# Naturally Occurring Proteasome Inhibitors from Mate Tea (*Ilex paraguayensis*) Serve as Models for Topical Proteasome Inhibitors

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**Proteasome inhibitors have emerged as a clinically important therapy for neoplastic disease, with velcade, an organoboron compound used extensively in multiple myeloma. Recently, (−)-epigallocatechin gallate has been found to be a potent inhibitor of the proteasomal chymotrypsin-like activity. Other compounds that inhibit angiogenesis and are active as chemopreventive agents, such as curcumin, also inhibit proteasome activity. We have screened natural product extracts using ras-transformed endothelial cells (SVR cells) as a bioassay, and found that extracts of mate tea (*Ilex paraguayensis*) inhibit the growth of these endothelial cells. The extract was fractionated and found to have novel cinnamate esters that inhibit proteasome activity. Based upon the structures of the compounds isolated from mate tea, we examined synthetic analogs of these compounds for proteasome activity. Cinnamic acid amides had no inhibitory activity against proteasomes, whereas cinnamate esters displayed the activity. Based upon these findings, preclinical and clinical trials of topical cinnamate esters as proteasome inhibitors are warranted for psoriasis and other inflammatory disorders.**

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Plant extracts are a major source of chemopreventive, antiangiogenic, and antitumor agents. These include potent agents that are present in small quantities, as well as compounds that form a major portion of human diets. Intensive investigation has identified a class of dietary available materials including green and black tea (*Camelia sinensis*), turmeric (*Curcuma longa*), and other agents that have been found to have chemopreventive and antitumor activities in experimental animals (Rao *et al*, 1995; Arbiser *et al*, 1998; Thaloor *et al*, 1998). Systems in which these agents have prevented the development of malignancy include the skin, oral cavity, forestomach, duodenum, colon, breast, and prostate (Huang *et al*, 1991, 1994; Singletary *et al*, 1998; Elmets *et al*, 2001; Gupta *et al*, 2001). The mechanisms through which these compounds prevent the development of malignancy are not completely understood. The lack of mechanism makes isolation and rational design of synthetic congeners difficult, as the structural features required for structure–function relationships are difficult to determine without adequate *in vitro* assays.

We developed immortalized and transformed endothelial cells through the sequential introduction of a temperature-sensitive SV40 large T antigen and oncogenic H-ras into murine microvascular endothelium (Arbiser *et al*, 1997). These cells have proven extremely valuable in elucidating mechanisms of the angiogenic switch (Klafter and Arbiser, 2000) (Arbiser, 2004). We now use ras-transformed endothelial cells (SVR cells) as a screening tool to isolate naturally occurring compounds that may have antitumor or antiangiogenic activities, or both (Arbiser *et al*, 1998, 1999). Using this assay, we partially purified extracts from the plant *Ilex paraguayensis*, which is consumed as a beverage in large quantities in South America (Anghileri and Thouvenot, 2000). These extracts were chemically characterized and found to contain caffeoyl esters of quinic acid. One of these fractions was found to be 3,5-dicaffeoylquinic acid (3,5-DCQ), which inhibits the chymotrypsin-like activity of a purified 20S proteasome and 26S proteasome in Jurkat T cell extracts. 3,5-DCQ treatment of intact Jurkat T cells caused growth arrest in the $G_2$/M phase of the cell cycle. In contrast, fraction T-2, identified as 5-caffeoylquinic acid (neochlorogenic acid) (5-CQ), contains much less proteasome-inhibitory activity and fails to induce $G_2$/M arrest in Jurkat T cells. This finding suggests that proteasome activity may depend on the number of cinnamate moieties. A cinnamate tetraester (pentaerythritol tetrakis (3,5-di-*tert*-butyl-4-hydroxyhydrocinnamate), PTTC) was tested and found to have activity against proteasomes. This compound and its derivatives may be useful in the topical and/or systemic treatment of psoriasis.

