SYNTHETIC PERACETATE TEA POLYPHENOLS AS POTENT PROTEASOME INHIBITORS AND APOPTOSIS INDUCERS IN HUMAN CANCER CELLS

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1. ABSTRACT

It has been suggested that proteasome activity is essential for tumor cell proliferation and drug resistance development. We have previously shown that natural and synthetic ester bond-containing tea polyphenols are selective inhibitors of the chymotrypsin-like activity of the proteasome. The most abundant catechin in green tea is (-)-epigallocatechin-3-gallate [(-)-EGCG], which has been found by many laboratories to exhibit the most potent anticancer activity. We have reported that (-)-EGCG is also the most effective proteasome inhibitor among all the natural green tea catechins tested. Unfortunately, (-)-EGCG is very unstable in neutral and alkaline conditions. In an attempt to increase the stability and thus the efficacy, we synthesized several (-)-EGCG analogs with acetyl protected –OH groups as prodrugs. Here we report, for the first time, that these acetylated synthetic tea analogs are much more potent than natural (-)-EGCG in inhibiting the proteasome in cultured tumor cells. Consistently, these protected analogs showed much higher potency than (-)-EGCG to inhibit proliferation and transforming activity and to induce

apoptosis in human leukemic, prostate, breast, and simian virus 40-transformed cells. Additionally, these protected analogs had greatly reduced effects on human normal and non-transformed cells. Therefore, these peracetate protected tea polyphenols are more efficacious than (-)-EGCG and possess great potential to be developed into novel anticancer drugs. Identification of the cytosolic metabolite(s) of peracetate-protected polyphenols in cultured tumor cells and examination of their *in vivo* tumor growth-inhibitory activity are currently underway in our laboratory.

2. INTRODUCTION

Tea, derived from *Camellia sinensis*, is the most highly consumed beverage in the world, next to water. The polyphenols found in green tea, *e.g.* (-)-epigallocatechin-3-gallate [(-)-EGCG], are associated with the beneficial effect of green tea. (-)-EGCG is the most abundant constituent in green tea, accounting for more than 10% of the weight of dry extract. (-)-EGCG is considered the most biologically active polyphenolic catechin in tea (1-3). Several epidemiological studies have shown that regular green tea consumption is linked to a decreased incidence of cancer (4-6). Furthermore, animal studies have also demonstrated that (-)-EGCG can reduce or inhibit tumor growth in a variety of tissue types including prostate (7), breast (8), skin (9), bladder (10), liver (11, 12), and lung (13).

The proteasome is an immense multi-catalytic, multi-subunit protease complex (14) responsible for regulating a variety of cellular processes, including cell cycle and apoptosis (15-17). The 20S subunit is the core complex responsible for ATP-independent degradation of proteins. It has been shown that tumor cell growth is highly dependent on the proteasome function, as its inhibition leads to growth arrest in the G_1 phase of the cell cycle and/or induction of apoptosis (18-21). Previously, our laboratories have shown that green tea catechins with the gallate ester bond, either natural or synthetic, are potent inhibitors of the chymotrypsin-like activity of the proteasome both *in vitro* and in cultured tumor cells (22-24).

Numerous studies have suggested that the pH environment plays an important role in (-)-EGCG stability. (-)-EGCG is relatively stable in acidic conditions (pH < 6.5) and rapidly degrades in neutral or alkaline conditions (25). This is attributed to the fact that the hydroxyl groups can donate protons under alkaline conditions, leading to formation of the more reactive phenoxide anions (25). In an effort to improve the stability and thus increase the efficacy of (-)-EGCG, we focused on designing a synthetic (-)-EGCG prodrug analog (26). The hydroxyl groups were converted to acetate groups, which once inside a mammalian cell could be potentially cleaved by esterases, leading to formation of the parent (-)-EGCG, and, theoretically, prolong the half-life and efficacy. We found that the peracetate-protected analog (compound 1; Figure 1) degraded slower than (-)-EGCG in neutral medium. Although compound 1 did not inhibit the activity of a purified 20S proteasome, it inhibited proteasome activity in

cultured leukemia Jurkat T cells, associated with induction of cell death (26).

Although compound **1** has improved proteasome-inhibitory and death-inducing activities in cultured Jurkat cells, the effect was moderate (26). Toward the goal of developing much more stable, potent and specific EGCG-related proteasome inhibitors and to characterize these compounds in various cancer and normal cell lines, we designed, synthesized and evaluated a serious of (-)-EGCG analogs, both unprotected and protected compounds (Figure 1). Here we report that several peracetate-protected EGCG analogs are much more potent proteasome inhibitors than (-)-EGCG and other unprotected counterparts when tested in various cancer and transformed cell lines, although the protected compounds are inactive to a purified 20S proteasome, suggesting possible conversion in biological systems. Additionally, the protected analogs induce a greater amount of cancer cell apoptosis, compared to their unprotected counterparts, and that this apoptotic induction is tumor cell-selective. Our study suggests that these protected green tea analogs are much more potent than (-)-EGCG in tumor cells and have great potential to be developed into novel anticancer agents. High performance liquid chromatography (HPLC) will help identify the formation of the cytosolic metabolite(s) after introduction of peracetate-protected polyphenols into cultured tumor cells. Additionally, we are examining the *in vivo* tumor growth-inhibitory effects of these protected compounds in nude mice bearing human breast tumors.

