

## Black Tea Polyphenols Inhibit Tumor Proteasome Activity

TASKEEN MUJTABA and Q. PING DOU

*The Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute and Departments of Oncology, Pharmacology and Pathology, School of Medicine, Wayne State University, Detroit, MI, U.S.A.*

**Abstract.** *Tea is a widely consumed beverage and its constituent polyphenols have been associated with potential health benefits. Although black tea polyphenols have been reported to possess potent anticancer activities, the effect of its polyphenols, theaflavins on the tumor's cellular proteasome function, an important biological target in cancer prevention, has not been carefully studied. Here black tea extract (T5550) enriched in theaflavins inhibited the chymotrypsin-like (CT) activity of the proteasome and proliferation of human multiple myeloma cells in a dose-dependent manner. Also an isolated theaflavin (TF-1) can bind to, and inhibit the purified 20S proteasome, accompanied by suppression of tumor cell proliferation, suggesting that the tumor proteasome is an important target whose inhibition is at least partially responsible for the anti-cancer effects of black tea.*

Black tea one of the world's most popular beverages, is an aqueous infusion of the dried leaves of the plant *Camellia sinensis*, rich in various polyphenols. Tea consumption has been associated with many health benefits including decreased incidence of cancer and its health-promoting effects have been intensively investigated (1). Substantial strides have been made to understand the molecular mechanisms responsible for the cancer-preventive property of tea; however, the conclusions are still elusive.

Theaflavins (TFs) and thearubigins are the major constituents found in black tea and are responsible for its unique taste and bright red-orange color. Thearubigins have higher molecular weight and are poorly characterized chemically and biochemically. The flavins have a benzotropolone ring structure and are a mixture of theaflavin

(TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3'-gallate (TF-2b), and theaflavin-3, 3'-digallate (TF-3) (Figure 1).

Over the past decade the polyphenolic constituents of black tea have been studied extensively to explore their biological properties as chemopreventive agents. Theaflavins are the bioactive flavonoids and TF-1 is generally considered to be the most bioactive and effective against carcinogenesis. Theaflavins in black tea have been reported to possess antibacterial, antitoxin, antiviral and antimutagenic properties (2-4). The antioxidant nature of tea components prevents DNA damage due to oxidative stress or mutagens, by interfering in signaling pathways, and suppressing transcription of certain onco-proteins involved in carcinogenesis. Katiyar and Mukhtar proposed that black tea could prevent carcinogenesis by inhibiting activities of cyclooxygenase and ornithine decarboxylase while enhancing the activities of enzymes such as glutathione peroxidase, catalase and quinoline reductase (5). Based on this evidence tea polyphenols have procured considerable research interest as chemopreventive agents.

Black tea polyphenols have been demonstrated to suppress proliferation and induce apoptosis in a variety of cancer cell lines by modulating various molecular targets (6-8). For instance in prostate cancer LNCaP cells, theaflavins have been reported to trigger apoptosis by inducing the expression of tumor suppressor p53 and down-regulating nuclear factor kappa B (NF- $\kappa$ B) and mitogen-activated protein kinase (MAPK) survival pathways (9). Similarly in human prostate cancer PC-3 cells, theaflavins have been reported to induce the expression of cell cycle modulators p21<sup>waf1/cip1</sup>, cdc25C and cyclin B causing cell cycle arrest in G<sub>2</sub>/M phase (10). In human breast cancer cells with mutated p53, theaflavins were reported to up-regulate Fas expression *via* activation of c-jun N-terminal kinase while simultaneously inhibiting Akt mediated survival pathway leading to loss of mitochondrial transmembrane potential, cytochrome release and apoptosis. (11). In breast cancers with functional p53 theaflavins have been shown to retard cell migration *via* p53 dependent reactive oxygen species (ROS) inducing phosphorylation of p53 by p38MAPK thereby down-regulating NF- $\kappa$ B and NF- $\kappa$ B dependent metalloproteinase expression (12). In JB6 mouse epidermal