Abbreviations: 5-CQ, 5-caffeoylquinic acid (neochlorogenic acid); 3,4-DCQ, 3,4-dicaffeoylquinic acid; 3,5-DCQ, 3,5-dicaffeoylquinic acid; [(−)-EGCG], epigallocatechin gallate; PTTC, pentaerythritol tetrakis (3,5-di-*tert*-butyl-4-hydroxyhydrocinnamate)

## Results

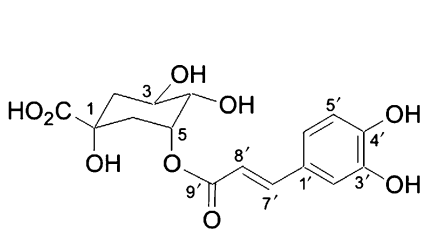
Aqueous filtered extract of mate tea exerted a strong inhibitory effect on SVR endothelium. In order to determine the component of mate tea responsible, the aqueous extract was fractionated by HPLC, and the ability of the fractions to inhibit SVR cell proliferation was assessed. The fractions that showed the most potent inhibitory effects were fractions T-2, T-5, and T-6. The structures of T-2, T-5, and T-6 were elucidated by proton NMR and mass spectroscopy (Fig 1). Fraction T-2 was found to be 5-CQ (neochlorogenic acid), fraction T-5 was found to be 3,5-DCQ, and fraction T-6 was found to be 3,4-dicaffeoylquinic acid (3,4-DCQ). The NMR spectra of the compounds are shown below.

**5-CQ[14] (T-2; neochlorogenic acid)** $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ 7.58 (1H, d, J = 15.9 Hz, H-7′), 7.05 (1H, d, J = 1.2 Hz, H-2′), 6.94 (1H, dd, J = 8.2, 1.5 Hz, H-6′), 6.78 (1H, d, J = 8.2 Hz, H-5′), 6.31 (1H, d, J = 15.9 Hz, H-8′), 5.37 (1H, br d, J = 4.8 Hz, H-5), 4.18 (1H, m, H-3), 3.66 (1H, dd, J = 8.6, 3.2 Hz, H-4), 2.17 (3H, m, H-6ax, H-6eq, H-2eq), 1.97 (1H, dd, J = 13.2, 10.4 Hz, H-2ax); $^1$C NMR ($CD_3OD$, 100 MHz): $\delta$ 178.4 (C, C-7), 169.2 (C, C-9′), 149.5 (C, C-4′), 147.0 (CH, C-7′), 146.8 (C, C-3′), 128.1 (C, C-1′), 123.0 (CH, C-6′), 116.6 (CH, C-5′), 115.9 (CH, C-2′), 115.2 (CH, C-8′), 75.5 (C, C-1), 75.0 (CH, C-4), 73.2 (CH, C-5), 68.3 (CH, C-3), 41.7 ($CH_2$, C-2), 36.8 ($CH_2$, C-6). HRESIMS m/z 377.0807 $[M + Na]^+$ (calculated for $C_{16}H_{18}O_9Na$, 377.0843).

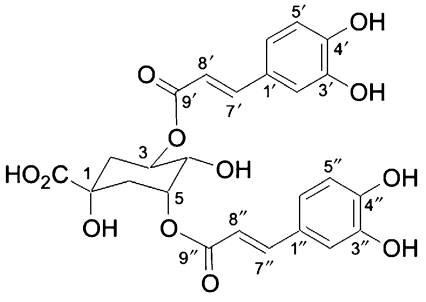
**3,5-DCQ[14] (T-5)** $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ 7.62 (1H, d, J = 16.0 Hz, H-7′ or H-7″), 7.58 (1H, d, J = 16.0 Hz, H-7′ or H-7″), 7.07 (2H, br s, H-2′,-2″), 6.97 (2H, m, H-6′, H-6″), 6.79 (1H, d, J = 8.0 Hz, H-5′, H-5″), 6.35 (1H, d, J = 16.0 Hz, H-8′ or H-8″), 6.27 (1H, d, J = 16.0 Hz, H-8′ or H-8″), 5.44 (1H, m, H-3), 5.40 (1H, br d, J = 5.9 Hz, H-5), 3.99 (1H, dd, J = 7.4, 3.1 Hz, H-4), 2.34-2.15 (4H, m, H-2, H-6); $^{13}$C NMR ($CD_3OD$, 100 MHz): $\delta$ 177.5 (C, C-7), 168.5 (C, C-9′ or C-9″), 168.3 (C, C-9′ or C-9″), 149.7 (2C, C-4′, C-4″), 147.4 (CH, C-7′ or C-7″), 147.2 (CH, C-7′ or C7″), 146.9 (2C, C-3′, C-3″), 128.0 (2C, C-1′, C-1″), 123.2 (CH, C-6′ or C-6″), 123.1 (CH, C-6′ or C-6″), 116.6 (2CH, C-5′, C-5″), 115.7 (1C each, d, C-2″), 115.5 (1C each, d, C-2′), 115.4 (1C each, d, C-8″), 115.2 (1C each, d, C-8′), 74.8 (C, C-1), 72.6 (CH, C-5), 72.2 (CH, C-3), 70.7 (CH, C-4), 37.7 ($CH_2$, C-2), 36.1 ($CH_2$, C-6). HRESIMS m/z 539.1160 $[M + Na]^+$ (calcd for $C_{25}H_{24}O_{12}Na$, 539.1116).