3. MATERIALS AND METHODS

3.1. Reagents
Fetal Bovine Serum was purchased from Tissue Culture Biologicals (Tulare, CA). Mixture of penicillin-streptomycin-L-glutamine, RPMI, and Dulbecco's modified Eagle's medium (DMEM) are from Invitrogen (Carlsbad, CA). Dimethyl sulfoxide (DMSO), *N*-acetyl-$_L$-cysteine (NAC), Hoechst 33342, 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), bovine serum albumin (BSA), and (-)-EGCG were purchased from Sigma (St. Louis, MO). Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) was obtained from Biomol (Plymouth Meeting, PA). Purified 20S proteasome from rabbit was acquired from Boston Biochem (Cambridge, MA). Amplex Red H_2O_2 assay kit was purchased from Molecular Probes (Eugene, OR). Monoclonal antibodies to Bax (H280) and Ubiquitin (P4D1) and polyclonal antibodies to IκB-α (C15), Caspase-3 (H277), and Actin (C11) as well as anti-goat, anti-rabbit, and anti-mouse IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody to Caspase-9 was purchases from Pharmingen Franklin Lakes, NJ). Monoclonal antibody to p27 (554069) was purchased from BD Biosciences (San Diego, CA). Vectashield Mounting Medium with DAPI was purchased from Vector Laboratories, Inc. (Burlingame, CA). The polyclonal antibody, specific to the poly(ADP-ribose) polymerase (PARP) cleavage site and FITC-conjugated, was acquired from Biosource (Camarillo, CA). CaspACE FITC-VAD-FMK marker was purchased from Promega (Madison, WI).
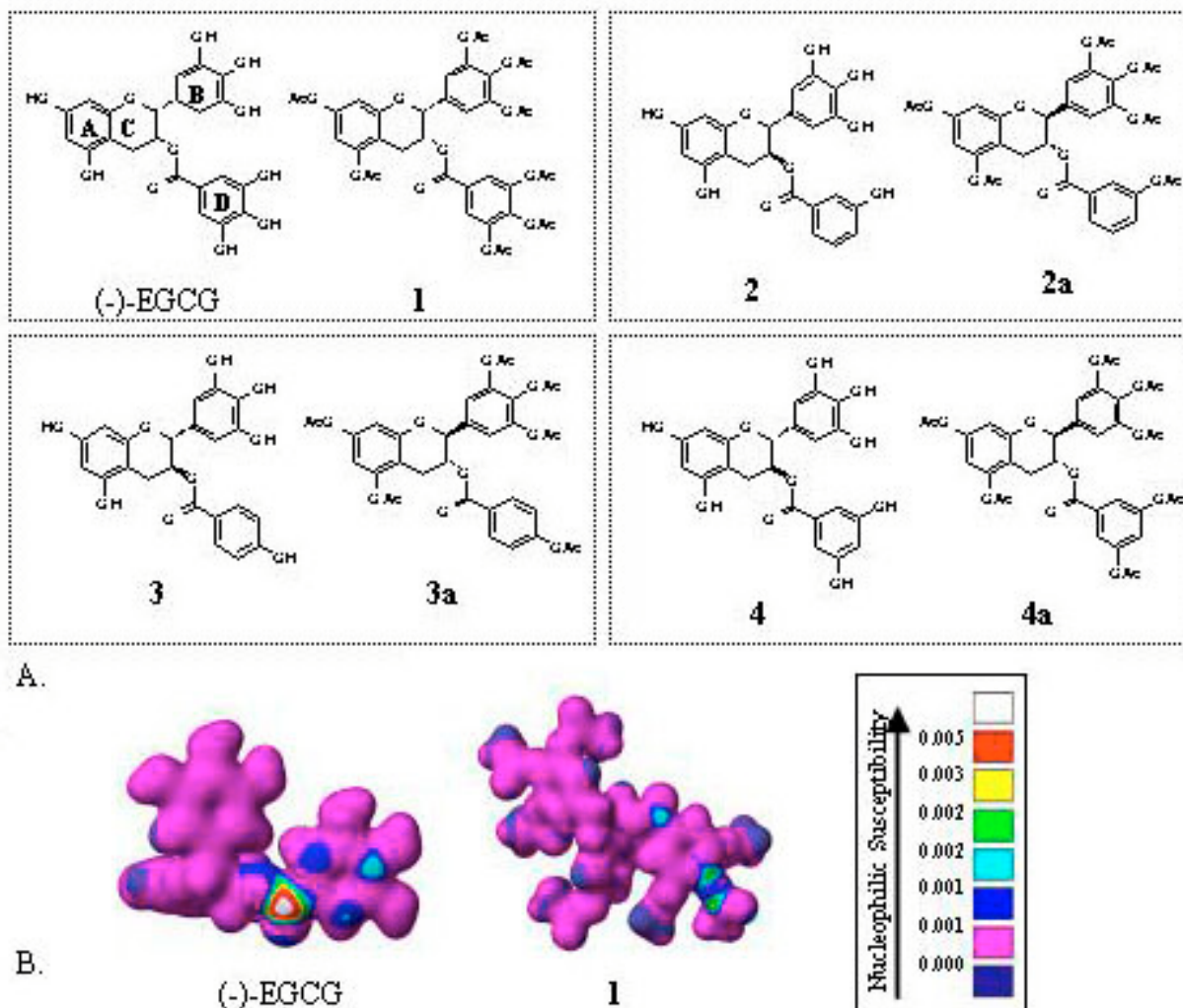
Figure 1. Synthetic tea polyphenols. A, Chemical structure of (-)-EGCG and synthetic tea polyphenols with and without protected hydroxyl groups (racemic compounds). B, Graphical analysis of polyphenol nucleophilic susceptibility (calculated as described in Material and Methods).

3.2. Synthesis of synthetic tea polyphenol analogs (Scheme 1)

3.2.1. General experimental conditions (chemistry)

All reactions were performed under an atmosphere of nitrogen in oven-dried glassware. Dichloromethane was collected from a solvent purification system. ^1H and ^{13}C NMR spectra were recorded at 500 and 100 MHz, respectively, unless otherwise noted. Thin-layer chromatography (TLC) was performed using commercially prepared 60 mesh silica gel plates visualized with short-wavelength UV light (254 nm). Silica gel 60 (230-400 mesh) was used for column chromatography. Melting points were determined in unsealed capillary tubes and are uncorrected. All commercially available reagents were used as received.

3.2.2. (2S*,3R*)-*trans*-5,7-Bis(benzyloxy)-2-[3,4,5-tris (benzyloxy) phenyl]chroman-3-yl 3-(benzyloxy)benzoate (6)

A quantity of $(COCl)_2$ (0.68 mL) was added to a solution of 3-(benzyloxy)benzoic acid (0.12 g, 0.53

mmol) in CH_2Cl_2 (3 mL). The mixture was refluxed for 2 hours. After which the excess $(COCl)_2$ and the solvent were removed by distillaton and the resulting residue was dried under vacuum overnight. The residue was redissolved in CH_2Cl_2 (3 mL) and added to a solution of **5** {Li, 2001 #77} (0.20 g, 0.26 mmol) and N,N-dimethylaminopyridine (DMAP, 0.08 g, 0.64 mmol) in CH_2Cl_2 (6 mL) at 0 ˚C. The mixture was then stirred at room temperature overnight. Saturated $NaHCO_3$ was added. The organic layer was separated and the aqueous layer was extracted with ethyl acetate. The organic layers were combined, dried (Na_2SO_4) and evaporated. The residue was purified by column chromatography (hexane: ethyl acetate 4:1) to afford the compound **6** as a white solid (0.22 g, 88%). Mp: 152.2 °C; MS *m/z* (ESI): 989 (MNa^+); HRMS: found, 989.3657; $C_{64}H_{54}O_9Na$ requires 989.3666; ^1H NMR (CDCl$_3$, 500MHz) δ 7.54-7.12 (m, 30H), 6.72 (s, 2H), 6.29 (d, *J*=4.5 Hz, 2H), 5.50 (q, *J*=7.0 Hz, 1H), 5.10 (d, *J*=6.5 Hz, 1H), 5.05-4.95 (m, 12H), 3.04 (dd, *J*=16.5, 6.0 Hz, 1H), 2.89
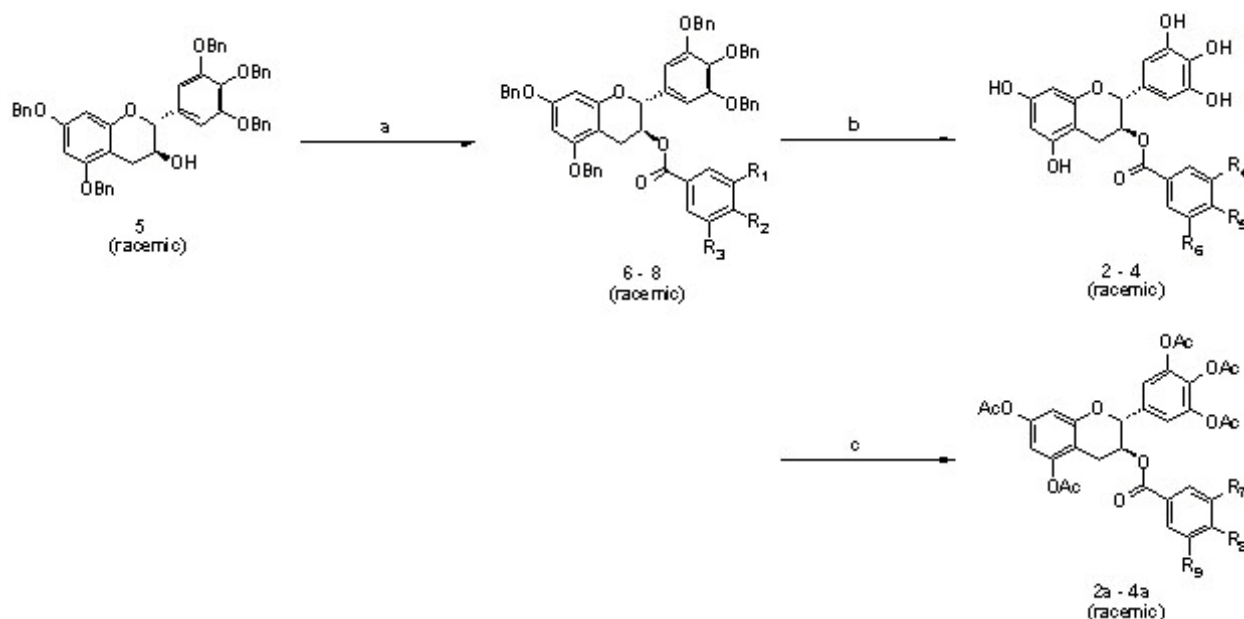
Scheme 1. Synthetic routes of the tea polyphenols. a.) truncated benzoyl chloride, DMAP, CH_2Cl_2, rt; b.) H_2, Pd(OH)$_2$, MeOH, THF, rt; c.) Ac_2O, pyridine, room temperature.

