

# Inhibitory effect of green tea extract and (-)-epigallocatechin-3-gallate on mammalian thioredoxin reductase and HeLa cell viability

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Received May 5, 2008; Accepted July 4, 2008

DOI: 10.3892/or\_00000169

**Abstract.** Mammalian cytosolic thioredoxin reductase (TrxR1) is an attractive target for developing cancer chemopreventive agents since its inhibition is associated with a reduced growth of cancer cells. However, the known inhibitors of this enzyme mostly have a toxic effect on human health. We report on a non-toxic inhibitor, green tea. TrxR1 was found to be inhibited by green tea extracts (Gte) with an IC<sub>50</sub> value of 256 µg/ml. Catechins, the major components of Gte, showed various inhibitory effects, in which (-)-epigallocatechin-3-gallate (EGCG) exhibited a stronger inhibition than any other catechins tested. The inhibition of TrxR1 by EGCG was close to competitive ( $K_i = 64 \mu\text{M}$ ) with substrate DTNB and was non-competitive ( $K_i = 92 \mu\text{M}$ ) with co-enzyme NADPH. The preincubation of TrxR1 with EGCG led to irreversible enzyme inactivation in a time-dependent manner, which was highly effective in the presence of NADPH. The inactivation included an equilibrium step used to form a reversible TrxR1-EGCG complex (*EI*) (dissociation constant  $K_i^* = 43 \mu\text{M}$ ), and an

isomerization step used to form an irreversible complex (*E\*I*) (rate constant  $k_3 = 4.8 \times 10^{-3} \text{ s}^{-1}$ ). We have identified thiol/selenol groups in the active site as reactive sites that mediated TrxR1 inhibition by EGCG. When cultured HeLa cells were treated with Gte or EGCG for 22-24 h, TrxR1 activity in cell extracts was significantly inhibited, accompanied by a reduction of cell viability in a concentration-dependent manner (IC<sub>50</sub> = 40 µg/ml for Gte and 107 µM for EGCG). The inactivation of TrxR1 by Gte/EGCG is most likely linked to a reduction of HeLa cell viability.

## Introduction

Thioredoxin reductase (TrxR) (EC 1.8.1.9) catalyzes the reduction of thioredoxin (Trx) by NADPH (1). TrxR, Trx and NADPH form a highly conserved Trx system present in living cells from archaea to human (2,3). This system possesses a large number of functions that affect cell growth and apoptosis. For instance, reduced Trx regulates nuclear factor κB (NF-κB) activation (4), and increases the DNA-binding activity of activator protein (AP)-1 via the nuclear signaling protein redox factor-1 (5). NF-κB and AP-1 are important transcriptional factors for cell proliferation. The Trx system also serves as an electron donor for ribonucleotide reductase, which is essential for DNA synthesis (6). The importance of the Trx system in cellular defense against oxidative stress is highlighted by transferring electron to Trx peroxidase (7), recycling vitamin C (8), sparing vitamin E (9), and inducing the expression of manganese superoxide dismutase (MnSOD) (10). Moreover, reduced Trx is a direct inhibitor of apoptosis signal regulating-kinase (ASK)1 that plays an important role in stress-induced apoptosis (11).

TrxR is the only known enzyme catalyzing reduction of the oxidized Trx. Mammalian TrxR is different in structure and properties from the enzymes in bacteria, fungi and plants (3,12,13). Mammalian cells express three isoforms of TrxR which are cytosolic (TrxR1), mitochondrial (TrxR2) and the testis-specific TGR (Trx GSH reductase) (14). The three isoforms are homodimeric selenoproteins, containing Cys-Sec pair in a conserved C-terminal-Gly-Cys-Sec-Gly motif

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**Abbreviations:** C, (+)-catechin; CL-TrxR, calf liver thioredoxin reductase; DNCB, 1-chloro-2,4-dinitrobenzene; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); EC, (-)-epicatechin; ECG, (-)-epicatechin gallate; EGCG, (-)-epigallocatechin-3-gallate; Gte, green tea extracts; 5-IAF, 5-(Iodoacetamide) fluorescein; PG, propyl gallate; rr-TrxR, rat recombinant thioredoxin reductase; Sec, selenocysteine; Sec-to-Cys TrxR, TrxR with Sec<sup>498</sup>-to-Cys replacement; Trx, thioredoxin; TrxR, thioredoxin reductase

**Key words:** thioredoxin reductase, green tea polyphenols, anticancer, (-)-epigallocatechin-3-gallate, selenoprotein

(15,16). Each subunit contains one tightly-bound FAD, one redox-active disulfide close to the N-terminus, and one redox-active selenenylsulfide at the C-terminus (17). Two subunits associate in a head-to-tail manner where the selenenylsulfide closely contacts the redox-active disulfide of the other subunit (13). The catalytic cycle of mammalian TrxR involves the electron transfer between the disulfide and the selenenylsulfide via selenolthiol/selenenylsulfide exchange reactions (17). The selenol of free selenocysteine has a  $pK_a$  of 5.2, and is fully ionized at a physiological pH. The selenocysteine residue in mammalian TrxR serves not only as a catalyst in the reduction of substrates (18), but also as a good target for certain inhibitors, such as motexafin gadolinium (19), cis-diamminedichloroplatinum (II) (20) and arsenic trioxide (21).