**3,4-DCQ[14] (T-6)** $^1$H NMR ($CD_3OD$, 400 MHz): $\delta$ 7.60 (1H, d, J = 15.9 Hz, H-7′or H-7″), 7.52 (1H, d, J = 15.9 Hz, H-7′ or H-7″), 7.03 (1H, br s, H-2′ or H-2″), 7.01 (1H, br s, H-2′ or H-2″), 6.91 (2H, m, H-6′, H-6″), 6.75 (1H, d, J = 8.0 Hz, H-5′,-5″), 6.29 (1H, d, J = 15.9 Hz, H-8′ or H-8″), 6.20 (1H, d, J = 15.9 Hz, H-8′ or H-8″), 5.64 (1H, m, H-3), 5.14 (1H, dd, J = 9.0, 2.6 Hz, H-4), 4.39 (1H, m, H-5), 2.32-2.11 (4H, m, H-2, H-6); $^{13}$C NMR ($CD_3OD$, 100 MHz): $\delta$ 176.8 (C, C-7), 168.7 (C, C-9′ or C-9″), 168.4 (C, C-9′or C-9″), 149.7 (2C, C-4′, C-4″), 147.7 (2CH, C-7′,7″), 146.8 (2C, C-3′, C-3″), 127.7 (2C, C-1′, C-1″), 123.3 (2CH, C-6′, C-6″), 116.6 (2CH, C-5′, C-5″), 115.3 (2CH, C-2′, C-2″), 114.8 (2CH, C-8′, C-8″), 76.3 (C, C-1), 75.8 (CH, C-4), 69.4 (CH, C-5), 69.1 (CH, C-3), 39.4 ($CH_2$, C-2), 38.4 ($CH_2$, C-6).

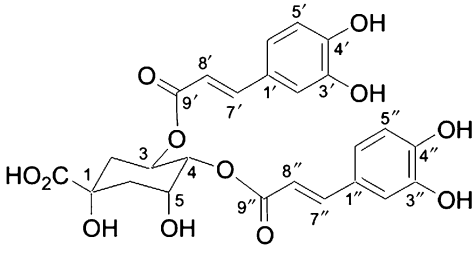
**Inhibition of purified 20S proteasome** The quinic acid esters resemble the proteasome inhibitor (−)-epigallocatechin gallate [(−)-EGCG] in that they contain hydroxylated aromatic carboxylic acids esterified to a polyhydroxylated aliphatic ring (Fig 2). Based upon this similarity, and the previous finding that EGCG is an inhibitor of proteasomes



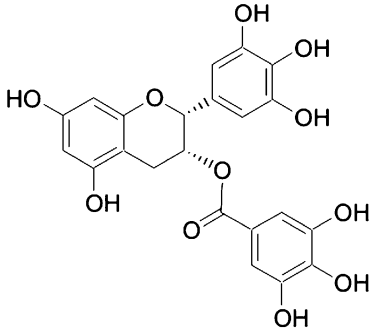
**T-2**: 5-caffeoylquinic acid (5-CQ) "neochlorogenic acid"