(dd, J=16.5, 6.5 Hz, 1H); ^{13}C NMR (CDCl$_3$, 100MHz) δ 165.20, 158.81, 158.51, 157.53, 154.68, 152.73, 138.20, 137.60, 136.76, 136.64, 136.25, 133.24, 131.14, 129.32, 128.49, 128.42, 128.40, 128.29, 128.00, 127.96, 127.91, 127.80, 127.69, 127.62, 127.51, 127.43, 127.38, 127.10, 122.13, 119.93, 115.29, 106.13, 101.23, 94.25, 93.74, 77.09, 78.38, 74.98, 71.07, 70.02, 69.81, 24.18.

3.2.3. (2S*,3R*)-trans-5,7-Bis(benzyloxy)-2-[3,4,5-tris(benzyloxy) phenyl]chroman-3-yl 4-(benzyloxy) benzoate (7)

The title compound was prepared in a similar manner as described for **6** using **5** (0.08 g, 0.1 mmol) and 4-(benzyloxy)benzoic acid (0.049 g, 0.22 mmol) giving **7** as a white solid (0.087 g, 90%). Mp: 145.0 °C; MS m/z (ESI): 989 (MNa$^+$); HRMS: found, 989.3666; $C_{64}H_{54}O_9Na$ requires 989.3666; ^{1}H NMR (CDCl$_3$, 500MHz) δ 7.91 (d, J=9.0 Hz, 2H), 7.47-7.22 (m, 30H), 6.95 (d, J=9.0 Hz, 2H), 6.74 (s, 2H), 6.31 (dd, J=5.5, 2.5 Hz, 2H), 5.52 (q, J=6.5 Hz, 1H), 5.14 (d, J=6.5 Hz, 1H), 5.07-4.97 (m, 12H), 3.02 (dd, J=17.0, 5.5 Hz, 1H), 2.87 (dd, J=16.5, 7.0 Hz, 1H); ^{13}C NMR (CDCl$_3$, 100MHz) δ 165.08, 162.49, 158.76, 157.53, 154.65, 152.69, 138.09, 137.63, 136.79, 136.70, 136.66, 136.00, 133.38, 131.61, 128.55, 128.49, 128.40, 128.29, 128.09, 127.96, 127.91, 127.78, 127.69, 127.62, 127.43, 127.38, 127.30, 127.09, 122.40, 114.33, 106.08, 101.30, 94.22, 93.67, 78.37, 77.09, 74.98, 71.05, 70.00, 69.95, 69.79, 69.26, 24.01.

3.2.4. (2S*,3R*)-trans-5,7-Bis(benzyloxy)-2-[3,4,5-tris(benzyloxy) phenyl]chroman-3-yl 3,5-bis(benzyloxy) benzoate (8)

The title compound was prepared in a similar manner as described for **6** using **5** (0.3 g, 0.4 mmol) and 3,5-bis(benzyloxy)benzoic acid (0.27 g, 0.81 mmol) giving **8** as a white solid (0.36 g, 85%). MS m/z (ESI): 1095 (MNa$^+$); HRMS: found, 1095.4059; $C_{71}H_{60}O_{10}Na$ requires 1095.4084; ^{1}H NMR (CDCl$_3$, 500MHz) δ 7.47-7.17 (m,

35H), 6.76 (s, 1H), 6.73 (s, 1H), 6.30 (d, J=4.5 Hz, 2H), 5.48 (q, J=7.0 Hz, 1H), 5.08 (d, J=7.0 Hz, 1H), 5.04-4.92 (m, 14H), 3.07 (dd, J=17.0, 5.5 Hz, 1H), 2.85 (dd, J=17.0, 7.0 Hz, 1H); ^{13}C NMR (CDCl$_3$, 100MHz) δ 165.14, 159.73, 158.98, 157.67, 154.88, 152.87, 137.77, 136.91, 136.82, 136.78, 136.29, 133.35, 131.86, 128.61, 128.56, 128.52, 128.41, 128.15, 128.10, 128.02, 127.94, 127.81, 127.76, 127.67, 127.51, 127.24, 108.52, 106.93, 106.30, 101.38, 94.43, 93.93, 78.57, 75.12, 71.17, 70.26, 70.15, 70.11, 69.92, 24.58.

3.2.5. (2S*,3R*)-trans-5,7-Bis(acetoxy)-2-[3,4,5-tris(acetoxy) phenyl]chroman-3-yl 3-(acetoxy)benzoate (2a)

Suspension of **6** (0.1 g, 0.1 mmol) in THF/MeOH (12 mL/12 mL) and Pd(OH)$_2$ (0.08 g, 20% on carbon) was placed under an H_2 atmosphere. The resulting mixture was stirred at room temperature until TLC showed that the reaction was completed. Then the reaction mixture was filtered through cotton to remove the catalyst. The filtrate was evaporated to afford the debenzylated compound which was used immediately in the next step without purification. The obtained debenzylated compound was dissolved in pyridine (4 mL) and acetic anhydride (2 mL) under air atmosphere. The resulting mixture was stirred at room temperature for overnight. After which, the acetic anhydride and pyridine were removed *in vacuo*. The resulting residue was taken up in 20 mL of CH_2Cl_2, and the solution was washed with 5 x 5 mL of H_2O and 5 mL of brine, dried over Na_2SO_4, and evaporated. The crude product was purified by column chromatography (hexane: ethyl acetate 1:1) to afford the compound **2** as a white powder (0.046 g, 66%). Mp: 71.6 °C; MS m/z (ESI): 701 (MNa$^+$); HRMS: found, 701.1541; $C_{34}H_{30}O_{15}Na$ requires 701.1482; ^{1}H NMR (CDCl$_3$, 500MHz) δ 7.79 (d, J=8.0 Hz, 1H), 7.64 (s, 1H), 7.42 (t, J=8.0 Hz, 1H), 7.28 (s, 1H), 7.17 (s, 2H), 6.69 (s, 1H), 6.64 (s, 1H), 5.46 (q, J=5.5 Hz, 1H), 5.32 (d, J=5.5

Hz, 1H), 3.02 (dd, *J*=17.0, 5.0 Hz, 1H), 2.80 (dd, *J*=17.0, 6.0 Hz, 1H), 2.32 (s, 3H), 2.29 (s, 3H), 2.27 (s, 3H), 2.26 (s, 9H); ^{13}C NMR (CDCl$_3$, 100MHz) δ 169.10, 168.81, 168.24, 167.47, 166.61, 164.61, 154.00, 150.42, 149.76, 149.28, 143.47, 135.74, 134.48, 130.72, 129.42, 127.18, 126.68, 122.79, 118.58, 109.84, 108.81, 107.54, 77.16, 69.17, 23.65, 20.96, 20.92, 20.65, 20.47, 20.10.

