Redox cycling of endogenous copper by thymoquinone leads to ROS-mediated DNA breakage and consequent cell death: putative anticancer mechanism of antioxidants

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Plant-derived dietary antioxidants have attracted considerable interest in recent past for their chemopreventive and cancer therapeutic abilities in animal models. Thymoquinone (TQ) is the major bioactive constituent of volatile oil of *Nigella sativa* and has been shown to exert various pharmacological properties, such as anti-inflammatory, cardiovascular, analgesic, anti-neoplastic, anticancer and chemopreventive. Although several mechanisms have been suggested for the chemopreventive and anticancer activity of TQ, a clear mechanism of action of TQ has not been elucidated. TQ is a known antioxidant at lower concentrations and most of the studies elucidating the mechanism have centered on the antioxidant property. However, recent publications have shown that TQ may act as a prooxidant at higher concentrations. It is well known that plant-derived antioxidants can switch to prooxidants even at low concentrations in the presence of transition metal ions such as copper. It is well established that tissue, cellular and serum copper levels are considerably elevated in various malignancies. Copper is an important metal ion present in the chromatin and is closely associated with DNA bases, particularly guanine. Using human peripheral lymphocytes and comet assay, we first show that TQ is able to cause oxidative cellular DNA breakage. Such a DNA breakage can be inhibited by copper-chelating agents, neocuproine and bathocuproine, and scavengers of reactive oxygen species. Further, it is seen that TQ targets cellular copper in prostate cancer cell lines leading to a prooxidant cell death. We believe that such a prooxidant cytotoxic mechanism better explains the anticancer activity of plant-derived antioxidants.

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Thymoquinone (TQ) is the bioactive constituent of the volatile oil of *Nigella sativa* (54%). It has been shown to exert anti-inflammatory, anti-oxidant and anti-neoplastic effects both *in vitro* and *in vivo*. Many investigators have shown that the growth inhibitory effects of TQ are specific to cancer cells.¹⁻⁴ TQ showed significant anti-neoplastic activity against human pancreatic adenocarcinoma, uterine sarcoma and leukemic cell lines, while being minimally toxic to normal cells.⁴ The multidrug-resistant variants of human pancreatic adenocarcinoma, uterine sarcoma and leukemic cell lines, which are over 10-fold more resistant to the standard anti-neoplastic agents doxorubicin and etoposide as compared with their respective parental controls, were equally sensitive to TQ.⁴ TQ also exerts anti-oxidant effects and inhibits inflammation in animal models and cell culture systems.⁵

TQ belongs to a family of quinones that can undergo enzymatic or non-enzymatic redox cycling with their corresponding semiquinone radicals to generate superoxide anion radicals.⁶ TQ has been shown to inhibit growth in various cancer cell lines, including prostate cancer cells, but the mechanism of TQ-induced growth inhibition has not been delineated. TQ has been shown to induce apoptosis by p53-dependent⁷ and p53-independent⁸ pathways. Although TQ is a known antioxidant at lower concentrations, a number of recent publications report the pro-oxidant effects of TQ.⁹⁻¹¹ Moreover, the antioxidant/prooxidant ability of TQ depends on the milieu where it is present.¹² Compared with normal cells, preneoplastic cells and neoplastic cells have been shown to contain elevated levels of copper,¹³ and may be more sensitive to electron transfer with antioxidants to generate reactive oxygen species (ROS). Therefore, DNA damage induced by antioxidants in the presence of redox-active metal Cu(II) may be an important pathway through which preneoplastic cells and neoplastic cells can be killed while normal cells survived.¹⁴ We have shown that various classes of plant polyphenols are able to cause oxidative breakage of cellular DNA either alone or in the presence of transition metal ions such as copper.¹⁴⁻¹⁸ We have hypothesized that the cytotoxic action

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**Abbreviations:** TQ, thymoquinone; ROS, reactive oxygen species; TBA, 2-thiobarbituric acid; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TBARS, TBA-reactive substance

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of these compounds against cancer cells involves mobilization of endogenous copper and consequent prooxidant action.\textsuperscript{19,20} Toward validation of our hypothesis that anticancer mechanism of plant polyphenols involves mobilization of endogenous copper possibly chromatin-bound copper and the consequent prooxidant action, considerable evidence has been deduced over the years.\textsuperscript{16–18,21} In this paper using human peripheral lymphocytes, we first show that TQ can cause oxidative DNA breakage in cells through mobilization of endogenous copper ions. As a further proof of concept, we show that TQ inhibits proliferation and induces apoptosis in prostate cancer cell lines by reactive oxygen species through the redox cycling of endogenous copper. It has been well documented that copper concentrations are significantly elevated in all types of malignancies. Thus, this mechanism better explains the anticancer activity of plant-derived antioxidants with varied structures.