**T-5**: 3,5-dicaffeoylquinic acid (3,5-DCQ)



**T-6**: 3,4-dicaffeoylquinic acid (3,4-DCQ)



**EGCG**: (-)-epigallocatechin-3-*O*-gallate

**Figure 1**
**Chemical structures of proteasome inhibitors isolated from mate tea.**

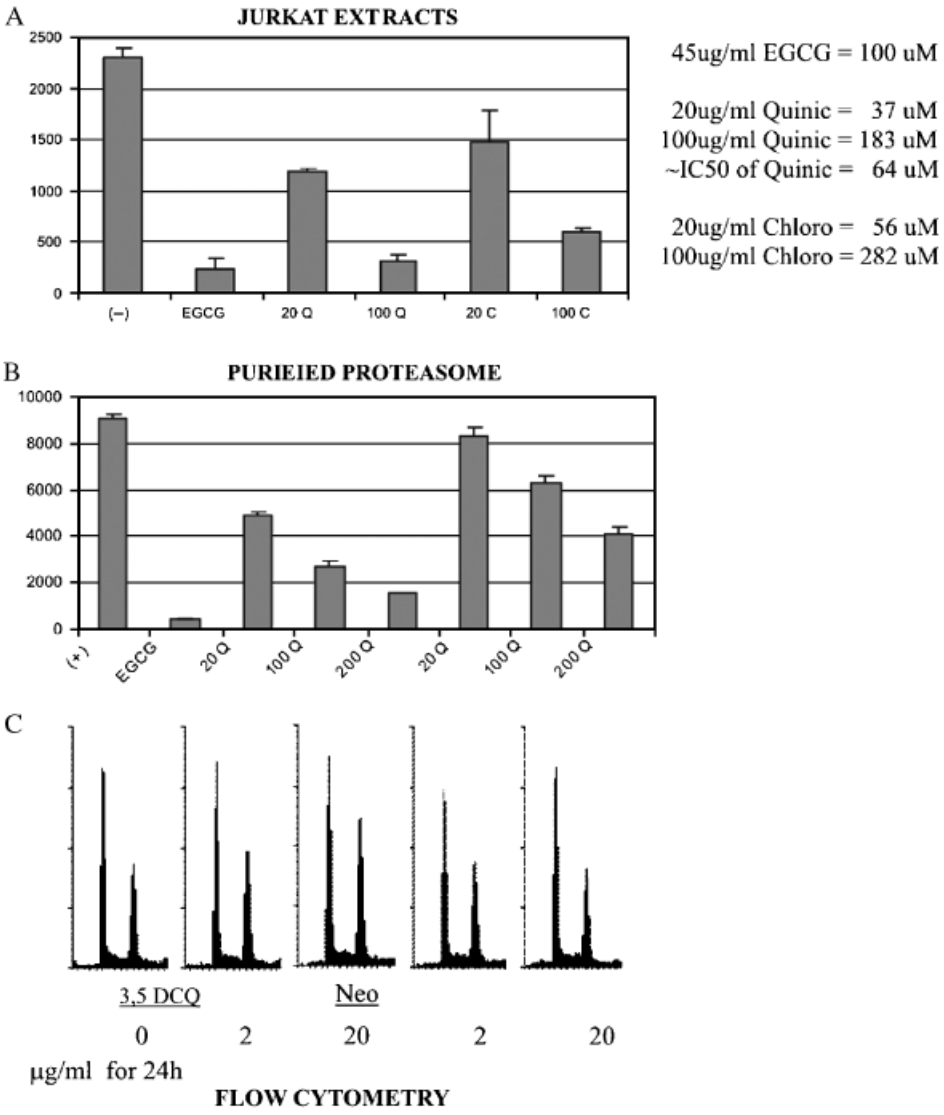



**Figure 2**
**Effect of mate tea-derived compounds on proteasome function *in vitro* and *in vivo*.** (*A*) Effect of 3,5-dicaffeoylquinic acid (3,5-DCQ) (Q) on proteasome function *in vitro* compared with neochlorogenic acid (Neo) (*C*). (*B*) Effect of 3,5-DCQ and Neo on proteasome function *in vivo* proteasome function. (*C*) Effect of 3,5-DCQ on proteasome function *in vitro* compared with Neo on cell cycle progression in Jurkat cells.

(Ren *et al*, 2000; Nam *et al*, 2001) (ref. 15 is not related to EGCG as a proteasome inhibitor), the ability of fractions T-2, T-5, and T-6 to inhibit proteasome function was assessed. To determine the ability of quinic acid esters to inhibit proteasome activity, a fluorescent substrate activity assay was performed with purified 20S proteasome. EGCG (Sigma Chemical Company, St Louis, Missouri) was used as a positive control for proteasome inhibition. To ensure complete inhibition of the proteasome, we used 100 μM EGCG. The compound 3,5-DCQ was tested against proteasome activity in three different concentrations: 20, 100, and 200 μg per mL, which correspond to 37, 183, and 366 μM, respectively (Fig 3). The $IC_{50}$ value for 3,5-DCQ was determined to be approximately 64 μM. In contrast, neochlorogenic acid was found to be much weaker, with an $IC_{50}$ value of ~ 564 μM for the purified 20S proteasome (Fig 3). The potency of fraction T6 (3,4-DCQ) was between that of 3,5-DCQ and neochlorogenic acid: at 100 μM, fractions T-5, T-6, and T-2 inhibited the proteasomal chymotrypsin-like activity by 60%, 40%, and 21%, respectively. These data suggest that 3,5-DCQ has the greatest proteasome-inhibitor activity in all the structurally related substances tested (Fig. 4).