3.2.6. (2*S**,3*R**)-*trans*-5,7-Bis(acetoxy)-2-[3,4,5-tris (acetoxy) phenyl]chroman-3-yl 4-(acetoxy)benzoate (3a)

The title compound was prepared in a similar manner as described for **2a** using **7** (0.15 g, 0.16 mmol) to afford **3a** as a white solid (92.6 mg, 88%). Mp: 189.4 °C; MS *m/z* (ESI): 701 (MNa$^+$); HRMS: found, 701.1467; C$_{34}$H$_{30}$O$_{15}$Na requires 701.1482; ^{1}H NMR (CDCl$_3$, 500MHz) δ 7.94 (d, *J*=9.0 Hz, 2H), 7.17 (s, 2H), 7.13 (d, *J*=9.0 Hz, 2H), 6.69 (d, *J*=2.0 Hz, 1H), 6.63 (d, *J*=2.0 Hz, 1H), 5.45 (q, *J*=6.0 Hz, 1H), 5.32 (d, *J*=6.0 Hz, 1H), 3.01 (dd, *J*=16.5, 5.0 Hz, 1H), 2.79 (dd, *J*=16.5, 6.0 Hz, 1H), 2.31 (s, 3H), 2.29 (s, 3H), 2.27 (s, 3H), 2.26 (s, 9H); ^{13}C NMR (CDCl$_3$, 100MHz) δ 168.81, 168.64, 168.26, 167.49, 166.62, 164.74, 154.48, 154.02, 149.76, 149.30, 143.49, 135.82, 134.50, 131.28, 126.76, 121.60, 118.59, 109.90, 108.80, 107.51, 77.33, 69.01, 23.64, 20.97, 20.63, 20.46, 20.00.

3.2.7. (2*S**,3*R**)-*trans*-5,7-Bis(acetoxy)-2-[3,4,5-tris (acetoxy) phenyl]chroman-3-yl 3,5-bis(acetoxy)benzoate (4a)

The title compound was prepared in a similar manner as described for **2a** using **8** (0.15 g, 0.14 mmol) to afford **4a** as a white solid (72 mg, 70%). Mp: 105.5 °C; MS *m/z* (ESI): 759 (MNa$^+$); HRMS: found, 759.1530; C$_{36}$H$_{32}$O$_{17}$Na requires 759.1537; ^{1}H NMR (CDCl$_3$, 500MHz) δ 7.54 (d, *J*=2.0 Hz, 2H), 7.17 (s, 2H), 7.13 (t, *J*=2.0 Hz, 1H), 6.69 (d, *J*=2.0 Hz, 1H), 6.64 (d, *J*=2.0 Hz, 1H), 5.45 (q, *J*=6.0 Hz, 1H), 5.29 (d, *J*=6.5 Hz, 1H), 3.01 (dd, *J*=17.0, 5.0 Hz, 1H), 2.79 (dd, *J*=17.0, 6.5 Hz, 1H), 2.30 (s, 6H), 2.28 (s, 3H), 2.27 (s, 3H), 2.26 (s, 3H), 2.25 (s, 6H); ^{13}C NMR (CDCl$_3$, 100MHz) δ 168.80, 168.62, 168.21, 167.45, 166.60, 163.78, 153.96, 150.78, 149.77, 149.27, 143.46, 135.55, 134.48, 131.26, 120.57, 120.29, 118.56, 109.79, 108.86, 107.57, 77.32, 69.48, 23.77, 20.94, 20.86, 20.64, 20.45, 19.99.

3.2.8. (2*S**,3*R**)-*trans*-5,7-Bis(hydroxy)-2-[3,4,5-tris(hydroxy) phenyl]chroman-3-yl 3-(hydroxy)benzoate (2)

Suspension of **6** (0.23 g, 0.24 mmol) in THF/MeOH (28 mL/28 mL) and Pd(OH)$_2$ (0.19 g, 20% on carbon) was placed under an H$_2$ atmosphere. The resulting mixture was stirred at room temperature until TLC showed that the reaction was completed. Then the reaction mixture was filtered through cotton to remove the catalyst. After evaporation, the residue was purified by column chromatography (ethyl acetate: CH$_2$Cl$_2$ 2:1) to afford the product **2** as a white solid (80 mg, 79%). MS *m/z* (ESI): 449 (MNa$^+$); HRMS: found, 449.0887; C$_{22}$H$_{18}$O$_9$Na requires 449.0849; ^{1}H NMR (CD$_3$OD, 500MHz) δ 7.38-6.97 (m, 4H), 6.42 (s, 2H), 5.97 (q, *J*=2.5 Hz, 2H), 5.40 (q, *J*=6.0 Hz, 1H), 5.03 (d, *J*=6.0 Hz, 1H), 2.85 (dd, *J*=16.5, 5.0 Hz, 1H), 2.74 (dd, *J*=16.5, 6.0 Hz, 1H); ^{13}C NMR (CD$_3$OD, 100MHz) δ 165.87, 157.00, 156.52, 156.03, 154.88, 145.42, 132.50, 130.91, 129.17, 120.29, 119.90,

115.45, 105.00, 98.07, 95.00, 94.12, 77.78, 70.15, 22.71.

3.2.9. (2*S**,3*R**)-*trans*-5,7-Bis(hydroxy)-2-[3,4,5-tris(hydroxy) phenyl]chroman-3-yl 4-(hydroxy)benzoate (3)

The title compound was prepared in a similar manner as described for **2** using **7** (0.24 g, 0.25 mmol) to afford **3** as a white solid (79 mg, 75%). MS m/z (ESI): 449 (MNa$^+$); HRMS: found, 449.0840; C$_{22}$H$_{18}$O$_9$Na requires 449.0849; ^{1}H NMR (CD$_3$OD, 500MHz) δ 7.75 (d, J=8.5 Hz, 2H), 6.77 (d, J=8.5 Hz, 2H), 6.42 (s, 2H), 5.95 (d, J=8.0, 2.5 Hz, 2H), 5.35 (q, J=6.0 Hz, 1H), 5.00 (d, J=6.0 Hz, 1H), 2.85 (dd, J=16.5, 5.0 Hz, 1H), 2.71 (dd, J=16.5, 6.5 Hz, 1H); ^{13}C NMR (CD$_3$OD, 100MHz) δ 165.88, 161.99, 156.54, 156.05, 154.95, 145.41, 132.46, 131.31, 129.25, 120.61, 114.57, 104.98, 98.14, 94.91, 94.04, 78.00, 69.81, 22.98.

3.2.10. (2*S**,3*R**)-*trans*-5,7-Bis(hydroxy)-2-[3,4,5-tris(hydroxy) phenyl]chroman-3-yl 3,5-bis(hydroxy) benzoate (4)

The title compound was prepared in a similar manner as described for **2** using **8** (0.17 g, 0.16 mmol) to afford **4** as a white solid (50 mg, 71%). MS *m/z* (ESI): 465 (MNa$^+$); HRMS: found, 465.0844; C$_{22}$H$_{18}$O$_9$Na requires 465.0798; ^{1}H NMR (CD$_3$OD, 500MHz) δ 6.85 (d, *J*=2.0 Hz, 2H), 6.44 (t, *J*=2.0 Hz, 1H), 6.41 (s, 2H), 5.96 (s, 2H), 5.40 (dd, *J*=10.5, 5.0 Hz, 1H), 5.05 (d, *J*=5.0 Hz, 1H), 2.80 (dd, *J*=16.5, 5.0 Hz, 1H), 2.73 (dd, *J*=16.5, 5.0 Hz, 1H); ^{13}C NMR (CD$_3$OD, 100MHz) δ 165.81, 158.10, 156.56, 156.06, 154.83, 145.43, 132.44, 131.49, 129.28, 107.34, 106.86, 104.81, 97.93, 94.88, 94.06, 77.60, 69.95, 22.28.