*Correspondence to:* Q.P. Dou, The Developmental Therapeutics Program, Barbara Ann Karmanos Cancer Institute, School of Medicine, Wayne State University, 540.1 HWCRC, 4100 John R Road, Detroit, MI 48201-2013, U.S.A. Tel: +1 3135768301 (Office), Fax: +313 5768307, e-mail: dou@karmanos.org

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cells theaflavins were reported to inhibit UV-induced activation of activator protein 1 (AP-1) transcription factor (13). Theaflavins were also reported to alter the balance between pro-apoptotic and anti-apoptotic Bcl-2 family of proteins to trigger apoptosis (14). Also, administration of black tea has been reported to suppress tumor formation and progression in the lung tissues of several mouse models (15-18). Previous reports showed that cancer cells are more sensitive to proteasome inhibition than normal cells and the tumor cellular proteasome now represents a validated and successful target in cancer therapy (19). However the effect of black tea polyphenols on the tumor cellular proteasome function has not been elucidated, with the exception of one abstract on black tea inhibiting the proteasome (20). In the present study, the effect of black tea extract on tumor cellular proteasome function was investigated.

## Materials and Methods

**Cell cultures.** Human multiple myeloma (Arp and Opm1) cells, kindly provided by Dr. Ramesh Batchu (Wayne State University), were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 µg/ml of streptomycin purchased from Invitrogen (Carlsbad, CA, USA). The cells were maintained at 37°C in 5% CO<sub>2</sub>.

**Chemicals.** Black tea extract (T5550) (>80% theaflavins), green tea extract (polyphenon 60), purified theaflavin (TF-1) and purified epigallocatechin gallate (EGCG) were obtained from Sigma Aldrich (St.Louis, MO, USA). All the chemicals were freshly prepared each time for use, by dissolvment in sterile water, except TF-1 that was dissolved in dimethylsulfoxide (DMSO).

**Inhibition of purified 20S proteasome activity.** Briefly, purified human 20S proteasome (35 ng) was incubated with 20 µM fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal CT-like activity) or 25 µM Bz-VGR-AMC (for the trypsin-like activity) from Calbiochem, (San Diego, CA, USA) in 100 µl assay buffer (20 mM Tris-HCl, pH 7.5) in the presence of each extract at various concentrations as indicated or the solvent for 2 h at 37°C, followed by measurement of hydrolysis of the fluorogenic substrates using a Wallac Victor3™ multi-label counter with 355-nm excitation and 460-nm emission wavelengths.

**Determination of cellular 26S proteasome activity in multiple myeloma cell extract.** The Arp and Opm1 multiple myeloma cells were treated with the tea extracts at various concentrations as indicated for 48 h at 37°C. Cell extracts from these cells were then used to measure the proteasomal chymotrypsin (CT)-like activity. Activity in the cell extract was measured following the protocol as described above (21).

**Cell proliferation and viability.** The multiple myeloma cells were grown in a 96-well plate and treated with indicated concentrations of the tea extracts for 24-72 hours followed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay to measure cell proliferation and cell viability as described elsewhere (22).

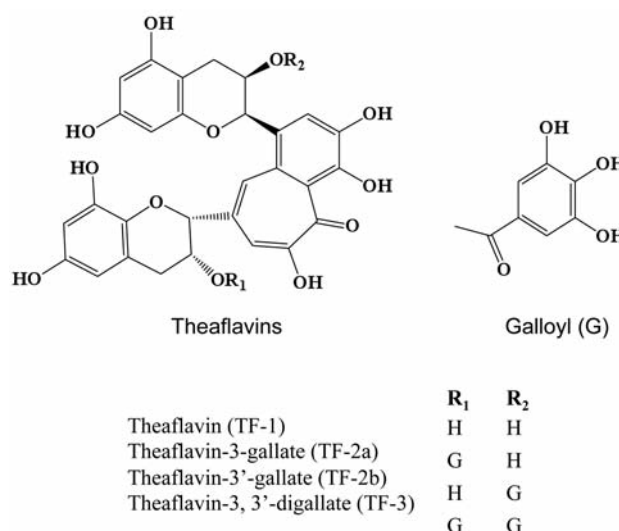


Figure 1. Chemical structures of black tea polyphenols.