**Inhibition of proteasome activity in Jurkat cell lysates** We then tested the abilities of 3,5-DCQ and neochlorogenic acid to inhibit the 26S proteasome activity in Jurkat cell extracts. We found that 3,5-DCQ at 20 μg per mL (37 μM) inhibited the proteasome activity by ~ 50%, and at 100 μg per mL (183 μM) inhibited the proteasome activity by ~ 85%, which was almost as potent as 100 μM EGCG. In this assay, neochlorogenic acid (5-CQ) was also able to inhibit the proteasome activity ( ~ 30% at 20 μg per mL or 56 μM and ~ 75% at 100 μg per mL or 282 μM), although its potency was weaker than that of 3,5-DCQ. These data further demonstrate that 3,5-DCQ is able to inhibit the chymotrypsin-like activity of the 26S proteasome.

**3,5-DC induces $G_2$/M block in tumor cells** To study whether the proteasome-inhibitory potencies of 3,5-DCQ and neochlorogenic acid are associated with growth-inhibitory activity *in vivo*, we treated Jurkat T cells with each compound 2 or 20 μg per mL for 24 h. After treatment, the cells were harvested and assayed by flow cytometry. 3,5-DCQ at 2 μg per mL produced a very slight arrest of Jurkat cells in the $G_2$/M phase of the cell cycle, whereas 20 μg per mL increased the $G_2$/M population by nearly 10%. In con-

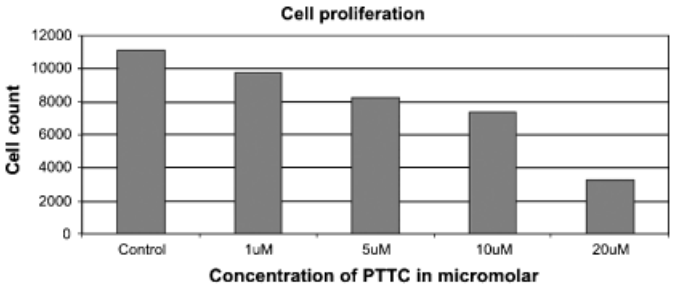



**Figure 3**
**Effect of pentaerythritol tetrakis (3,5-di-tert-butyl-4-hydroxyhydrocinnamate) on proliferation of SVR cells.** Note that the inhibition of endothelial cell proliferation is concentration dependent.

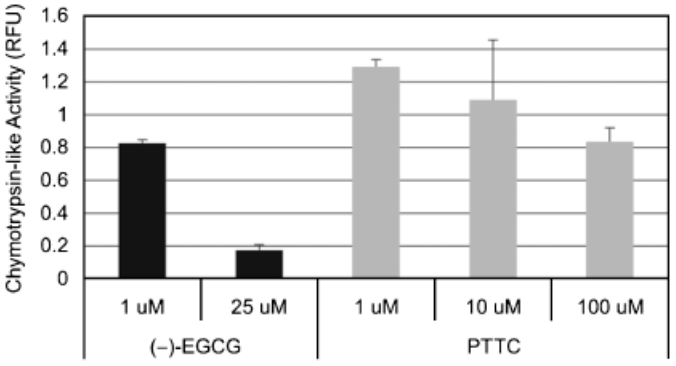



**Figure 4**
**Pentaerythritol tetrakis (3,5-di-tert-butyl-4-hydroxyhydrocinnamate) (PTTC) inhibits chymotrypsin-like proteasome inhibitor.** Note that PTTC has inhibitory activity of the proteasome at high doses. Epigallocatechin gallate is used as a positive control.

trast, neochlorogenic acid at the same concentrations had no effects. These data suggest that 3,5-DCQ inhibits the proteasome in intact tumor cells, resulting in $G_2$/M arrest.