3.3. Cell cultures

Human Jurkat T and LNCaP cells were cultured in RPMI supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The non-transformed natural killer cells (YT line) were grown in RPMI medium containing with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM MEM sodium pyruvate, and 0.1 mM MEM nonessential amino acids solution. Human breast cancer MCF-7 cells, normal (WI-38) and simian virus 40 (SV40)-transformed (VA-13) human fibroblast cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin. All cell cultures were maintained in a 5% CO$_2$ atmosphere at 37° C.

3.4. Nucleophilic susceptibility surface analysis

Colorimetric analysis of the electron density surface susceptible to nucleophilic attack was performed using the Cache Work-system version 3.2 (Oxford Molecular Ltd) as previously described (22).

3.5. Cell extract preparation and western blotting

Whole cell extracts were prepared as described previously (19). Analysis of Bax, IκBα, p27, PARP, and ubiquitinated protein expression were performed using monoclonal or polyclonal antibodies according to previously reported protocols (19).

3.6. Inhibition of purified 20S proteasome activity by (-)-EGCG or synthetic tea polyphenols

Measurement of the chymotrypsin-like activity of the 20S proteasome was performed by incubating 0.5 μg of

purified rabbit 20S proteasome with 40 µM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC, with or without various concentrations of natural and synthetic tea polyphenols as described previously (22).

3.7. Inhibition of proteasome activity in intact cells by natural or synthetic tea polyphenols

Cells were grown in 24 well plates (2 ml/well) to 70-80% confluency, followed by treatment with an indicated compound at 25 µM for 24 h. Suc-Leu-Leu-Val-Tyr-AMC (40 µM) was then added into the cells for 2.5 h and the chymotrypsin-like activity was measured as described above.

3.8. Immunostaining of apoptotic cells with anti-cleaved PARP conjugated to FITC

Immunostaining of apoptotic cells was performed by addition of a FITC-conjugated polyclonal antibody that recognizes cleaved PARP and visualized on an Axiovert 25 microscope (Zeiss; Thornwood, NY). Briefly, cells were grown to ~80% confluency in 60 mm dishes, and then treated with an indicated compound at 25 µM for 24 h. Following treatment, both suspension and adhering cells were collected and washed twice in PBS/pH 7.4. The cells were washed for 1 min in PBS between all steps listed below. Cells were then fixed in ice-cold 70% ethanol, permeabilized in 0.1% Triton-X-100 and blocked for 30 min in 1% BSA at room temperature. Incubation with the primary FITC-conjugated-p85/PARP antibody was for 30 min at 4ºC in the dark with mild shaking. Cell suspension was then transferred to glass slides in the presence of Vector Shield mounting medium with DAPI. Images were captured using AxioVision 4.1 and adjusted using Adobe Photoshop 6.0 software. Cell death was quantified by counting the number of apoptotic cells over the total number of cells in the same field. Data are mean of duplicate experiments ±SD.

3.9. Trypan blue assay and apoptotic morphology changes

The trypan blue dye exclusion assay was used to ascertain cell death in Jurkat T cells treated with natural and synthetic polyphenols as indicated (22, 23). Apoptotic morphology was assessed using phase-contrast microscopy as described previously (23, 27).

3.10. MTT assay

MTT was used to determine effects of polyphenols on overall proliferation of tumor cells. Cells were plated in a 96-well plate and grown to 70-80% confluency, followed by addition of analogs for 24 h. MTT (1 mg/ml) in PBS was then added to wells and incubated at 37ºC for 4 hours to allow for complete cleavage of the tetrazolium salt by metabolically active cells. Next, MTT was removed and 100 µl of DMSO was added, followed by colorimetric analysis using a multilabel plate reader at 560 nm (Victor[3]; Perkin Elmer). Absorbance values plotted are the mean from triplicate experiments.

3.11. Soft agar assay

Cells (2 x 10^{4}) were plated in soft agar on 6-well

plates in the presence of (-)-EGCG (25 µM), a protected tea analog (25 µM) or DMSO (control) to determine cellular transformation activity as described previously (23).

3.12. Nuclear staining

After each drug treatment, both detached and attached populations of VA-13 and WI-38 lines were stained Hoechst 33342 to assess apoptosis. Briefly, cells were washed 2X in PBS, fixed for 1 h with 70% ethanol at 4ºC, washed 3X in PBS, and stained with 50 µM Hoechst for 30 min in the dark at room temperature. Detached cells were plated on a slide and attached cells were visualized on the culture plate with a fluorescent microscope at 10X or 40X resolution (Zeiss, Thornwood, NY). Images were obtained using an AxioVision 4.1 and adjusted using Adobe Photoshop 6.0.

3.13. Measurement of H_2O_2 formation

Production of hydrogen peroxide (H_2O_2) was determined in Jurkat T cells treated with tea polyphenols (25 µM) for 3 h. H_2O_2, indicative of oxidation, was measured according to manufacturer's specifications. To determine if N-acetyl-$_L$-cysteine (NAC) could inhibit H_2O_2 production induced by polyphenol treatment, cells were pretreated for 2 h with 5 µM NAC, followed by addition of 25 µM of each indicated tea polyphenol and incubated at 37ºC for 2 h.

4. RESULTS

To further discover stable, potent and specific polyphenol proteasome inhibitors as tumor-specific apoptotic inducers, we designed and synthesized a number of (-)-EGCG analogs with deductions to the phenolic hydroxy groups on the gallate moiety (D-ring), namely **2**, **3**, and **4** (racemic compounds; Figure 1A). In addition, **2a**, **3a**, and **4a** (racemic compounds) were also synthesized to include protected hydroxyl groups as acetates (peracetates) to elucidate whether protection of the reactive –OH groups would aid in improving polyphenol stability.

4.1. Acetylated synthetic tea polyphenols do not inhibit the purified 20S proteasome chymotrypsin-like activity

We have previously shown that natural (-)-EGCG is a potent inhibitor of the chymotrypsin-like activity of the proteasome (22). This inhibition is associated with the susceptibility to nucleophilic attack of the ester bond carbon in (-)-EGCG (22). Atomic orbital analysis of (-)-EGCG and its protected analog (**1**) shows that only (-)-EGCG displays a significant area of nucleophilic susceptibility over the ester bond carbon, while **1** and other peracetate-protected compounds have little potential for undergoing nucleophilic attack (Figure 1B and data not shown). Thus, **1** and other protected analogs should be inactive inhibitors to purified proteasome. To test this hypothesis, up to 25 µM of all protected and unprotected compounds were incubated with a purified rabbit 20S proteasome and a fluorogenic substrate for chymotrypsin activity for 30 min. The half-maximal inhibitory concentration or IC_{50} was then determined (Table 1). (-)-EGCG showed to be the most potent with an IC_{50} of 0.2 µM, as we reported previously (22, 24), followed by **2**

Table 1. Inhibition of proteasome activity by synthetic tea polyphenols [1,2]

Unprotected	IC$_{50}$ (μM)	Protected	IC$_{50}$ (μM)
(-)-EGCG	0.2 ± 0.1	**1**	n.a.[3]
2	9.9 ± 1.1	**2a**	n.a.[3]
3	14.9 ± 0.8	**3a**	n.a.[3]
4	14.1 ± 5.8	**4a**	n.a.[3]

[1] Inhibition of purified 20S rabbit proteasome was measured as described under "Materials and Methods". [2] Results obtained from experiments performed in triplicate. [3] n.a. indicates that the inhibitory activity of the purified 20S proteasome at μM was <35%.