Results

Formation of complexes involving calf thymus DNA/Cu(II) with TQ. Figure 1a shows the effect of addition of increasing molar base pair ratios of calf thymus DNA on the fluorescence emission spectra of TQ excited at 255 nm. Such an addition resulted in a dose-dependent enhancement of the fluorescence. However, there was no significant shift in the $\lambda_{\text{max}}$ emission, suggesting a simple mode of binding of DNA and TQ. Similarly, binding of copper ions to TQ was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of the compound. The result shown in Figure 1b indicates binding as addition of Cu(II) causes quenching of TQ fluorescence. The control (native DNA alone and copper alone) when excited at the same wavelength (255 nm) did not interfere with the emission spectrum, thus confirming the binding of TQ to copper ions as well as DNA.

Detection of TQ-induced Cu(I) production by bathocuproine. The production of Cu(I), as a result of reduction of Cu(II) by TQ, was analyzed using bathocuproine, which is a Cu(I)-specific sequestering agent. Bathocuproine binds specifically to the reduced form of copper, Cu(I), but not to the oxidized form, Cu(II).\textsuperscript{22} The Cu(I)-bathocuproine complex exhibits an absorption maximum at 480 nm. As shown in Figure 2, neither Cu(II) nor TQ interfere with the maxima, TQ + Cu(II) react to generate Cu(I), which complexes with bathocuproine as evidenced by the peak appearing at 480 nm. The result shows that TQ is able to reduce Cu(II) to Cu(I) and may contribute to the redox cycling of the metal.

Generation of hydroxyl radicals by TQ. Rahman \textit{et al.}\textsuperscript{23} have previously shown that during the reduction of Cu(II) to Cu(I), reactive oxygen species such as hydroxyl radicals are formed, which may serve as the proximal DNA cleaving agent. Therefore, the capacity of TQ to generate hydroxyl radical in the presence of Cu(II) was examined. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of 2-thiobarbituric acid (TBA)-reactive material, which forms a colored adduct with TBA whose absorbance is read at 532 nm.\textsuperscript{24} The result given in Figure 3 clearly shows that increasing concentration of the TQ leads to a progressive increase in the formation of hydroxyl radicals.

Cleavage of pBR322 DNA treated with TQ and Cu(II). To examine the efficacy of TQ-Cu(II) system in DNA cleavage, in Figure 4, the ability of TQ to cause cleavage of supercoiled plasmid pBR322 DNA in the presence of copper ions was tested. As can be seen from the ethidium bromide-stained
agarose gel pattern, 30 μM copper alone does not cause significant DNA cleavage. Although TQ alone at relatively higher concentration of 150 μM leads to some DNA cleavage, increasing concentrations of TQ (50–150 μM) in the presence of 30 μM copper leads to progressive increase in the generation of open circular topological structures of plasmid DNA, demonstrating that TQ is capable of DNA degradation in the presence of copper ions, suggesting that an antioxidant-DNA-Cu system is physiologically feasible and could be of biological importance.

DNA breakage in isolated human peripheral lymphocytes using comet assay. The corresponding comet tail length is plotted as a function of compound concentration in Figure 5. It is seen that although TQ alone causes breakage of cellular DNA, the degree of such breakage is significantly enhanced in the presence of Cu(II). Cu(II) (25 μM) controls were similar to untreated lymphocyte without any significant DNA breakage. The results clearly establish that TQ-Cu(II) system is capable of DNA breakage in isolated lymphocytes.

The DNA breakage capacity of TQ in whole lymphocytes and nuclei was also compared. As in the lysed version of the comet assay, membrane and cytoplasmic barrier are eliminated, it would be reasonable to assume that the compounds are able to directly interact with the cell nuclei. Thus, considerably greater DNA breakage should be observed in the lysed version as compared with the standard version where intact lymphocytes are used. Increasing concentrations (0–40 μM) of TQ (Figure 5b) was tested for DNA breakage in intact lymphocytes and compared with that observed with lymphocyte nuclei. It is seen that the rate of tail formation is considerably greater in the case of lysed version, suggesting that TQ is able to directly interact with the nuclei when lysed version of comet assay is used.

