced a similar cellular response. The common remarkable observation was the decreases of Trx2 upon the triphenylmethane treatments (Supplemental Fig. 3).

We applied a redox Western Blot method based on an electrophoretic mobility shift assay [28] to analyze the redox state of Trx1 and Trx2 as a result of BG treatment (Fig. 5). The method could detect the redox state of the five thiols in Trx1 and two thiols in Trx2. In control HeLa cells without treatment, most of Trx1 was in the fully reduced form (51%) or with one cysteine oxidized (34%) probably reflecting mixed disulfide S-X protein [28]. Only 12% of Trx1 was in the oxidized form with one disulfide. No Trx1 was observed in the highly oxidized forms without -SH or with only one -SH. The treatment with 0.25–2.0 μ M BG for 24 h caused Trx1 redox state to change. The fully reduced was dramatically decreased (Fig. 5B), while the fully oxidized Trx1 and the forms with only one, two, or three free thiols increased. In control cells, more than 90% of Trx2 was fully reduced, whereas treatment with BG resulted not only a decrease of the amount of Trx2, but also in dramatic oxidation of Trx2 (Fig. 5).

Treatment of HeLa cells with BG was shown to be time dependent (Fig. 6A) and significant cell death was observed after the treatment for 16 or 24 h. Treatment with 2.0 and 4.0 μ M of BG for 24 h caused more than 90 percent cell death, while treatment with 1.0 and 0.5 μ M resulted in a cell viability decrease to 60% of control. We therefore determined the Trx, TrxR and RNR protein levels upon the treatment with BG at different times. As shown in Fig. 6B, TrxR1 and Trx1 displayed a slight elevation with increasing incubation time on treatment with 1.0 μ M BG. A decrease of Trx2 and RNR R2 were early events, and observed after treatment for 3 h. HO-1 was found to be induced after treatment for 6 h. Treatment with 1.0 μ M BG induced a profound decrease of RNR R2 but no change of RNR R1 (Fig. 6B). In contrast, treatment with 2.0 μ M BG neither caused the induction of HO-1 nor a large decrease of Trx2 (data not shown).

We used quantitative real-time PCR to measure mRNA levels of Trx, TrxR and RNR R1 and R2 proteins (Fig. 6C). RNR R1 mRNA was kept at similar levels upon 1.0 μ M BG treatment, whereas RNR R2 mRNA decreased with the increase of BG and treatment time. Treatments with 1.0 μ M BG for 16 h induced an increase of TrxR1, Trx1 and TrxR2 mRNA levels. At 9 h, 1.0 μ M BG did not lead to an obvious change of Trx1 mRNA, but caused a decrease of Trx2 mRNA, and a significant increase of TrxR1 and TrxR2 mRNAs (Fig. 6C).

Effects of BG on Trx system in different organelles

Since Trx systems are present in different organelles, we performed fluorescence staining to detect the subcellular location of BG. BG treatment resulted in green fluorescent cells, which overlapped with the MitoTracker-Red mitochondrial staining (Fig. 7A). This result showed that BG accumulated in mitochondria. Thus, we isolated the mitochondria and nuclei after the treatment with BG to examine the time course of BG effects on Trx systems in different organelles. Treatment with 1.0 μ M BG induced a slight increase of TrxR1 both in mitochondrial and cytosolic fractions, which is consistent with the observation in whole cell lysates (Fig. 7B). TrxR activity in cytosol was kept at the similar level, same as the result in the whole cell lysates, while TrxR activity in mitochondria was increased above two fold of control after 6 h treatment (Fig. 7C). The BG treatment caused an increase of Trx1 in cytosol and nucleus. Moreover, 3 to 9 h BG treatment caused a decrease of Trx2 in all the organelles from 3 h, indicating that Trx2 change in the organelles is an early cellular event, which is consistent with the observation in cell lysates. But cytosolic Trx2 increased

at 16 h, which may be attributed to the release of Trx2 from mitochondria to cytosol and probably caused by mitochondrial membrane disruption. This is supported by the release of other apoptosis factors from mitochondria to cytosol such as apoptosis inducing factor (AIF) and cytochrome c (Fig. 7B). As an early event, cytochrome c expression level was increased in mitochondria upon the BG treatment for 6 h. Taken together, BG treatment induced significant changes of Trxs, including Trx1 translocation from cytosol to nucleus and Trx2 translocation from mitochondria to cytosol as well as the decrease of total Trx2 level.