Increased levels of the Trx system were observed in certain human carcinoma tissues compared to normal ones, which appears to promote the growth and chemotherapy resistance of cancer cells (22,23). The inhibition of TrxR by antisense RNA (24) or chemical agents, such as diaryl chalcogenides (25), (2,2':6',2''-terpyridine)platinum(II) complexes (26), and 1,2-[bis (1,2-benzisoselenazolone-3 (2H)-ketone)]ethane (27), usually reduces cell growth and induces apoptosis in the investigated cell lines. These findings make TrxR an attractive target for the development of drugs to treat and prevent cancer (28). However, side effects and toxicity of the above inhibitors are serious, which underscores the need for less toxic TrxR1 inhibitors.

Tea [*Camellia sinensis* (Theaceae)] is one of the most widely consumed beverages in the world. Consumption of green tea has been associated with many health benefits, including the prevention of cancer (29). As an anticancer reagent, green tea has obvious advantages over other anticancer substances since it can be taken at a high dose without toxicity. The anticancer effects of green tea have been attributed to the biological properties of the polyphenols in it, especially EGCG (30). Several molecules have been proposed as a target for EGCG, such as mitogen-activated protein kinases, cyclin-dependent kinases, activator protein 1, NF- $\kappa$ B, topoisomerase I, matrix metalloproteinases (31) and a metastasis-associated laminin receptor (32), but whether green tea may inhibit TrxR1 has never been examined. In this study, we provide evidence that green tea as TrxR1 inhibitor can reduce HeLa cell viability.

## Materials and methods

**Materials.** Calf liver TrxR1 (CL-TrxR1) was prepared as described in (15). The rat recombinant TrxR1 (rr-TrxR1) was purified as described previously (33), and human thioredoxin (hTrx) was prepared according to the method described by Ren *et al* (34). EGCG was purchased from ICN Biomedicals, Inc. Other catechins, NADPH, DTNB, 5-IAF, insulin, DTT and HEPES were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Chinese longjing green tea leaves were from Zhang Yi Yuan tea shop (Beijing, P.R. China). The other reagents were local products of analytical grade.

**Preparation of green tea extracts (Gte).** Dried green tea leaves were crushed and extracted with 50% ethanol at a ratio of 1:20

(w:v) at room temperature for 3 h. The extracts were centrifuged at 1500 x g for 15 min, and the supernatant was used in the subsequent experiments as Gte with a concentration of 24 mg/ml.

**Kinetic studies on the inhibition of TrxR1 by Gte and tea catechins.** DTNB reduction assay (35) was employed in measuring TrxR1 activity *in vitro*. The activity was measured using an Amersham Pharmacia Ultrospec 4300 pro UV-Vis spectrophotometer at 20°C, and monitored by an increase of absorbance at 412 nm due to TNB<sup>-</sup> anion formation.

To study the concentration-dependent inhibition, the TrxR1 was preincubated with various concentrations of Gte, EGCG or other tea catechins in the presence or absence of 0.2 mM NADPH for 5 min, respectively. The reaction was initiated by the addition of a 500  $\mu$ l assay mixture containing 1 mM DTNB, 0.1 M potassium phosphate, pH 7.5 and 1 mM EDTA.

To investigate the steady-state kinetic mechanism, the kinetic parameters for DTNB reduction were determined by measuring the initial rate of TrxR1 activity at various DTNB or NADPH concentrations in the presence and absence of EGCG. Three concentrations of EGCG were tested. The inhibition constant ( $K_i$ ) of the competitive inhibition was determined from re-plots of the apparent  $K_M$  (obtained from Lineweaver-Burk plots at each concentration of EGCG) versus EGCG concentration. The inhibition constant ( $K_i$ ) of the non-competitive inhibition was determined from re-plots of the apparent  $1/V_{max}$  (obtained from Lineweaver-Burk plots at each concentration of EGCG) versus the concentration of EGCG.

For the time-dependent inactivation, rate and dissociation constants were assessed using the method described by Kitz *et al* (36). EGCG at 0, 0.35, 0.5, 0.6, 0.9 and 1.4 mM was pre-incubated with NADPH-reduced enzyme (0.33  $\mu$ M) at 20°C, respectively. At the indicated time intervals, aliquots (16  $\mu$ l) of the mixture were transferred into the cuvette containing 500  $\mu$ l of DTNB assay mixture to test the remaining TrxR1 activity.

**Reversibility of EGCG time-dependent inhibition.** To characterize whether the inhibition is reversible, the NADPH-reduced rr-TrxR1 was incubated with EGCG at a ratio of 214 nmole/nmole (EGCG/TrxR) at 20°C. A control was prepared under the same condition except without EGCG. The incubation mixture set aside after 1 min of incubation was assumed as a 0% inhibition sample. An aliquot mixture was assayed for TrxR1 activity at various time intervals. After 30 min of incubation, the mixture was applied to Sephadex G-25 column, and eluted with 50 mM potassium phosphate, pH 7.5 and 1 mM EDTA, in order to separate EGCG from the enzyme. The fractions containing proteins were combined and assayed for TrxR1 activity and protein concentration.

**Effect of EGCG on the labeling of TrxR with 5-IAF.** rr-TrxR1 was pre-incubated with NADPH at a concentration ratio of 1:70 for 2 min. The reduced rr-TrxR1 (8.45  $\mu$ M) was incubated with EGCG (182  $\mu$ M or 3.69 mM, being 10- or 200-fold over the concentration of rr-TrxR1 subunits) for 3 h. A control was set with 10% DMSO substituting for EGCG.