The DNA breakage induced by TQ in intact lymphocytes and lymphocyte nuclei has been assessed in the presence of Cu(I)-specific chelators neocuproine and bathocuproine (Figures 5c and d). Incubation of lymphocytes with neocuproine (a cell membrane permeable copper chelator) inhibited DNA degradation in intact lymphocytes. Bathocuproine disulphonate (the water soluble membrane impermeable analog of neocuproine), which is unable to permeate through the cell membrane, did not cause such inhibition. This study has further shown that TQ is able to degrade DNA in cell nuclei and that such DNA degradation is inhibited by neocuproine as well as bathocuproine disulphonate (both of which are able to permeate the nuclear pore complex), suggesting that nuclear copper is mobilized in this reaction. It may be mentioned that Fe^{3+} and Cu^{2+} ions are the most redox active of the metal ions present in living cells. Also, copper and zinc are the major metal ions present in the nucleus. To examine the possible involvement of iron and zinc in TQ-induced DNA breakage, the effect of desferrioxamine mesylate (a Fe(II)-specific chelator)
and histidine (a zinc-specific chelator) has been examined on DNA breakage in lymphocytes and lymphocyte nuclei. It is seen that although desferrioxamine mesylate is able to inhibit TQ-induced DNA breakage in whole lymphocytes to a certain degree, it did not provide any protection on TQ-induced DNA breakage in cell nuclei (results not shown). Moreover, histidine was unable to inhibit TQ-induced DNA breakage in whole lymphocytes as well as lymphocyte nuclei. These results indicate that TQ-induced DNA breakage in lymphocytes involves mobilization of endogenous copper, possibly chromatin-bound copper and that Cu(I) is an intermediate in the pathway. Thus, demonstrating and copper-mediated cellular DNA breakage property of antioxidants is possible in vitro.

Effect of scavengers of ROS on TQ-induced DNA breakage. Table 1 gives the results of an experiment where various scavengers of ROS, such as superoxide dismutase (SOD), catalase and thiourea, were tested for their effect on TQ-induced DNA breakage in intact lymphocytes and lymphocyte nuclei. SOD and catalase remove superoxide and H₂O₂, respectively, and thiourea removes hydroxyl radicals. All scavengers caused a significant inhibition of DNA breakage as evidenced by decreased tail lengths. It may be mentioned that because of the site-specific nature of the reaction of hydroxyl radicals with DNA it is difficult for any trapping molecules to intercept them completely. Thus, complete inhibition of DNA breakage in the presence of scavengers of ROS is not seen. The results indicate that superoxide anion and H₂O₂ are essential components in the pathway that leads to the formation of hydroxyl radical and other species, which would be the proximal DNA cleaving agents. Thus, it is suggested that a mechanism involving reactive oxygen species is responsible for cellular DNA breakage induced by TQ.

Table 1 Effect of scavengers of reactive oxygen species on TQ-induced DNA breakage in intact lymphocyte and lymphocyte nuclei

| Treatment               | Whole lymphocyte | Lysed cells |
|-------------------------|------------------|-------------|
|                         | Tail length  % Inhibition | Tail length | % Inhibition |
| None                    | 2.23 ± 0.09 1   | 2.39 ± 0.07 1 |
| TQ (40 µM)              | 29.28 ± 1.42 2  | 42.86 ± 2.04 3  |
| - SOD (100 µg/ml)       | 12.26 ± 0.82 3  | 16.28 ± 0.94 4  |
|                         |      58          | 62          |
| - Catalase (100 µg/ml)  | 13.41 ± 0.88 3  | 15.41 ± 0.83 3  |
|                         |      54          | 64          |
| - Thiourea (1 mM)       | 15.46 ± 1.35 3  | 15.80 ± 1.35 3  |
|                         |      51          | 65          |

Whole lymphocytes and lymphocyte nuclei were layered with reaction mixture containing TQ (40 µM) in the presence of scavengers of ROS (at indicated concentrations) at 4 °C for 1 h and processed further for comet assay as described in 'Materials and Methods'.