(IC$_{50}$ 9.9 μM). IC$_{50}$ values of compounds **3** and **4** were found be 14-15 μM (Table 1). In contrast, the protected analogs were much less active: <35% inhibition at 25 μM (Table 1). This is consistent with the atomic orbital analysis data (Figure 1B).

4.2. Protected tea analogs exhibit greater proteasome-inhibitory potency in intact tumor cells

To determine what effects the synthetic tea analogs had on cellular proteasome activity, Jurkat T cells were treated with 25 μM of each synthetic compound for either 4 or 24 h, with (-)-EGCG as a control (Figure 2A and B). Inhibition of the proteasome should result in accumulation of polyubiquitinated proteins which are detectable by a specific antibody to ubiquitin. Western blot analysis shows that after 4 h of treatment, the acetate-protected analogs induced more ubiquitinated proteins than unprotected counterparts (Figure 2A, *Lanes 3, 5, 7, 9 vs. 2, 4, 6, 8*), indicating that proteasome activity is abrogated.

We then determined the effects of these synthetic tea compounds on Bax and IκBα, two well-known proteasome targets (18 , 28). Previously by performing a coupled immunoprecipitation and Western blotting assay, we identified an ubiquitinated form of Bax with molecular mass 55 kDa (p55) (18). After Jurkat T cells were treated for 4 h, Western blots for Bax revealed the appearance of a p55 band, similar to the previously reported ubiquitinated Bax (18), which was accumulated to a high level by the protected compounds **2a**, **3a** and **4a**, and a low level by **1** (Figure 2A, *lanes 3, 5, 7, and 9*). Previously we have also reported that the green tea polyphenol proteasome inhibitor (-)-EGCG was able to accumulate a candidate ubiquitinated IκB-α of ~56 kDa (p56) (22). Levels of a p56 band, detectable by the specific antibody to IκB-α, significantly increased with 4 h treatment by all the protected compounds **2a**, **3a**, **4a**, and **1** (Figure 2A, *lanes 3, 5, 7, and 9*).

After 24 h treatment, the ubiquitinated Bax band is still found in the cells treated with **2a 3a**, and **4a** (Figure 2B, *Lanes 9, 5, 7*). Accumulation of p27, another proteasome target (29), is also found in Jurkat cells treated with the protected analogs **2a**, **3a** and **4a** for 24 h (Figure 2B, *Lanes 9, 5, 7*). However, the ubiquinated IκBα band is absent in these cells (data not shown), possibly due to deubiquitination or very low expression. Actin was used as a loading control in this experiment (Figure 2A and B).

In a kinetics experiment, Jurkat T cells were treated with a pair of analogs, **4** and **4a**. It was found that the unprotected analog **4** induced accumulation of ubiquitinated proteins with the highest expression after 8 h of treatment (Figure 2C). Conversely, the protected **4a** showed increased ubiquitinated protein accumulation as early as 2 h and lasting up to 8 h (Figure 2C). To determine if acetate-protected analogs are potent proteasome inhibitors in other cancer cell systems, prostate cancer LNCaP cells were treated for 24 h with 25 μM of (-)-EGCG, **1**, **2a**, or **3a**, with DMSO as a control. Indeed, ubiquitin-conjugated proteins were observed, with the greatest increase found in cells treated with **2a** and **3a** (Figure 2D). These data suggest that the protected forms of (-)-EGCG analogs are potent proteasome inhibitors in intact tumor cells.

### 4.3. Protected analogs are more potent apoptosis inducers than unprotected compounds

It has been shown that proteasome inhibition can induce apoptosis in a wide variety of cancer cells, but not in normal and non-transformed cells (19 , 30 , 31). We then treated Jurkat T cells with 25 μM of each of the selected polyphenols for 24 h to investigate their abilities to induce apoptotic cell death. The trypan blue incorporation assay revealed that **2a**, **3a** and **4a**, but not others, induced death in 99, 57, and 83% of Jurkat cells, respectively (Figure 3A). Similarly, Western blot analysis showed that only **2a**, **3a**, and **4a** induced apoptosis-specific PARP cleavage after 24 h (Figure 3B). An immunofluorescent staining assay that detects only the cleaved PARP fragment (p85; green) showed that SV40-transformed VA-13 cells are highly sensitive to apoptosis induced by **2a** with 73% apoptotic cells after 24 h treatment (Figure 3C and D). The unprotected **2** induced much less apoptosis (21%), while VP-16, which was used as a positive control, induced apoptosis in 92% of cells (Figure 3C and D). Counterstain with DAPI, which binds to the minor groove in A-T rich regions of DNA (32), was decreased drastically in apoptotic cells (Figure 3C), consistent with DNA fragmentation in late stage of apoptosis. To determine the involvement of caspase-3 and −9, Western blotting was performed with lysates of Jurkat cells treated with (-)-EGCG, **4**, and **4a** for 24 h (Figure 3E). Only the protected compound **4a** promoted the cleavage of both procaspase-3 and −9, indicating the involvement of both caspase-9 and caspase-3 in the apoptotic cell death induced by the protected analogs (Figure 3E).

4.4. Inhibition of solid tumor cell proliferation by protected polyphenols

We then treated breast cancer MCF-7 cells with 5 or 25 μM of peracetate-protected analogs for 24 h, followed by MTT analysis to determine their effects on cell growth. Compound **1** at 25 μM inhibited cellular proliferation by 40% (Figure 4A). The protected compounds **2a**, **3a**, and **4a** caused 50% inhibition at 5 μM and 70% at 25 μM (Figure 4A).

We also treated human prostate cancer LNCaP cells for 24 h with each selected tea polyphenol at 25 μM, followed by determining the apoptotic morphological changes.
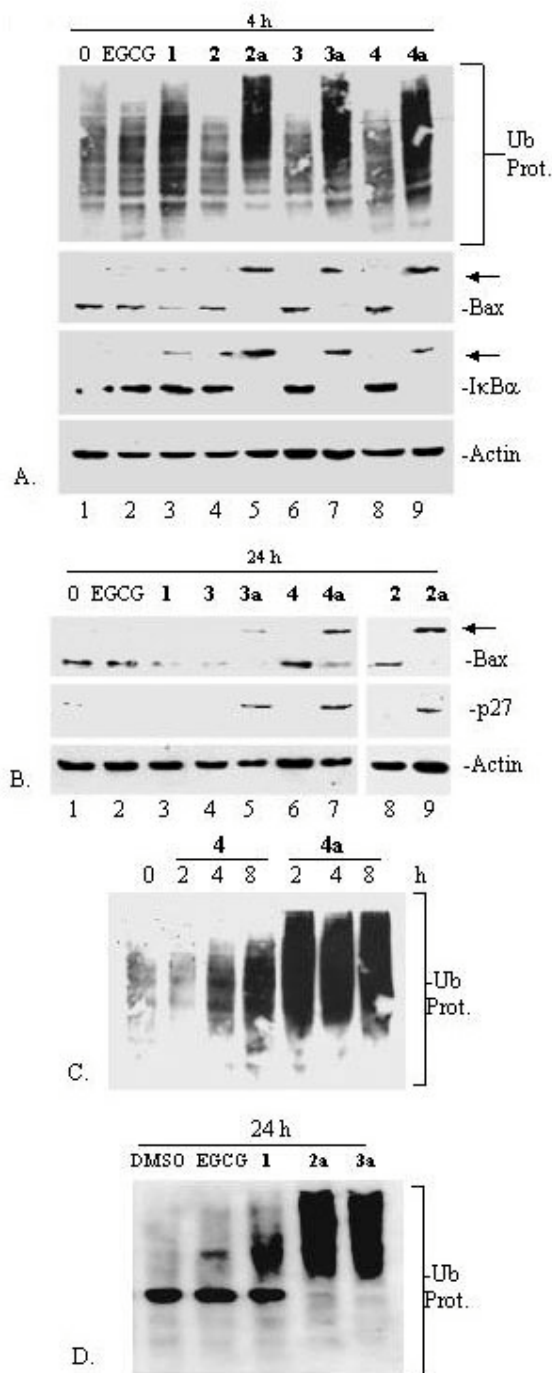
Figure 2. Peracetate-protected analogs are potent proteasome inhibitors in cultured cells. Jurkat T cells were treated with 25 μM of each indicated polyphenol for 4 (A), up to 8 (C), or 24 h (B), or LNCaP cells were treated with 25 μM of indicated compound for 24 h (D), followed by Western blot analysis using specific antibodies to Ubiquitin, Bax, IκBα, p27 and Actin. The bands indicated by an *arrow* are possible ubiquitinated forms of Bax and IκBα. A, *Lane 4*, Ub-IκBα band may be result of spillage from *Lane 5*. Data shown are representative from three independent experiments.