**Nucleophilic susceptibility analysis and molecular docking of TF1 to proteasome β5 subunit.** A CAChe workstation (Perkin Elmer Life and analytical Sciences, Shelton, CT, USA) was used to build the chemical structures and the nucleophilic susceptibility analysis was determined using the softwares PM5 geometry and wave function in water. Surface analysis was performed, in which a colored 'bull's-eye' with a white center denotes atoms that are highly susceptible to nucleophilic attack. The PDB files generated in the CAChe were imported to AutoDock for molecular docking as described previously (23). *In silico* docking was performed on a Linux Red Hat 9.0 based platform using AutoDock 3.0, according to a previously published protocol (24). The eukaryotic yeast 20S proteasome used in this docking study was selected from the protein databank (Ref. no 1JD2).

## Results

**Effects of tea extracts from green tea and black tea on the activities of both purified 20S and cellular 26S proteasome.** Both polyphenon 60 (green tea extract) and T5550 (black tea extract) markedly inhibited the CT-like activity of the purified 20S proteasome at the two tested concentrations (10 and 20 µg/ml, Figure 2A). When the cellular extracts from treated human Arp and Opm1 multiple myeloma cells were tested, in contrast to the green tea extract polyphenon 60, the black tea extract, T5550, potently inhibited the CT-like activity of the cellular proteasome in both the Arp and Opm1 multiple myeloma cells (Figure 2B and C).

**Effect of tea extracts on cell proliferation.** After 24 hr treatment both polyphenon 60 and T5550 inhibited the cell proliferation in Arp and Opm1 cells in a dose-dependent manner at relatively higher concentrations than the one required for inhibition of the purified proteasome system. The black tea extract was more potent compared to polyphenon 60 from green tea (Figure 2D and E).