Figure 5 DNA breakage by TQ in human peripheral lymphocytes. (a) Whole lymphocyte cells were incubated in microfuge tubes with reaction mixture at 4 °C for 1 h in dark. Reaction mixture contained RPMI (400 µl), Ca²⁺ and Mg²⁺-free PBS, increasing concentrations of TQ (0–40 µM), alone (○) and with fixed concentration of Cu(I) (25 µM) (□) and processed further for comet assay. (b) Comparison of DNA breakage by TQ in intact lymphocytes and lymphocyte nuclei as measured by comet assay. Whole lymphocyte (□)/lymphocyte nuclei (■) embedded in agarose were incubated with the reaction mixture containing indicated concentrations of TQ (0–40 µM) at 4 °C for 1 h and processed further for comet assay. Whole lymphocyte (□)/lymphocyte nuclei (■) incubated with reaction mixture (2.0 ml) containing TQ (40 µM) and indicated concentrations of membrane permeable copper chelator (Neo, neocuproine); membrane impermeable copper chelator (Batho, bathocuproine) at 4 °C for 1 h and processed further for comet assay. Values reported are mean ± S.E.M. of three independent experiments. Error bars denote ± S.E.M. **P < 0.05 when compared with 1 (untreated control cells)
Effect of preincubation of lymphocytes with thiourea/neocuproine on TBA-reactive substance (TBARS). As mentioned above, it is suggested that lymphocyte DNA breakage induced by the TQ is the result of the generation of ROS in situ. Oxygen radical damage to deoxyribose or DNA is considered to give rise to TBA-reactive material. In the experiment shown in Figure 6, the formation of TBARS as a measure of oxidative stress in lymphocytes with increasing concentrations of TQ has been determined. The effect of preincubating the cells with neocuproine and thiourea was also studied. It was seen that there is a dose-dependent increase in the formation of TBARS in lymphocytes. However, when cells were preincubated with neocuproine and thiourea there was a considerable decrease in the rate of formation of TBARS by TQ. The results given in Figure 5 along with the above results of comet assay indicate that both DNA breakage and oxidative stress in cells is inhibited by Cu(I) chelation and scavenging of reactive oxygen. Therefore, it can be concluded that the formation of ROS by TQ in lymphocytes involves its interaction with intracellular copper as well as its reduction to Cu(I). In order to examine the possibility of auto-oxidation of TQ in cell culture media and further generation of ROS, we determined the formation of H₂O₂ by the TQ in the incubation medium (RPMI) and found the rate of H₂O₂ production by TQ is significantly negligible (result not shown). The cellular DNA breakage by TQ antioxidant observed in our studies is not the result of extracellular production of H₂O₂ in the incubation medium.

Inhibition of cell proliferation and induction of apoptosis in prostate cancer cell lines treated with TQ. Cells from prostate cancer cell lines PC3, LNCaP, DU145 and C42B were incubated with TQ alone or in the presence of copper chelator neocuproine (Neo), iron chelator desferrioxamine mesylate (DM) and zinc chelator histidine (His). All results are expressed as percentage of control ± S.E. of triplicate determinations from three independent experiments. *P < 0.01 when compared with untreated (control) cells.
were treated with varying concentrations of TQ (0–20 μM) for 72 h. As can be seen from the Figure 7a, TQ caused a clear dose-dependent inhibition of cell proliferation in all cell lines tested. Further experiments were then carried out using 5 μM TQ on prostate cancer cell lines PC3 and LNCaP as at this concentration these cell lines were found to exhibit maximum sensitivity to TQ treatment. We have shown in Figure 7 that the DNA breakage effect of TQ is inhibited in the presence of copper-specific chelators in comet assay, thus in order to verify that the cytotoxic action of TQ in cancer cells is also copper mediated, prostate cancer cell lines PC3 and LNCaP were treated with 5 μM TQ in the presence of metal-specific chelators. As can be seen in the Figure 7b, neocuproine (Cu-specific chelator) significantly inhibits the cytotoxic effect of TQ as compared with desferrioxamine (Fe-specific chelator) and histidine (Zn-specific chelator) in both the cell lines.

The results obtained from DNA-Histone ELISA (Figure 7c) clearly show that the exposure of prostate cancer cells to increasing concentrations of TQ (0–10 μM) leads to a rise in absorbance at 405 nm, because of enhanced internucleosomal fragmentation. Further, in the presence of neocuproine, TQ-induced apoptosis was significantly inhibited (Figure 7b), whereas desferrioxamine and histidine were unable to protect the cells. Thus, again suggesting the role of copper in TQ-mediated cell death.