Again, the protected analogs **2a**, **3a**, and **4a** caused dramatic round-up, detachment, and cellular fragmentation (Figure 4B). The protected compound **1** induced mild morphological changes (Figure 4B), while (-)-EGCG treatment led to enlarged, flattened cells (Figure 4B), indicating growth arrest (33).

Soft agar assay is used to determine the transforming activity of tumor cells. Abrogation of colony formation is linked to G_1 arrest and/or apoptosis (23). LNCaP cells were added to soft agar in 6-well plates, and were then treated one time at initial plating with 25 μM of (-)-EGCG or a protected analog (Figure 4C). After 21 days, colony formation was evaluated. Cells treated with (-)-EGCG showed a significant decrease in colony formation compared to the control cells treated with DMSO, as reported previously (23) (Figure 4C and D). Protected polyphenols also inhibited tumor cell transforming activity, with **2a** and **4a** being the most potent inhibitors of colony formation (Figure 4D).

4.5. Preferential induction of apoptosis in tumor cells by protected analogs

The ability to induce apoptosis in tumor cells, but not normal cells is an important measure for novel anti-cancer drugs. We have previously shown that (-)-EGCG and other proteasome inhibitors preferentially accumulate p27, induces G_1 growth arrest and apoptosis in SV40-transformed (VA-13) over normal (WI-38) human fibroblasts (19 , 22). To determine whether the protected compounds also affect normal cells, we treated both VA-13 and WI-38 cells with 25 μM of (-)-EGCG, **2** or **2a** for 24 h and examined the proteasome activity, nuclear morphological changes, and detachment. We found that there was a differential effect on the chymotrypsin-like activity of the proteasome in VA-13 *over* WI-38 cells (Figure 5A). In VA-13 cells, treatment with (-)-EGCG and compound **2** caused a 45-48% decrease in the proteasome activity, and compound **2a** inhibited 92% of the proteasomal activity (Figure 5A). Conversely, the proteasome activity in WI-38 cells was decreased by only 5% or less with all three polyphenol treatments.

Next we examined apoptotic nuclear morphology (Figure 5B). While treatment of (-)-EGCG and **2** for 24 h exhibited little or no apoptosis, 24 h treatment with **2a** markedly induced detachment and apoptosis in the transformed VA-13 cells (Figure 5B). In contrast, normal WI-38 fibroblasts treated with all the compounds did not undergo apoptosis and very little detachment was visible (Figure 5B).

After 36 h treatment, we did notice that (-)-EGCG initiated morphological changes and detachment of VA-13 cells (Figure 5C). Again all of the protected analogs induced these events in transformed (VA-13), but not in normal (WI-38) cells (Figure 5C). Similarly, when leukemic (Jurkat T) and normal, non-transformed natural killer (YT) cells were treated with (-)-EGCG and **2a** for 24 h, only Jurkat cells underwent apoptosis as evidenced by PARP cleavage (Figure 5D).
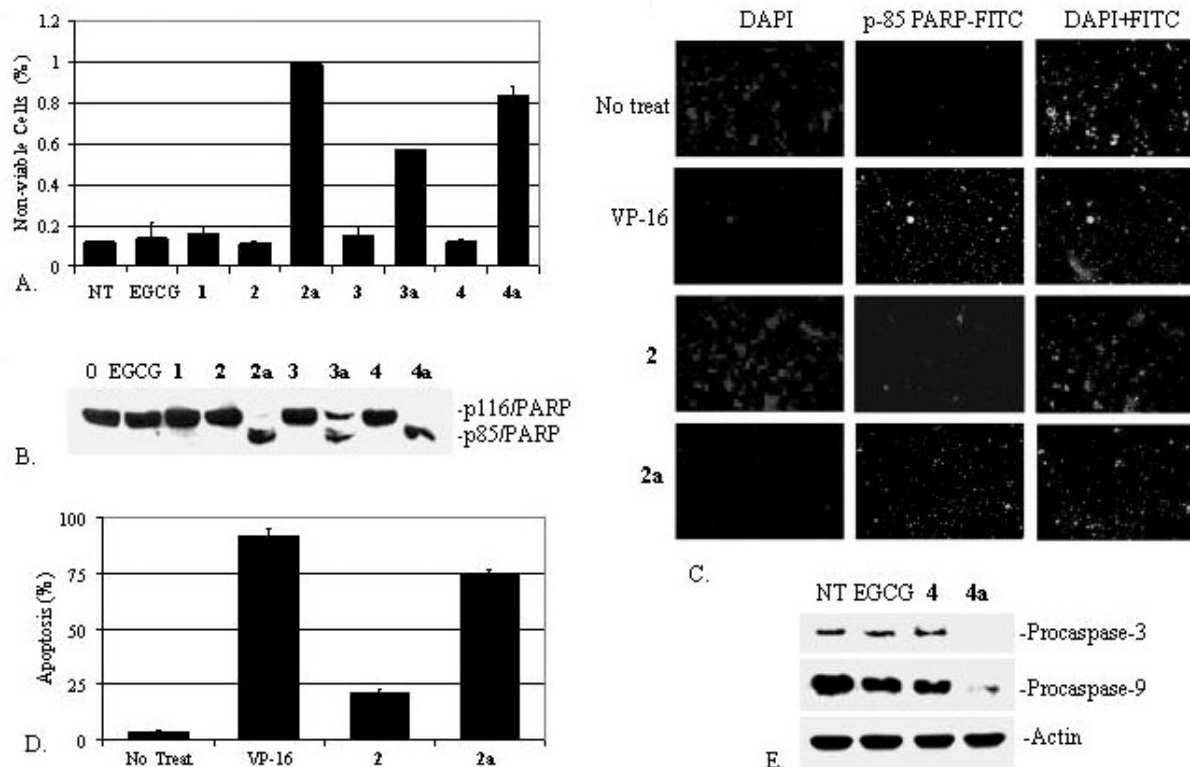
Figure 3. The acetylated polyphenol analogs are more potent apoptosis inducers than their unprotected counterparts. Jurkat T cells (A, B, and E) or VA-13 (C and D) cells were treated with 25 μM of indicated polyphenols for 24 h. A, trypan blue incorporation assay. The data represented are as the mean number of dead cells over total cell population ±SD. B, Western blot for PARP cleavage. C, Fluorescent microscopy studies of late-stage apoptosis using a specific antibody to the p85 cleaved PARP fragment conjugated to FITC. Counterstaining with DAPI is used as a control for non-apoptotic cells. Images were obtained with AxioVision software utilizing an inverted fluorescent microscope (Zeiss, Germany). D, Quantification of apoptotic cells (in C) was calculated by counting the number of apoptotic cells *over* the total number of cells in the same field. Data are mean of duplicate experiments ±SD. E, Western blot for levels of procaspase-3, and -9.