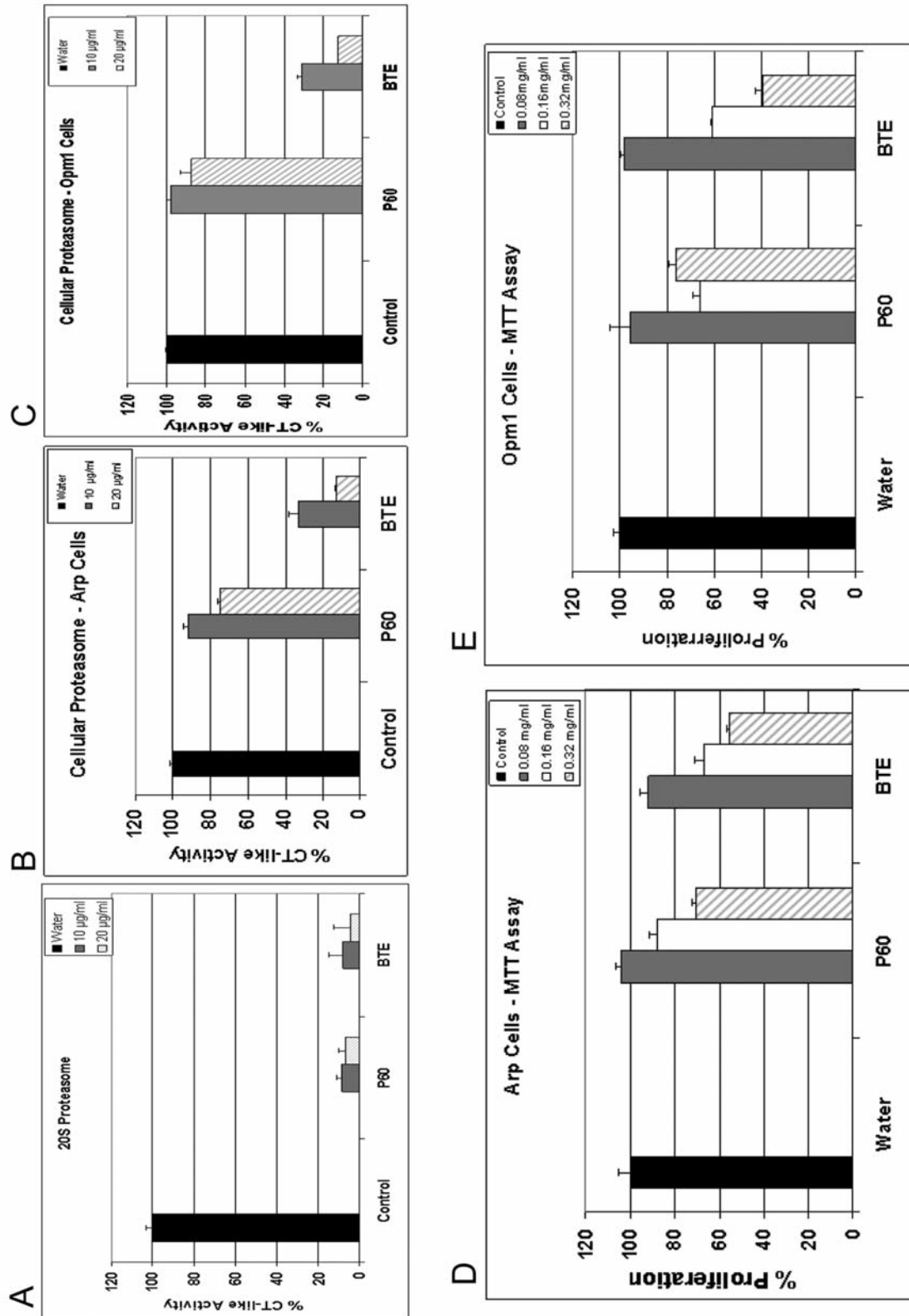


Figure 2. Effect of tea extracts on tumor proteasome activity and cell proliferation. Chymotrypsin-like (CT-like) activity of the purified 20S proteasome (A). CT-like activity of the proteasome from Arp multiple myeloma cells (B). CT-like activity of the proteasome from Opm1 multiple myeloma cells (C). Proliferation after 24h incubation of Arp cells (D). Opm1 cells (E). P60: polyphenon 60, green tea extract; T5550: black tea extract.