**TQ-induced apoptosis in prostate cancer cell lines is mediated by ROS.** As observed in above, TQ-induced DNA breakage in lymphocytes is inhibited by scavengers of ROS. In order to examine the involvement of ROS in TQ-mediated apoptosis in cancer cells, the effect of scavengers of ROS–SOD, catalase and thiourea – was tested on TQ-induced cytotoxic action against cancer cell lines. All three scavengers of ROS significantly inhibited the apoptotic activity of TQ in both the cancer cell lines (Table 2). From the data it is concluded that the cytotoxic action of TQ is mediated by ROS.

**Discussion**

Cancer is a genetic disease that involves a number of inherited genetic alterations leading to changes in cell physiology, following progression and the development in to malignant tumors. Recently, Curtis et al.27 have suggested that breast cancer can be sub-categorized in to 10 sub-groups depending upon the inherited and acquired mutational variants. This leads to the suggestion that it is quite unlikely for the development of a single drug or even a combination of drugs targeting genetic abnormalities for the management or prevention of cancer. However, in all types of cancer there are certain metabolic changes that have been found to be common. The abundance of certain metabolic enzymes and transporters has been suggested to result in poor clinical outcome of the treatment of various forms of cancer. Elevated levels of copper have been reported in studies estimating the levels of different metal in tumors.

It is generally understood that antioxidants counteract ROS production and inhibit the ROS-induced oxidative DNA damage and therefore reduce the risk of cancer. However, some data in the literature suggests that the antioxidant properties of such compounds may not fully account for their chemopreventive and therapeutic properties.25,28 Many such plant-derived antioxidants have been shown to be capable of inducing apoptosis through the induction of ROS in different cancer cell lines.36–38 Over the last several years, this laboratory has extensively characterized oxidative DNA cleavage reactions mediated by polyphenolic antioxidants in the presence of copper ions.31–34 The above results provide evidence for the reduction of Cu(II) to Cu(I) by TQ and the possible formation of a ternary complex of the TQ-Cu(II)-DNA is formed. The major conclusions that are drawn from these results are (i) TQ is able to cause DNA breakage in the absence of added copper ions in lymphocytes presumably through mobilization of endogenous copper ions; this redox cycling of copper leads to the generation of various ROS, particularly the hydroxyl radical, which may serve as the proximal DNA-cleaving agent; (ii) TQ induces a dose-dependent increase in the cell proliferation of prostate cancer cell lines; the compound-induced cell proliferation inhibition and apoptosis induction in cancer cells are reversed in the presence of copper chelator, neocuproine, confirming the role of copper in the cytotoxic action of the antioxidants; (iii) further, the redox cycling of copper in the presence of TQ leads to the generation of ROS as evidenced by the decrease in apoptosis induction in the presence of scavengers of ROS. Thus, providing further evidence and identifying copper as a molecular target for the cancer cell inhibition property of antioxidant TQ.

It has been reported that several chemopreventive agents that are antioxidants at some concentrations become pro-oxidants at other concentrations.35 Several lines of evidence suggest that TQ has potent anion scavenging abilities in different models.36,37 However, TQ with its respective semiquinone radicals may generate superoxide anion radicals.6 Evidence in the literature shows that various quinones (e.g., furanonaphthoquinones, pyrrolo[2,1–c][1,4] benzodiazepine-anthraquinone, 2-acylamino-1,4-napthoquinone and 1,4-napthoquinone) induce apoptosis in cancer cells by generating ROS.38–40 Recent studies have shown that TQ-induced apoptosis in colon cancer and prostate cancer cell lines is mediated by ROS.11,12

**Table 2** Effect of ROS scavengers on TQ-induced apoptosis in cancer cell lines

| Cell lines | Apoptosis (folds) | Effect of scavengers (% inhibition) |
|------------|-------------------|------------------------------------|
| PC3        |                   |                                    |
| Untreated  | —                 |                                    |
| TQ         | 2.8               |                                    |
| + TU       | 1.29*             | 53.93                              |
| + SOD      | 1.59*             | 43.21                              |
| + Cat      | 1.72              | 38.57                              |
| LNCaP      |                   |                                    |
| Untreated  | —                 |                                    |
| TQ         | 3.3               |                                    |
| + TU       | 1.19*             | 63.94                              |
| + SOD      | 1.67*             | 49.39                              |
| + Cat      | 1.82*             | 44.85                              |

PC3 and LNCaP prostate cancer cell lines were incubated with different ROS scavengers, and treated with TQ. TQ, 5 μM; TU, 700 μM thiourea; SOD, 100 μg/ml superoxide dismutase; Cat, 100 μg/ml catalase.