Mechanism of the Degradation of Trx2

To see how Trx2 is degraded upon the treatment of BG, we treated Hela cells with BG together with MG132, an inhibitor of both proteasome and mitochondrial Lon protease [36,37] (Fig 8A). The presence of MG132 prevented the Trx2 degradation in some degree. However, no oligo-ubiquitinated Trx2 was detected after the immunoprecipitation with Trx2 antibody (data not shown), indicating that Trx2 decrease is not an ubiquitination-dependent proteasome degradation process. In addition, Trx2 is located mitochondrial matrix, in which mitochondrial Lon protease is an important protein quality control enzymes by degrading the oxidized protein [38,39]. We thus analyzed the mRNA level of Lon protease (Fig. 8B). Lon protease mRNA was elevated upon the treatment of 1 μ M BG. The treatment with BG only for 3 h caused Lon protease mRNA increase to 1.5 fold of control. The treatment for 9 h resulted in a 2.5 fold increase. Treatment with higher concentration of BG alleviated the elevation (Fig. 8B). These results suggest that Trx2 degradation is a Lon protease mediated process.

Discussion

It is well known that alteration of mitochondrial structure and function is a hallmark of cancer cells different from normal cells [40] and thus targeting mitochondria emerges a reasonable approach for tumor therapy [41]. Here we show that some tumor cells are more dependent on the mitochondrial Trx2 than normal fibroblast cells. This is evident by the observations that fibroblast cells had lower level of Trx2 (Fig. 1C); Trx2 knock-down resulted in mRNA level changes of other Trx system proteins, a decrease of cell viability, and a higher sensitivity against the mitochondrial targeting drug BG in HeLa cells, but not in untransformed fibroblast cells (Figs. 2 and 3). In addition, BG treatment caused a sharp decrease of Trx2. These results strongly suggest that mitochondrial Trx2 system may become a novel target for cancer therapy.

Cationic triphenylmethanes are ubiquitous compounds in the environment, both as drugs and environmental contaminants [1]. Triphenylmethanes are treated as harmful agents and may increase the risk of the incidence of some cancers in mice and rats at the higher concentration [42,43]. However, very interestingly, the incidence of some cancers such as leukemia was significantly decreased in rats exposed to malachite green, a triphenylmethane with a structure similar with BG [43]. Moreover, the treatment with BG and GV resulted in a 95.7% and 92.6% decrease in tumor size of bEnd.3 hemangiomas in mice, respectively [4]. These results show that cationic triphenylmethanes may serve as pharmacophores for the development of drug to disrupt Trx2 system.

The cytosolic Trx1 system and mitochondrial Trx2 system are essential for cell viability [16] and play critical roles in cellular regulation and apoptosis by providing electrons to RNR, Prxs, and MSRs enzymes [44,45]. RNR is essential for DNA synthesis, while MSRs and Prxs are critical for repair of oxidized methionine residues and the removal of H₂O₂ to keep redox balance. Moreover, mitochondria are the organelles responsible for respiration in cells and the reduced Trx2 level in Trx2^{+/-} mice resulted in a decreased activity of electron transport chain complexes and ATP production [46]. Thus, a dramatic decrease of Trx2

caused by cationic triphenylmethanes may lead to an impairment of mitochondrial function in tumor cells.

The cellular response upon BG treatment involves the regulation at mRNA and protein level in a concentration dependent manner. Treatment with low concentration of BG (below 1 μM) results in cellular detoxification responses to up-regulate the antioxidant enzymes including TrxRs, HO-1 and NQO1, etc (Fig. 9). The observation is supported by the results that Trx1 translocation to nucleus and TrxR1 mRNA level was elevated after 6 h treatment (Fig. 9). However, treatment with higher concentration of BG (2 μM) did not cause a change of HO-1 and NQO-1 (Fig. 4 B and C), which may indicate that the cellular response mediated by the Nrf2 or other oxidation sensitive transcription factors was blocked.

The Trx1 and Trx2 systems are located in different subcellular organelles, and they have different specific functions. Trx1 is mainly located in the cytosol (Fig. 7B), where RNR is also mainly located [47]. Trx1 is suggested to be the main electron donor for RNR in S-phase [35]. In mitochondria, where Trx1 is thought to be located only in the inter-membrane space, Trx2 plays critical roles for maintaining the reducing environment. One novel property of BG is that the consequence on cytosolic and mitochondrial Trx system is distinct (Fig. 9). Upon treatment with 1.0 μM BG, the Trx1 level in nucleus was increased, whereas Trx2 in mitochondria was decreased. Mitochondrial accumulation of triphenylmethanes resulted in degradation of Trx2, and the mRNA levels of TrxR1 and Trx1 were elevated more than those of TrxR2 and Trx2 (Fig. 6C). Treatment of BG did not induce changes of TrxR activity in the cytosol, but caused the TrxR activity in mitochondria increase to nearly two fold of the control (Fig. 7C). In addition, the treatment of triphenylmethanes induced a decrease of RNR R2, which is a cell cycle regulated essential protein for DNA synthesis in S-phase. RNR R2 is also an anticancer target for cancer therapy [48,49].