## Discussion

Controlled protein degradation plays a major role in the regulation of multiple genes, including transcriptional activators, oncogenes, and tumor suppressor genes (Hershko *et al*, 2000). An organoboron compound, velcade (PS-341, bortezomib), has entered the clinic for the treatment of multiple myeloma and other malignancies, based upon evidence that constitutive activation of NFκB is required for tumorigenesis (Richardson *et al*, 2002).

Other drugs may interact with the NFκB/IκB system to explain their effects. Glucocorticoids, which are the mainstay of anti-inflammatory activities, as well as first-line treatment for several hematopoietic malignancies, including multiple myeloma, also influence NFκB/IκB activation by inducing IκB protein. Glucocorticoids, however, have other undesirable effects not attributable to proteasome inhibition, such as atrophy of connective tissue and muscle. A recent study of an animal model of psoriasis, derived from transplantation of psoriatic human skin to an immunocompromised mouse, showed that systemic administration of a proteasome inhibitor caused regression of psoriasis (Zollner *et al*, 2002). Thus, proteasome inhibition is a valid target for human psoriasis.

Topical therapies are the mainstay of human psoriasis treatment. The most common therapy for psoriasis is topical glucocorticoids (Wrone-Smith and Nickoloff, 1996; Stern, 1997; Lebwohl, 2004; Nickoloff and Nestle, 2004). These drugs have anti-inflammatory effects in psoriasis, but are associated with side effects. Prominent among these side effects are dermal atrophy, due to the known inhibitory effect of glucocorticoids on collagen synthesis and deposition. This often necessitates discontinuation of therapy. Other topical agents include retinoids and vitamin D derivatives. All of these are potentially irritating and less effective than topical glucocorticoids. Thus, more effective therapies with novel mechanisms of activity are needed in psoriasis.

Velcade is the only specific proteasome inhibitor currently used in humans. Other chemopreventive agents, however, such as curcumin and EGCG, have proteasome inhibitory activity (Nam *et al*, 2001; Jana *et al*, 2004). We have previously demonstrated that curcumin has anti-angiogenic activity *in vivo*, and curcumin ointments have been shown to have benefit in human psoriasis (Arbiser *et al*, 1998). Curcumin is a dimer of two cinnamoyl moieties fused to a middle carbon, whereas EGCG is an ester of the polyphenol catechin, with gallic acid (a hydroxylated benzoic acid). Thus, the requirements for proteasome-inhibitory activity are not fully understood. Upon fractionation of antiangiogenic components of mate tea, we found that the presence of two cinnamate esters was more potent as a proteasome inhibitor than a single ester. One possibility is that the difference between the monoester (caffeoylquinic acid) and the 3,5-diester is simply that of hydrophobicity. In order to make a topically available agent, several criteria must be met. First, the agent must be lipophilic. Second, it should not be allergenic. Attempts to make an ointment using EGCG have not been successful due to irritation.[1] Unsaturated cinnamates have been found to cause allergic reactions in some individuals (Hausen *et al*, 1992). We thus chose a saturated cinnamate that is highly lipophilic and has a known human safety record as a candidate for treatment of human skin. We found that PTTC, a commercially available antioxidant, fulfilled the criteria of having multiple cinnamoyl ester groups and appropriate hydrophobicity. PTTC is extensively used in industrial processes, and it has not been shown to be carcinogenic, or toxic orally to rats ($LD50 > 5000$ mg per kg in the rat), or dermally to rabbits ($LD_{50} > 3160$ mg per kg in the rabbit) (Material Safety Data Sheet, Great Lakes Chemical Corporation—Durad AX32, MSDS number 00771). Thus, our study of mate tea derivatives suggests that proteasome inhibitors can be synthesized by varying the alcohol as well as producing multiple ester groups. The development of polycinnamate esters as proteasome inhibitors may lead to the development of topical proteasome inhibitors, which may be used in inflammatory and neoplastic disorders, without the side effects of topical glucocorticoids.

### Materials and Methods

**Preparation of mate tea extracts** Powdered mate tea (Chimarrao Laranjeiras Puraerva, Cascavel, Brazil) was supplied by Glenn and

[1]D. Alberts, personal communication.

Lynn Cohen (Atlanta, Georgia). Mate tea (100 g) was extracted by boiling in 500 mL water for 30 min. PTTC was obtained from Aldrich Chemical Co (St Louis, Missouri). Once cool, the crude aqueous extract was first filtered through a 0.45 μm filter and further filtered to exclude materials of greater than 3000 MW.