**SPANDIDOS PUBLICATIONS** inhibition mixture was mixed with IAF solutions of 2.8  $\mu$ M and 6.5, respectively, for 5 h at 37°C, with 2.8  $\mu$ M rr-TrxR1 subunits versus 275  $\mu$ M IAF. The mixtures were then subjected to SDS-PAGE in a 10% gel and the bands were analyzed by Pharmacia Biotech Image Master VDS. Band leader version 3.0 was used for quantification of the fluorescence intensity.

**Cell culture and preparation of cell extracts.** HeLa, a human cervical carcinoma cell line, were cultured routinely in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS (PAA, Pasching, Austria), 2 mM glutamine (PAA) and 100 U/ml penicillin/streptomycin (PAA). The cells were incubated at 37°C in a 90% humidified atmosphere containing 5% CO<sub>2</sub>.

HeLa cells were preincubated for 24 h with designed concentrations of Gte or EGCG. The control group contained the same amount of 50% ethanol instead of the inhibitors. Cells were harvested and washed twice with cold buffer containing 50 mM potassium phosphate, pH 7.5, 2 mM EDTA and 1 mM PMSF. After centrifugation (7,000 rpm for 10 min at 4°C), the cell pellets were washed once with the same buffer and centrifuged again. The resulting pellets were homogenized by sonication in the above buffer. The homogenates obtained were centrifuged at 10,000 rpm for 30 min. The resulting supernatants were heated at 55°C for 5 min and centrifuged at 10,000 rpm for 30 min. The supernatants were concentrated with Ultrafree-MC filter (M<sub>r</sub> cut-off 30 kDa) at 6,000 rpm to 200  $\mu$ l, and washed with 50 mM potassium phosphate, pH 7.5 and 1 mM EDTA twice. The resulting materials were used as the cell extracts.

**Cell viability assays.** Cell viability was determined by using the cell proliferation kit II (Roche Applied Science, Mannheim, Germany). In brief, cells were seeded in 96-well plates to a density of 50% about 24 h before treatment. The cells were washed with phosphate buffer (PBS, 20 mM sodium phosphate and 150 mM NaCl, pH 8.0) and incubated with different concentrations of Gte or EGCG in 115  $\mu$ l of medium for 24 h. Gte was diluted with 50% ethanol into 8 mg/ml. A stock solution of 50 mM EGCG in sterile water was diluted into 1 mM in 50% (v/v) ethanol before use. The amount of viable cells was analyzed in a microplate reader at 490 nm. The absorbance of the sample control without inhibitors or the reagent control without cells was used as a 100 or 0% standard, respectively.

**Assay of TrxR1 activity in cell extracts.** TrxR1 activity in the cell extracts was measured by a Trx-coupled insulin reduction assay (37). The reaction volume was 120  $\mu$ l, which contained 5  $\mu$ M human Trx, 100  $\mu$ M insulin, 0.2 mM NADPH and 5 mM EDTA in 0.1 M potassium phosphate buffer, pH 7.5. The reaction was started by the addition of the cell extracts. After 30 min incubation at 20°C, the reaction was terminated with 500  $\mu$ l of 1 mM DTNB in 8 M guanidine hydrochloride, 50 mM potassium phosphate, pH 7.5 and 1 mM EDTA. A blank, prepared by the addition of the cell extracts after the reaction had been stopped with the DTNB-guanidine-hydrochloride solution, was run for each sample. A<sub>412 nm</sub> of each sample was measured against the sample blank. This blank corrects for SH groups in cellular proteins. The results are

Table I. IC<sub>50</sub> values of Gte, some catechins and analogue of the galloyl group.<sup>a</sup>

The reagents	IC <sub>50</sub> values
Gte	0.256 mg/ml
Gallated catechin	
EGCG	26.0 $\mu$ M
ECG	17.0 $\mu$ M
Ungallated catechin	
C	257.0 $\mu$ M
EC	365.8 $\mu$ M
The analogue of the galloyl group	
PG	379.0 $\mu$ M

<sup>a</sup>To determine IC<sub>50</sub> values, CL-TrxR was incubated with different concentrations of the above inhibitors in the presence of NADPH for 5 min, and the remaining TrxR activity was measured by DTNB assay as described in the text.

expressed as the net absorbance at 412 nm/mg protein. The protein concentration was measured by the CD-protein assay procedure (Bio-Rad). With pure TrxR1 as a standard, 0.2 A<sub>412 nm</sub> corresponded to 37 ng of TrxR.

## Results

**Inhibitory effects of Gte and five green tea polyphenols on TrxR1.** Gte inhibited the purified TrxR1 in a dose-dependent manner with an IC<sub>50</sub> of 256  $\mu$ g/ml (Table I). Catechins are major components of Gte. Among the catechins tested, EGCG and (-)-epicatechin gallate (ECG) were better inhibitors than (-)-epicatechin (EC), (+)-catechin (C) and propyl gallate (PG), respectively (Table I). EC and C lack the galloyl group, and PG is a galloyl moiety analogue that contains the propyl and galloyl groups without A, B and C rings of catechin (Fig. 1). Therefore, A, B and C rings of catechin and the galloyl group are essential structures required for the inhibitory capacity of the tea catechins. As EGCG is an effective inhibitor, we evaluated its effect on TrxR1 activity and reaction mechanism in more detail.