The triphenylmethane dyes belong to lipophilic cations which can easily pass through the lipid bilayers [50]. The plasma membrane potential gradient ($\Delta\psi$) is 30–60 mV, while the mitochondrial membrane potential is 150–180 mV. The membrane potential gradients drive these lipophilic cations to move and accumulate in the matrix of mitochondria [50]. Our results are consistent with the previous reports that mitochondria of *Trypanosoma cruzi* are a target of gentian violet [51] and more than 90% of some triphenylmethane cations can bind with the isolated mitochondrial fraction [52].

A remarkable observation in this study is that Trx1 and Trx2 were oxidized and Trx2 was degraded upon the treatment of BG. Though cytosolic and mitochondrial TrxR activities were not decreased in the cells treated with BG, a purified TrxR1 protein can be inhibited by BG *in vitro*. BG reacted with TrxR active site cysteine/seleocysteine and the reactivity was enhanced with an increase of pH (data not shown). Considering the pH value in mitochondrial matrix is 8.0, which is higher than pH in cytosol, 7.2, BG should be more reactive with the free thiols of mitochondrial proteins and produced more non-native oxidized proteins. Furthermore, we show that Trx2 degradation is probably mediated by enhancing the activity of mitochondrial Lon protease system. As a quality control enzyme, Lon protease will degrade the oxidized Trx2 caused by the treatment of BG (Fig. 9) at the low concentration (below 1 μM). At the higher concentration (above 2 μM), degradation of Trx2 was diminished. This may be due to Trx2 is highly oxidized and cannot be degraded by Lon protease. The same property was observed when aconitase is oxidized by H_2O_2 and degraded by Lon-protease [53]. Moreover, the elevation of Lon protease was found to be reduced upon the treatment with higher concentration of BG. The discrepancy of the degradation of Trx2 may also explain the concentration dependence of cell toxicity of BG. At higher concentration (above 2 μM), the oxidized Trx2 cannot be removed by Lon protease and thus caused the cell death rapidly, which is supported by the protection role of

Lon protease in cell viability [54]. Another reason for the cell death upon the treatment of higher concentration of BG is that the up-regulation of cellular antioxidant enzymes is blocked.

In summary, our results demonstrate that the viabilities of some tumor cells are more dependent on the mitochondrial Trx system than those of normal cells. Moreover, the mitochondrial Trx system was disrupted by triphenylmethane dyes such as brilliant green and gentian violet. Mitochondrial Trx2 was oxidized by and triphenylmethane dyes and degraded by mitochondrial Lon-protease. The results strongly suggest that the mitochondrial Trx system is a novel cancer therapy target and also demonstrate a mechanism for the anticancer activity of cationic triphenylmethane dyes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

AIF	apoptosis inducing factor
ASK1	apoptosis signal-regulating kinase 1
BG	brilliant green
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GV	gentian violet
HO-1	heme oxygenase-1
IAA	iodoacetic acid
IAM	iodoacetamide
MBG	methyl brilliant green
MSR	methionine sulfoxide reductase
Prx	peroxiredoxin
PS	proton sponge dye
RNR	ribonucleotide reductase
ROS	reactive oxygen species
Sec (U)	selenocysteine
Trx	thioredoxin
TrxR	thioredoxin reductase

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Fig. 1.

The toxicity of BG to HeLa and fibroblast cells and the cellular protein level of Trx system. (A) The toxicity of BG to HeLa cells; (B) the toxicity of BG to fibroblast HeLa cells. HeLa or normal human fibroblast cells were treated with different concentration of BG for 24, 48 and 72 h, and then the cell toxicity was detected by MTT assay. Error bars represent the standard deviation of 4–8 experiments. (* $p < 0.05$, treatment with same concentration of BG to HeLa vs fibroblast cells, student's t-test). (C) Protein levels of Trx1, TrxR1 and Trx2 in HeLa and fibroblast cells were analyzed by Western Blotting.

Fig. 2. Down regulation of Trx1, Trx2 and TrxR1 by siRNA technique. HeLa (A, C) or fibroblast (B, D) cells were split into 6 well plate and transfected with Trx1, Trx2, TrxR1 and control siRNA for 72 h. Cells were harvested and protein (A, B) and mRNA (C, D) level were analyzed by Western Blotting and Quantitative real-time PCR. Error bars for mRNA measurement represent the standard deviation of duplicate experiments.