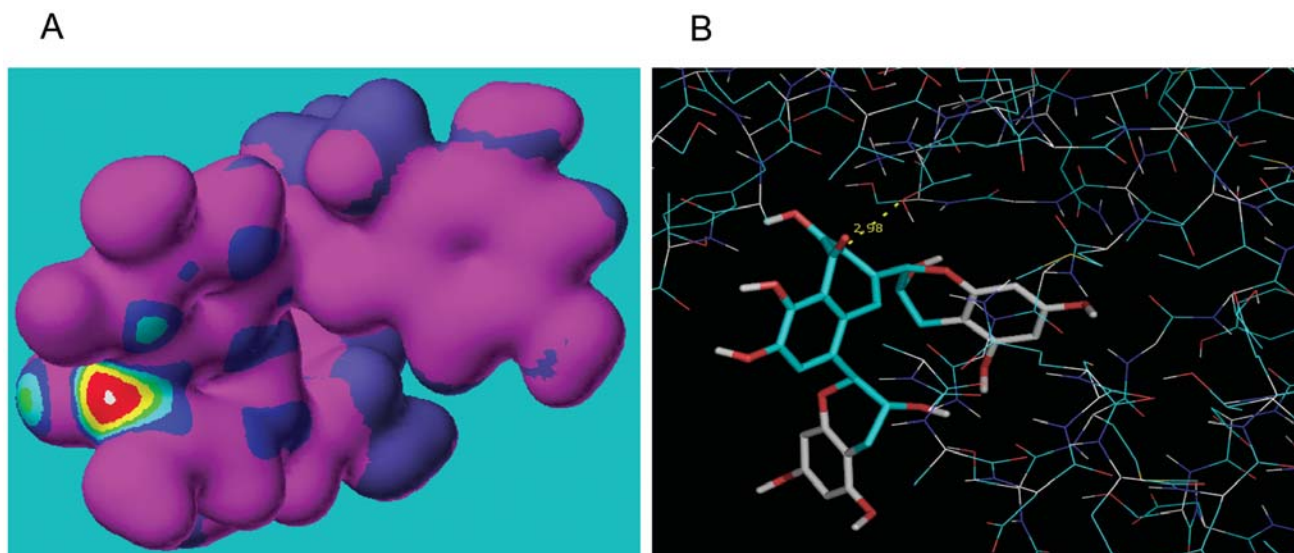


Figure 3. Computational analysis of theaflavin (TF-1) structure. TF-1 structure showing nucleophilic susceptibility regions (A), Ball and stick model showing the distance between N-terminal threonine in  $\beta 5$  subunit of proteasome and theaflavin (B).

**Computational docking of TF-1 structure.** The computational docking studies of purified theaflavin1 (TF-1) showed high nucleophilic susceptibility regions in its structure. The distance between the N terminal threonine of  $\beta 5$  and carbonyl carbon of TF-1 was about 2.98 Å which is comparable to EGCG from green tea. The lowest docking energy was found to be ( $\pm$ ) 10.8 Kcal/mol which is in the acceptable range for binding to the  $\beta 5$  subunit of the proteasome (Figure 3).

**Effect of TF-1 on proteasome activities and tumor cell proliferation.** Based on the results from the computational docking studies the potential of purified TF-1 to inhibit proteasome CT-like and trypsin-like activities of the purified 20S proteasome was investigated. TF-1 inhibited the CT-like and trypsin-like activities of the purified 20S proteasome in a dose-dependent manner (Figure 4A and B). At lower concentrations the inhibition of the CT-like activities of the proteasome was lower with TF-1 compared to purified epigallocatechin gallate (EGCG) but at higher concentrations it was comparable to EGCG (Figure 4A). TF-1 inhibited the trypsin like activity of the purified 20S proteasome more potently than EGCG from green tea (Figure 4B). TF-1 inhibited cell proliferation in a dose-dependent manner with the addition of drug after every 24 hr for 72 hours in both the Arp and Opm1 multiple myeloma cells. Inhibition by TF-1 was comparable to inhibition by EGCG (Figure 4C-D). The cell proliferation data also suggested that the proteasome is a target of TF-1 and proteasome inhibition by TF-1 contributes to its tumor growth-inhibitory effects.

## Discussion

The black tea extract rich in theaflavins was a potent inhibitor of the purified and cellular proteasome and its inhibitory potency was comparable to green tea polyphenol EGCG we reported previously (23). Using computational docking TF-1 was found to be susceptible to nucleophilic attack by the N-terminal threonine of the proteasome  $\beta 5$  subunit. TF-1 did indeed inhibit the proteasome function and cell proliferation in a dose-dependent manner but it was not as potent as EGCG obtained from green tea (Figure 4A) or the black tea extract itself (Figure 2A) indicating that the other theaflavins from black tea could be important for the potent biological activities of the black tea extract. Due to the costs related to the purified TF-1 the details of proteasome inhibition following TF-1 treatments were not pursued. Future studies should further investigate the molecular targets following proteasome inhibition by black tea polyphenols. Thus, the polyphenolic constituents present in black tea are concluded to exert their biological effects by inhibiting the proteasome and could be used as potential chemopreventive agents against cancer. Henning *et al.*, showed that the relative bioavailability of theaflavins was 70% higher than the one of EGCG in human prostate tissues (25). As theaflavins are more bioavailable they could be more efficient as chemopreventive agents than EGCG.