**Inhibitory kinetics of EGCG on TrxR1.** The active site of TrxR1 contains one disulfide and one selenenylsulfide that are NADPH-reducible. The TrxR1 activity, after being treated with EGCG at different concentrations, decreased slightly in the absence of NADPH (the upper curve in Fig. 2A), but decreased significantly in the presence of NADPH (the lower curve in Fig. 2A) with an IC<sub>50</sub> of 26  $\mu$ M. As shown in Fig. 2B, TrxR1 inhibition by EGCG was dependent on the time of incubation. In the presence of NADPH, the rate of activity decrease was much faster (the lower curve in Fig. 2B) than that in the absence of NADPH (the upper curve in Fig. 2B). It is thus apparent that the active site is involved in the inhibition produced by EGCG.

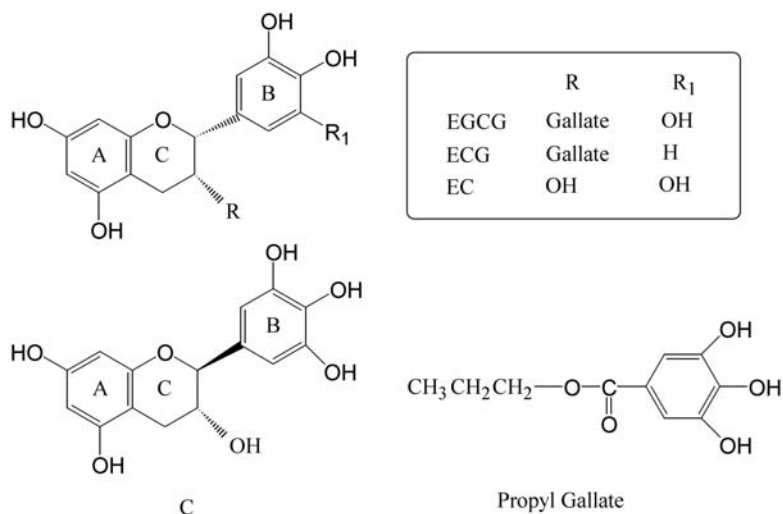


Figure 1. Structures of the green tea polyphenols. The ring nomenclature is defined as A, C, B or D (for gallate) rings and used throughout the text.

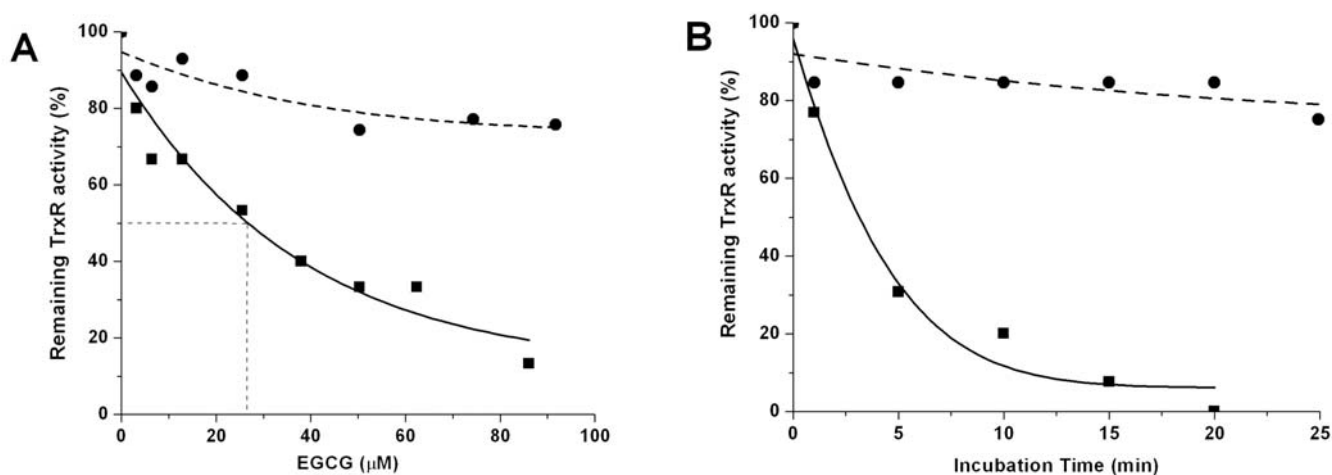
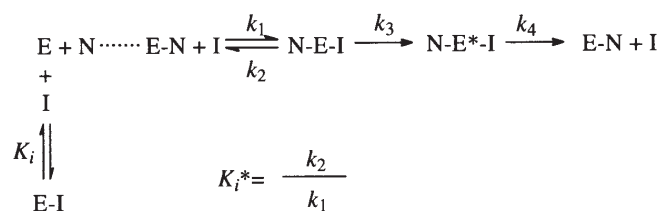


Figure 2. Dose- and time-dependent inhibition of TrxR1 by EGCG. (A) NADPH-reduced (■) or non-NADPH-reduced (also called oxidized) (●) TrxR (10.4 nM) was incubated with the indicated concentrations of EGCG for 5 min at 20°C prior to assay of TrxR1 activity. The remaining TrxR1 activity is expressed as a percentage of the activity in the absence of EGCG. (B) EGCG (4.45 μM) was pre-incubated with TrxR1 (1 nM) in the presence (■) or absence (●) of NADPH (0.57 μM) prior to measuring TrxR1 activity at the indicated time intervals. The remaining TrxR1 activity is expressed as a percentage of the activity at zero time of incubation with EGCG.