Fig. 3. Roles of Trx systems in protecting cells against BG treatment. Following the transfection with Trx1 siRNA, TrxR1 siRNA, Trx2 siRNA and control siRNA for 48 h, HeLa cells (A) and fibroblast cells (B) were treated with 0.5 μ M BG for 24 h. The cell viability was measured by MTT method. Error bars represent the standard deviation of three experiments. (* $p < 0.05$, ** $p < 0.01$ treatment with Trx1 siRNA, TrxR1 siRNA, or Trx2 siRNA vs control siRNA, student's t-test).

Fig. 4.

The cellular response induced by triphenylmethane dyes. (A) HeLa cells were treated with BG for 24 h. Then Trx system and RNR proteins were analyzed by Western blotting, GAPDH was used as the loading control. (B) Fibroblast cells were treated with BG for 24 h. Then Trx system proteins were analyzed by Western blotting. (C) Quantitative real-time PCR analysis of the mRNA level of NQO1. HeLa cells were treated with 1.0 or 2.0 μ M BG for different time, then the total RNA was extracted and used for Quantitative real-time PCR analysis. Actin was used as a control. The data represent the average of four experiments and error bars represent standard deviation.

Fig. 5.

Redox state change of Trx1 and Trx2 caused by triphenylmethane dyes. (A) HeLa cells were treated with BG for 24 h. Then redox state of Trx1 and Trx2 were analyzed by redox Western blotting. Hela cells without treatment was used as the control. The preparation of the markers of fully reduced (Red), oxidized (Ox) and the intermediated (Inter) Trx1 and Trx2 was described in material and methods. (B) Densitometric analysis of the fraction of Trx1 and Trx2 bands with different redox state. The data were obtained with Quantity One software.

Fig. 6.

Time course of the effects of BG on the protein and mRNA levels of Trx systems and RNR. (A) Time course of toxic effects of BG on HeLa cells. HeLa cells were treated with different concentration of BG for different time. Then HeLa cell viability was measured by MTT assay. Error bars represent the standard deviation of quadruplicate experiments. (* $p < 0.05$, treatment with BG vs control without treatment, student's t-test). (B) Time course of effects of BG on Trx systems and RNR protein level upon the treatment of 1.0 μ M BG. (C) Quantitative real-time PCR analysis of the mRNA level of Trx system and RNR. HeLa cells were treated with 1.0 μ M BG for different time, then the total RNA was extracted and used for Quantitative real-time PCR analysis. Actin was used as a control. The data represent the average of three experiments and error bars represent standard deviation.

Fig. 7.

The effects of BG on Trx systems in different organelles. (A) HeLa cells were treated with 1 μ M BG for 1 h and then MitoTracker-Red was added to the culture medium 20 min before fixation. (B) Time course of the effect of BG on subcellular Trx system. After the treatment with 1 μ M BG for different times, HeLa cells were harvested for the isolation of mitochondrial and nuclear fractions. Protein levels in cytosol, mitochondrial and nuclear fractions were detected by Western Blotting. (C) TrxR activity in cytosolic and mitochondrial fraction was measured by fluorescent labeled insulin coupled Trx reduction assay.

Fig. 8.

Trx2 degradation is probably mediated by mitochondrial Lon Protease. (A) The effects of MG132 on the degradation of Trx2. HeLa cells were treated with 100 nM BG and 100 nM MG132 for 48 h and then Trx2 and Prx3 proteins were analyzed by Western blotting, GAPDH was used as the loading control. (B) Quantitative real-time PCR analysis of the mRNA level of mitochondrial Lon protease. HeLa cells were treated with 1.0 or 2.0 μ M BG for different time, then Lon protease mRNA was measured as described as above.

Fig. 9.

The scheme of the effects of low concentration of brilliant green (below 1 μ M) on the cellular Trx systems. The high mitochondrial membrane potential can drive lipophilic triphenylmethane cations moving and accumulating to the matrix of mitochondria. BG reacts and oxidized mitochondrial Trx2 which was further degraded Lon Protease and caused a sharp decrease of mitochondrial Trx2. Thus, the activities to reduce MSRs, Prxs, p66^{Shc} will be inhibited and more reactive oxygen species (ROS) will be produced, which may lead to a mitochondria mediated cell death. In cytosol, BG led to Trx1 translocation to nucleus and the cellular detoxification response to up-regulate the antioxidant enzymes including TrxRs and Trx1. The cellular response was probably mediated by the Nrf2 or other oxidative sensitive transcription factors.