Accumulating evidence from several studies have suggested that tea polyphenols possess potent anticancer effects and consistently the current study suggests that the combined effect of all the polyphenolic constituents in the



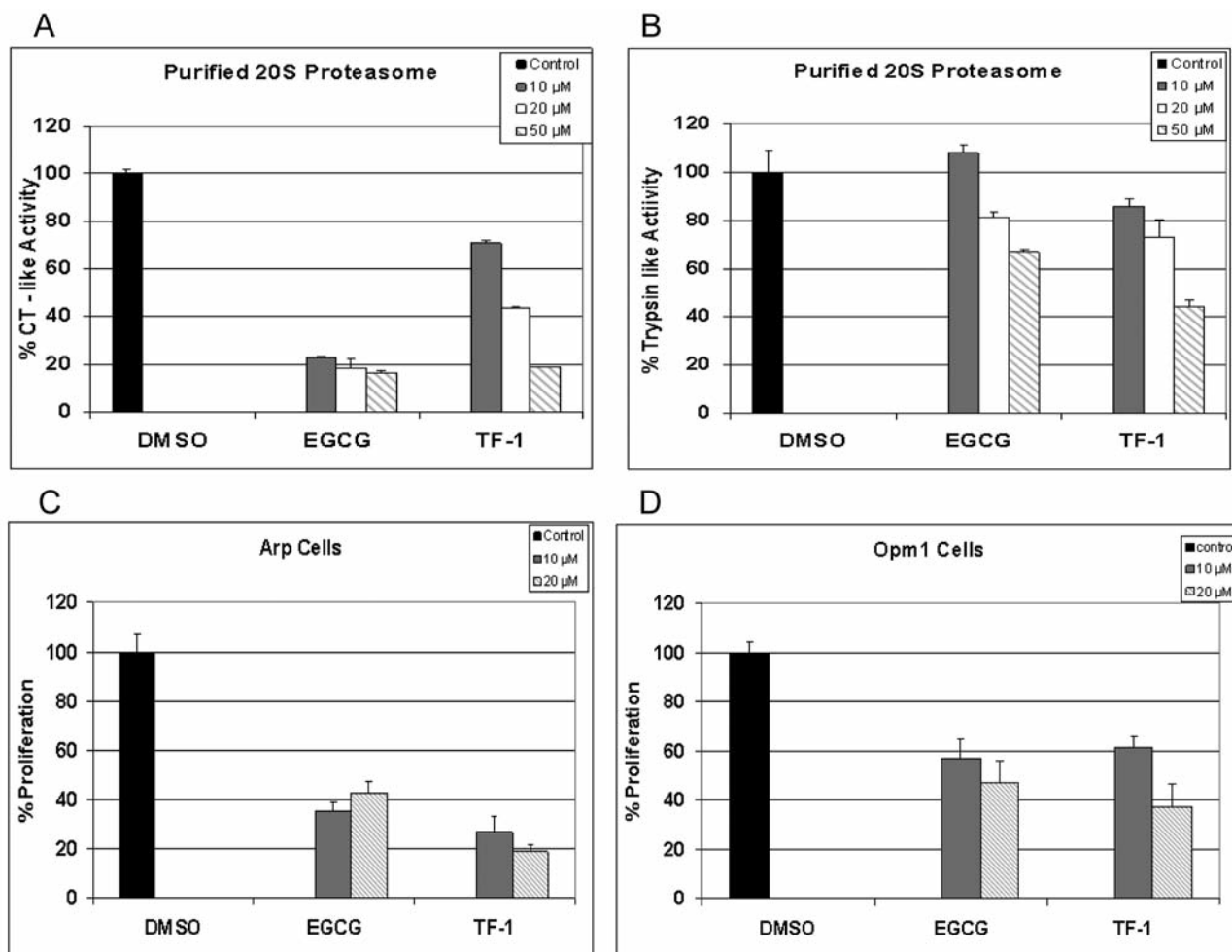


Figure 4. Effect of purified theaflavin (TF-1) on proteasome activities and cell proliferation. CT-like activity of the purified 20S (A). Trypsin-like activity of the purified 20S proteasome (B). Proliferation of Arp myeloma cells (C) and Opm1 myeloma cells (D).

black tea extract as a chemopreventive agent should not be ignored. These *in vitro* results need to be validated in *in vivo* systems. The bioactive black tea constituents may yield potential health benefits and hence there is large scope for future research in this field.

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