Initial kinetic analyses revealed that EGCG was close to a competitive inhibitor towards substrate DTNB (Fig. 3A) with a  $K_i$  value of 64 μM, but was a non-competitive inhibitor against the co-enzyme NADPH (Fig. 3B) with a  $K_i$  value of 92 μM. To the NADPH pre-reduced TrxR1, the time-dependent reduction in activity caused by EGCG was irreversible (Fig. 4). The inactivation mechanism was probed by measuring the rates of inactivation in the presence of different EGCG concentrations. As shown in Fig. 5A, plots of natural logarithm [ln] of remaining activity against incubation time (t) give a series of straight lines at different concentrations of EGCG, displaying first-order kinetics at the EGCG concentrations used. The slopes represent the apparent first-order rate constants ( $k_{app}$ ) for inhibition by the corresponding EGCG concentration. The plot of  $k_{app}$  versus EGCG concentration was a hyperbola, whereas its double-reciprocal plot was linear (Fig. 5B). These results indicated that an inactivation mechanism

included the following steps: EGCG (I) initially binds to NADPH-reduced TrxR1 (E-N) to form an equilibrium complex (N-E-I), which in turn isomerizes to inactivated enzyme-inhibitor complex (N-E\*-I) (Equation 1).



where  $k_3$  is the rate constant for conversion of the reversible complex (N-E-I) to the inactivated enzyme [N-E\*-I] at a high EGCG concentration, and  $K_i^*$  is the dissociation constant for the initial reversible complex [N-E-I]. As we did not obtain



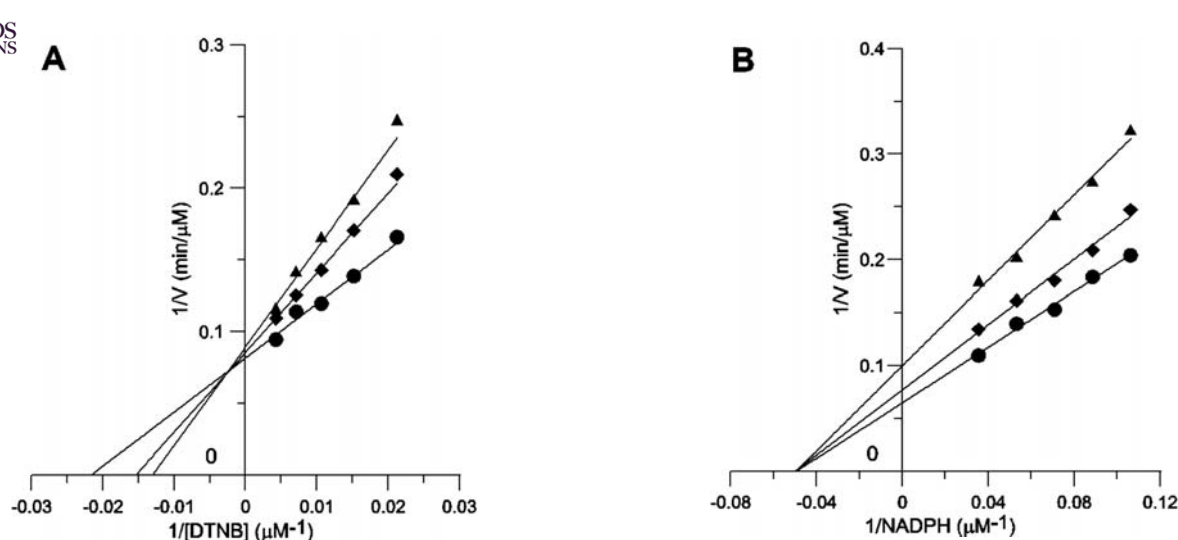


Figure 3. Effect of EGCG on steady-state kinetics of TrxR1. The concentration of TrxR1 used for this study was 11 nM. (A) DTNB was the variable substrate. The fixed concentration of NADPH was 0.2 mM. The concentrations of EGCG were 0 (●), 25.6 (■) and 51.2  $\mu$ M (▲), respectively. (B) The concentration of NADPH was variable. The concentration of DTNB was fixed at 1 mM. The concentrations of EGCG were 0 (●), 25.6 (■) and 51.2  $\mu$ M (▲), respectively. Re-plots of the apparent  $K_M$  or  $1/V_{max}$  (derived from the double-reciprocal plots) versus the concentration of EGCG gave the inhibition constant  $K_i$ .

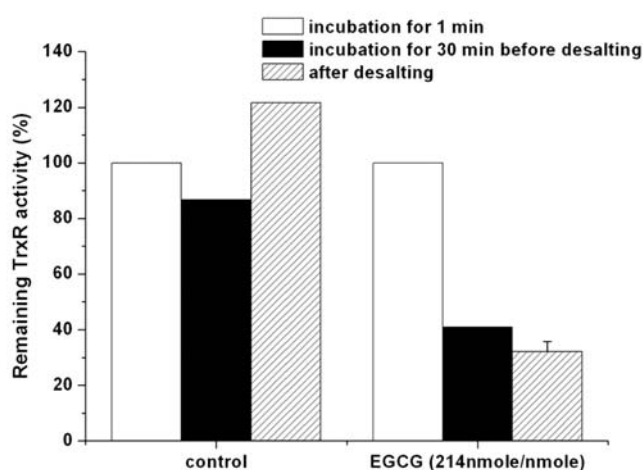


Figure 4. Reversibility of EGCG time-dependent inhibition of NADPH-reduced TrxR1, which was incubated at 20°C with buffer (in control) or with EGCG, respectively (see text). Each incubation mixture was analyzed for TrxR1 activity at the indicated stages of 1 min of incubation (white bars), 30 min of incubation before gel-filtration (black bars) and after gel-filtration (shaded bars). The activity is expressed as a percentage of the activity at 1 min of incubation. The data after gel-filtration in the EGCG-treated group were the mean  $\pm$  range of two independent experiments.

any recovery of TrxR1 activity after gel filtration,  $k_4$  must be very small and can be neglected. The amount of remaining activity was dependent on  $k_3$ .