*P<0.05 compared with treated control.
stilbenes, and ascorbic acid (known to possess anticancer properties), which also exhibit prooxidant DNA-damaging properties. The generation of oxygen radicals in the proximity of DNA is well established as a cause of strand scission. It is generally recognized that such reactions with DNA are preceded by the association of the ligand with DNA, followed by the production of oxygen radicals at that site. The results presented here show that TQ is capable of binding to DNA as well as copper, and thus it would be reasonable to assume that a similar mechanism operates in the case of TQ-Cu(II)-mediated DNA cleavage. Thus, the mechanism proposed by us is an alternative, non-enzymatic and copper-dependent pathway for the cytotoxic action of certain anticancer agents that are capable of mobilizing and reducing endogenous copper. Such a common mechanism better explains the anticancer effects of antioxidants with diverse chemical structures as also the preferential cytotoxicity toward cancer cells.

Materials and Methods

Materials. TQ, DMSO, agarose (normal melting and low melting), phosphate-buffered saline Cu²⁺ and Mg²⁺ free, RPMI 1640 media, histopaque 1077, metal chelators (neocuproine, bathocuproine disulfonic acid, deferoxamine mesylate and histidine), calf thymus DNA, cupric chloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were commercial products of analytical grade.

Preparation of stock solutions of TQ. TQ was dissolved in DMSO just before use as stock of 3 mM. Upon addition to reaction mixtures, in the presence of buffers and at the concentrations used, the compounds remained in solution and did not lead to any appreciable change in pH. To test any effect of solvent on the DNA breakage, DMSO solution was added to the cells at the final concentration of 2% (v/v), which was the highest concentration of DMSO used in the test compound-treated reaction medium. No difference was observed with or without DMSO indicating that the DMSO at the tested concentrations did not influence the results.

Hydroxyl radical generation. Hydroxyl radical production by increasing concentrations of TQ in the presence of 50 μM Cu(II) was detected by the method of Quinlan and Gutteridge. Calf thymus DNA (100 μg) was used as a substrate and the malondialdehyde generated from deoxyribose radicals was assayed by recording the absorbance at 532 nm.

Isolation of lymphocytes. Fresh heparinized blood samples (2.0 ml) from non-smoking healthy donors were obtained by venipuncture and diluted suitably in Ca²⁺ - and Mg²⁺-free PBS for each experiment. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma Diagnostics, St. Louis, MO, USA), and the cells were finally suspended in RPMI 1640.

Viability assessment of lymphocytes. The lymphocytes were checked for their viability before the start and after the end of the reaction using Trypan blue exclusion test. The viability of the cells was found to be greater than 95%.

Alkaline single-cell gel electrophoresis (comet assay). Treatment of whole lymphocytes with TQ and the subsequent comet assay was performed essentially as described earlier. As the DNA breakage had to be compared with that in lymphocyte nuclei, the treatment of cells with TQ was done on slides rather than in microcentrifuge tubes.

Cell lines and reagents. Prostate cancer cell lines PC3, LNCaP, DU145, C42B were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA). The media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in a 5% CO₂-humidified atmosphere at 37 °C. Stock solution of TQ (25 mM) was made by dissolving in DMSO and fresh stocks were made for every individual assay. The stock solutions of various chelators of metal ions—neocuproine/deferoxamine mesylate/histidine—were made in phosphate-buffered saline (PBS) at a final concentration of 50 mM and were always made fresh just before experiments.

Cell growth inhibition studies by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Prostate cancer cells were seeded in 96-well microtiter culture plates. After overnight incubation, normal growth medium was removed and replaced with a fresh medium containing DMSO (vehicle control) or different concentrations of test compounds as indicated for individual experiments. Various chelators were added in individual assays as mentioned in respective experiments. After 3 days of incubation, 25 μl of MTT solution (5 mg/ml in PBS) was added to each well and incubated further for 2 h at 37 °C. Upon termination, the supernatant was aspirated and the MTT formazan, formed by metabolically viable cells, was dissolved in DMSO (100 μl) by mixing for 30 min on a gyratory shaker. The absorbance was measured at 595 nm on Ultra Multifunctional Microplate Reader (TECAN, Durham, NC, USA). Each treatment had eight replicate wells and the amount of DMSO in reaction mixture never exceeded 0.1%. Moreover, each experiment was repeated at least three times.

Statistical analysis. The statistical analysis was performed as described by Tice et al. and is expressed as ± S.E.M. of three independent experiments. A Student’s t-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. P-values < 0.05 were considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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