The filtered aqueous extract was lyophilized to a dry powder, which was dissolved in distilled water and analyzed by HPLC, and five fractions were collected. HPLC fractions were lyophilized. Each fraction was reconstituted into 10 mg per mL solutions, and the ability to inhibit proliferation of SVR cells was tested.

**Cell proliferation assays** SVR cells ($1 \times 10^4$) were plated for 24 h in a 24-well plate. The medium was then changed to DMEM containing purified extract at a concentration of 10 μg per mL. Cells were exposed to the drug for 72 h and were counted with a Coulter Counter (Coulter, Hialeah, Florida) according to the method of LaMontagne *et al* (2000).

**General spectroscopic and spectrometric methods** The NMR spectra were recorded in $CD_3OD$ on a Bruker DRX 400 spectrometer operating at 400 MHz for $^1H$ and 100 MHz for $^{13}C$, running gradients, and using residual solvent peaks as internal references. The HRESIMS data were acquired on a Bruker BioAPEX 30es (NCNPR, University of Mississippi).

**Chemical characterization of mate tea components** The HPLC fractions were found to be 5-CQ (neochlorogenic acid), 3,5-DCQ, and 3,4-DCQ, respectively. The $^1H$ and $^{13}C$ NMR spectroscopic data for the compounds isolated by HPLC were identified as three known quinic acid derivatives. The relative number of caffeoyl ester groups in each metabolite was evident from the number of characteristic ester carbonyl carbon resonances observed in the $^{13}C$ NMR spectrum of each compound. The $^{13}C$ NMR spectrum of 5-CQ contained one carbon resonance for the free carboxylic acid (178.4 ppm for C-7) and one carbon signal for the single ester carbonyl (169.2 ppm for C-9′). The di-substituted nature of the two dicaffeoylquinic acid derivatives was evident from the presence of two separate ester carbonyl resonances in the $^{13}C$ NMR spectrum of 3,5-DCQ (168.5 for C-9′, 168.3 ppm for C-9″) and 3,4-DCQ (168.7 for C-9′, 168.4 for C-9″). The substitution patterns of the caffeoyl ester moieties were identified, based upon the characteristic downfield chemical shifts (1 ppm or greater) of the oxygen-bearing α-methine proton signals in the $^1H$ NMR spectrum of each of the caffeoyl-substituted quinic acid derivatives. Since all were previously reported known compounds, a detailed structure elucidation of each metabolite was not required. All $^1H$ and $^{13}C$ NMR chemical shifts were identical to those previously published (not 14). In addition, the molecular compositions of 5-CQ ($C_{16}H_{18}O_9$) and 3,5-DCQ ($C_{25}H_{24}O_{12}$) were confirmed by high-resolution ES-IMS analysis of the sodium adducts of each compound, respectively.

**Inhibition of purified 20S proteasome activity by HPLC-purified mate tea fractions** The chymotrypsin-like activity of purified 20S proteasome was measured as previously described (Nam *et al*, 2001). Briefly, purified prokaryotic 20S proteasome (0.5 μg) was incubated with 20 μM fluorogenic peptide substrate, Suc–Leu–Leu–Val–Tyr—AMC, for 30 min at 37°C in 100 μL of assay buffer (50 mM Tris-HCl, pH 7.5), with or without a mate tea fraction at indicated concentrations. After incubation, production of hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups was measured using a multi-well plate VersaFluor Fluorometer with an excitation filter of 380 nm and an emission filter of 460 nm (Bio-Rad, Hercules, California).

**Inhibition of proteasome activity in Jurkat T cell extracts by HPLC-purified mate tea fractions** Whole-cell extracts (20 μg) of Jurkat T cells were incubated for 60 min at 37°C with 20 μM of fluorogenic peptide substrate Suc–Leu–Leu–Val–Tyr–AMC in 100 μL of the assay buffer, with or without a mate tea fraction at indicated concentrations. The hydrolyzed AMC were quantified as described above.

**Flow cytometry** Cell cycle analysis based on DNA content was performed, as described previously (Nam *et al*, 2001). The cell cycle distribution is shown as the percentage of cells containing $G_1$, S, $G_2$, and M DNA judged by propidium iodide staining.

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