The natural logarithm  $[\ln]$  of the remaining activity is given by Equation 2:

$$\ln \frac{[E]}{[E_{total}]} = - \frac{k_3 \cdot t}{1 + \frac{K_i^*}{[I]}}$$

where  $[E]$  is free TrxR1 concentration,  $[E_{total}]$  is total TrxR1 concentration,  $t$  is incubation time, and  $[I]$  is EGCG concentration. The semi-logarithmic plots of the remaining enzyme activity  $[E]/[E_{total}]$  versus  $t$  appeared to be first-order, and the slopes represent  $k_{app}$  for inhibition by the corresponding EGCG concentration.

In Equation 3, for  $[I] \gg [E_{total}]$ , let

$$k_{app} = \frac{k_3}{1 + \frac{K_i^*}{[I]}}$$

A double-reciprocal plot of the  $k_{app}$  versus  $[I]$  gave Equation 4:

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_i^*}{k_3} \cdot \frac{1}{[I]}$$

Values for  $k_3$  ( $4.8 \times 10^{-3} \text{ s}^{-1}$ ) and  $K_i^*$  (43  $\mu$ M) were therefore obtained from the y intercept, and the curve slope of the plot, respectively.

*EGCG interferes with incorporation of 5-IAF into thiols and/or selenol in the active site.* An active site of TrxR1 contains three cysteine residues (Cys<sup>59</sup>, Cys<sup>64</sup> and Cys<sup>497</sup>) and one selenocysteine residue (Sec<sup>498</sup>), which are joined in two NADPH-reducible bridges. As with free thiol, free selenol may react with 5-IAF (a sulfhydryl-directed fluorescent reagent). The possible effects of EGCG on 5-IAF incorporation into NADPH-reduced TrxR were determined at pH 6.5 or 8.5, respectively, because the  $pK_a$  value is  $\sim 5.2$  for selenol and is  $>8$  for thiol (38). It turned out that the difference of the fluorescence intensity of TrxR at pH 8.5 or 6.5 was very small (Fig. 6), indicating that not only selenol but also thiol groups

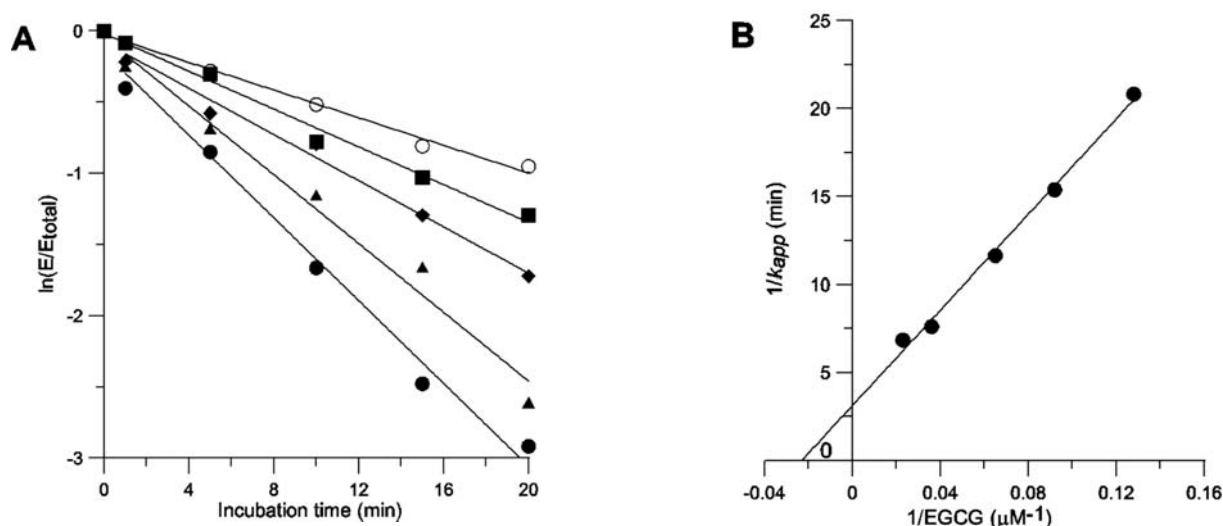


Figure 5. Time-dependent inactivation of NADPH-reduced TrxR1 by EGCG. (A) TrxR1 ( $0.33 \mu M$ ) was incubated at  $20^\circ C$  with 0.35 ( $\circ$ ), 0.5 ( $\blacksquare$ ), 0.6 ( $\blacklozenge$ ), 0.9 ( $\blacktriangle$ ) and 1.4 ( $\bullet$ ) mM EGCG in the presence of NADPH. At indicated time intervals (0, 1, 5, 10, 15 and 20 min), aliquots of the incubation mixture were removed, and the remaining activity was determined by a 32-fold dilution into the assay mixture. The data are fitted to Equation 2 (see text). The  $[E]/[E]_{total}$  was calculated from the ratio of the remaining activity to that of zero incubation time. The slope of the individual line gives the apparent rate constant  $k_{app}$  at the corresponding EGCG concentration. (B) A double-reciprocal plot of the  $k_{app}$  versus EGCG concentrations according to Equation 4 (see text). The dissociation constant  $K_i^*$  of the reversible N-E-I complex was derived from the  $x$  intercept, and the rate constant  $k_3$  was from the  $y$  intercept.

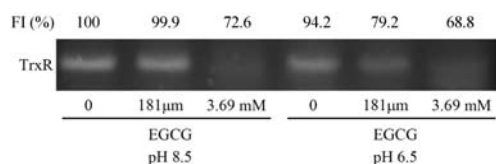


Figure 6. Effect of EGCG on 5-IAF labelling of TrxR1. The NADPH pre-reduced rr-TrxR1 was incubated with increasing concentrations of EGCG respectively, and labelled with IAF at pH 8.5 (left) or pH 6.5 (right) as described in Materials and methods. As the concentration of EGCG increases, the fluorescent intensity (FI) representing the extent of 5-IAF labelling decreases.

were modified by 5-IAF at the two pH conditions. Once NADPH-reduced TrxR1 was treated with EGCG, incorporation of the 5-IAF fluorescence into TrxR1 decreased at the two pH conditions as the concentration of EGCG increased (Fig. 6). These results suggested that selenolate and thiolate were modified by EGCG, which blocked the subsequent binding of 5-IAF.

*The inhibitory effects of Gte and EGCG on TrxR1 activity in HeLa cells and their viability.* TrxR1 inactivation is frequently related to the growth/proliferation inhibition or apoptotic induction in cell lines (39). It is of note to determine whether Gte or EGCG would affect cancer cell viability. HeLa cells, cultured with Gte or EGCG for 24 h, showed a dose-dependent decrease in cell viability with  $IC_{50}$  values of  $40 \mu g/ml$  for Gte and  $107 \mu M$  for EGCG, respectively (Fig. 7A and B). After a 22 h treatment of HeLa cells, Gte, at  $149 \mu g/ml$ , decreased the TrxR specific activity in HeLa cell extracts by 90%, and EGCG, at  $100 \mu M$ , decreased the TrxR specific activity in HeLa cell extracts by 70% (Fig. 7C). Thus, the reduction of HeLa cell growth by Gte and EGCG appeared to be associated with the inhibition of TrxR1 activity.

## Discussion

TrxR1 is overexpressed in certain tumor cells compared to levels in its equivalent normal cells, and its high levels can add to the problem of chemotherapy resistance (40). Tumor cells that grow rapidly require a higher concentration of DNA than normal cells, and TrxR supports DNA synthesis (41,42). Thus, tumor cells should be more sensitive to inhibitors of TrxR. Our findings that TrxR1 was inhibited by Gte and EGCG, the most abundant catechin in Gte (43), are in line with the reports about the prevention of carcinogenesis by drinking green tea.

The individual catechins tested exhibited different inhibitory effects on TrxR1, and their inhibition was in the decreasing order of  $ECG \geq EGCG > C > PG \approx EC$  (Table I), which is related to their structure. The gallated catechins showed stronger inhibitory effects as compared to ungallated catechins, but gallic acid ester itself (the case of PG) was not an effective inhibitor. These results indicate that the catechins that have gallate ester at the  $C_3$  position in the C-ring are essential for the potent inhibition of TrxR1. Similar to EGCG, ECG was also a potent inhibitor, indicating that the hydroxyl groups on B-ring appear not to be important. Although EGCG is a weaker inhibitor than arsenic trioxide [an effective cancer therapeutic drug (21)] for TrxR1, EGCG has advantages in the treatment and prevention of cancer because it has few adverse side effects, often associated with TrxR1 inhibitors such as arsenic trioxide which is currently in clinical use.

A pronounced difference in the inhibition potency exhibited by EGCG with native and NADPH-pre-reduced TrxR1 indicates that EGCG binds to the active site of the enzyme, further evidence for which comes from the interference of 5-IAF incorporation by EGCG. NADPH-reducible thiol/selenol groups appeared to be direct targets for EGCG. Normally these thiol/selenol groups are involved in an electron

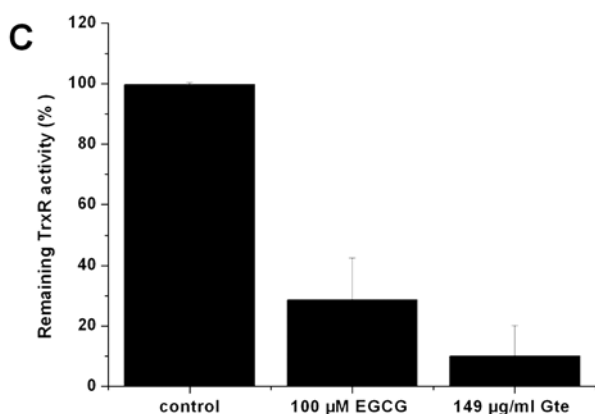
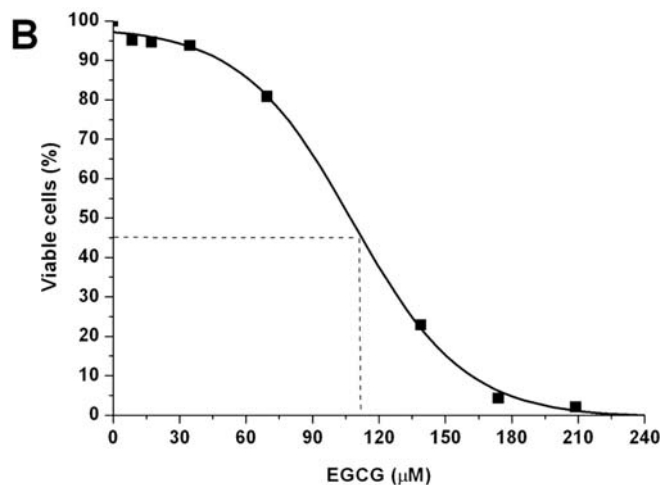
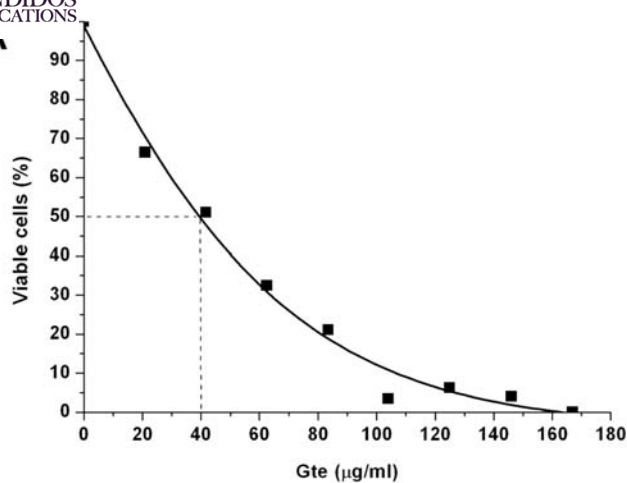


Figure 7. Effects of Gte and EGCG on HeLa cell viability and cellular TrxR1 activity. HeLa cells were cultured in the medium containing different concentrations of Gte (A) or EGCG (B) for 24 h, respectively. The  $IC_{50}$  values of cell viability are expressed as the concentration of Gte or EGCG with 50% of viable cells. (C) HeLa cells were treated with 100  $\mu$ M EGCG or 0.149 mg/ml Gte for 22 h, respectively. The cells were harvested to make cell extracts, and cellular TrxR1 activity was measured as described in Materials and methods. TrxR1 activities are expressed as a percentage of the control sample without treatment of Gte or EGCG.

transfer from NADPH to substrate via selenolthiol and selenenylsulfide exchange reactions (17). EGCG binding to these residues blocks the reaction of a highly reactive selenolate with substrate, making TrxR1 inactive as a reductant.

Our results showed the importance of time-dependent inhibition to the potency of EGCG against TrxR1. The kinetic character of time-dependent inhibition of TrxR1 by EGCG is featured by the formation of a reversible complex followed by a chemical modification that irreversibly inactivates the enzyme (the lower curve in Fig. 2B). A time-dependent inactivation of TrxR1 appears due to the irreversible reaction of EGCG with the thiol and selenol groups at the active site that is conserved in the three isoforms of mammalian TrxR. Our modelling of EGCG into an active site of TrxR1 will be described in a later study.

EGCG reduces HeLa cell viability in a concentration-dependent manner ( $IC_{50} = 107 \mu$ M). EGCG has been reported to have pro-oxidant activity (44). The  $H_2O_2$  generation, pro-oxidant and apoptotic effects induced by EGCG have been associated with its inhibition on cancer cell growth (45). Our results showed that the reduction of HeLa cell viability by EGCG or Gte was accompanied by the inhibition of TrxR1 activity in HeLa cell extracts with EGCG or Gte. It is well known that TrxR1 plays a pivotal role in keeping the balance of cellular redox status via catalyzing the reduction of a broad range of substrates, such as Trx (14), protein disulfide isomerase (46), selenodiglutathione (47), *S*-nitrosoglutathione (48), ebselen (49), dehydroascorbate (8), hydrogen peroxide (35) and alkyl hydroperoxides (50). Therefore, TrxR1

inhibition by EGCG or Gte increases ROS concentration, reduces the supply of deoxyribonucleotides and DNA synthesis, as well as activates ASK1 to induce apoptosis (51), which appears to be a direct mechanism for EGCG or Gte to inhibit the growth of tumor cells. These data provide strong evidence that one of the major sites of action of EGCG and Gte in cells is TrxR, and may explain the observation that the administration of EGCG, an antioxidant compound, caused a decrease in the plasma antioxidant activity (52).

Under the fasting condition, the oral administration of tea catechin capsules at 800 mg dose levels may increase the human plasma levels of EGCG up to  $3.37 \pm 1.65 \mu$ M (53). This plasma level of EGCG is relatively lower than the concentration required to inhibit TrxR1, but EGCG/green tea are very safe, which is an important advantage, and green tea can be consumed in very high doses over a very long period of time without any toxic effects. Since the inhibition of TrxR1 activity is considered an important mechanism for the prevention/treatment of cancer by chemopreventive agent(s) (42), further elucidation of the mechanisms, by which EGCG inhibits mammalian TrxR, would provide useful information for developing non-toxic inhibitors of TrxR1 to be used as potential therapeutic agents.

#### Acknowledgements

This study was supported in part by a grant (Project 30470374) from the National Natural Science Foundation of China, from the Graduate University of Chinese Academy of Sciences



(Project KYQD200509) and a fellowship from Hegelen Stiftelse of Sweden.

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