4.6. Proteasome inhibition and apoptosis are not mediated by oxidative damage induced by synthetic tea polyphenols

There have been conflicting reports about the oxidative effect of (-)-EGCG and the correlation with its anti-cancer properties. (-)-EGCG has been shown to be a powerful anti-oxidant (34 , 35), while others report production of H_2O_2 by (-)-EGCG (36 , 37). To determine whether the protected or unprotected analogs induced formation of H_2O_2 and, if so, whether this activity is coupled with the proteasome-inhibitory and apoptosis-inducing effects of the synthetic tea analogs, we performed several experiments. First, Jurkat T cells were treated with 25 μM of each of the tea polyphenols (Figure 1), followed by colorimetric detection of H_2O_2 after a 3 h-incubation. We found that (-)-EGCG produced ~400 nM of H_2O_2. Synthetic analogs, **1**, **2a**, and **4a** also induced H_2O_2 levels comparable to that of (-)-EGCG. Second, to verify that the tea polyphenols were causing production of H_2O_2, we pretreated Jurkat T cells with 5 μM of *N*-acetylcysteine (NAC) for 2 h, followed by introduction with (-)-EGCG or a synthetic compound for 2 h. We found that NAC did indeed inhibit production of H_2O_2 by (-)-EGCG or the synthetic compounds. However, NAC pretreatment did not affect the abilities of the tea polyphenols to inhibit the

proteasome and induce apoptosis in Jurkat T cells (data not shown), indicating that production of H_2O_2 is not required for either proteasome inhibition or apoptosis. Finally, to ascertain whether H_2O_2 has an effect on the proteasome-inhibitory properties of protected/unprotected tea analogs and (-)-EGCG, we treated purified 20S proteasome with H_2O_2 and measured the effects on the proteasomal chymotrypsin-like activity. H_2O_2 at up to 10 μM caused very little inhibition (~25%).

5. DISCUSSION

Recently, in an effort to enhance the stability of green tea polyphenols such as (-)-EGCG at biological pH, we synthesized a protected form of (-)-EGCG, and found that it was more stable and potent than natural (-)-EGCG although the effect was moderate (26). To further evaluate the proof of concept of whether acetylated EGCG analogs act as prodrugs for proteasome inhibitors and apoptosis inducers, we designed, synthesized and evaluated a series of (-)-EGCG analogs, both unprotected and protected compounds (Figure 1).

We observed, as expected, that the protected compounds were inactive to a purified 20S proteasome,
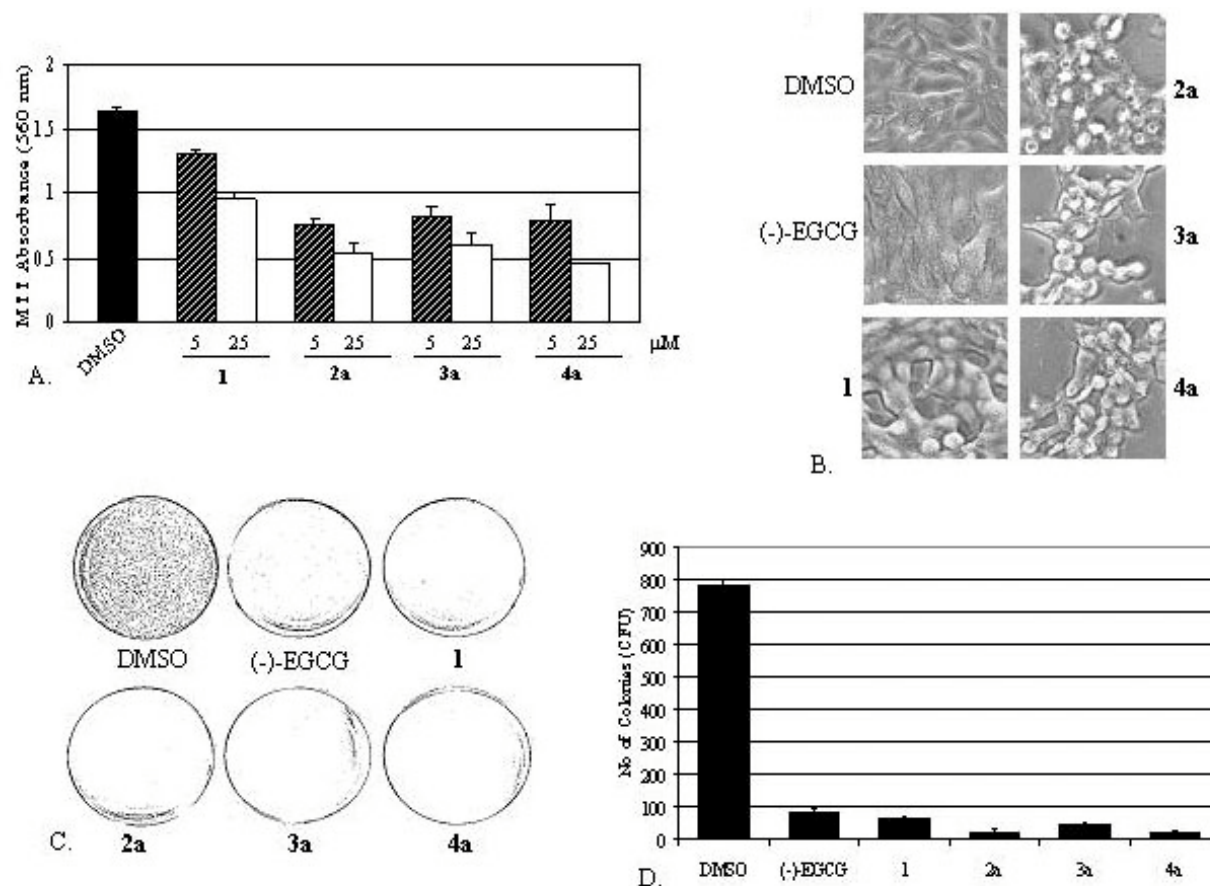
Figure 4. Effects of synthetic acetylated polyphenols on breast and prostate cancer cells. A, MTT assay. Breast cancer MCF-7 cells were treated with each indicated compound at 5 or 25 μM for 24 h. B, Morphological changes. Prostate cancer LNCaP cells were treated with 25 μM of (-)-EGCG or a protected analog for 24 h, followed by morphological assessment. Images were obtained using a phase-contrast microscope at 40X magnification (Leica, Germany). C, Soft agar assay. LNCaP cells were plated in soft agar with the solvent DMSO or 25 μM of (-)-EGCG or protected analogs. Cells were cultured for 21 days without further addition of drug. Data shown are representative scanned wells from triplicate experiments. D, Colonies in C were quantified with an automated counter and presented as mean values ±SD.

compared to their unprotected counterparts (Table 1). By performing electron density surface analysis, our laboratory previously showed that the ester carbon of (-)-EGCG is the likely site of nucleophilic attack (Figure 1B) (24). The β5 subunit of the proteasome, specific for chymotrypsin-like activity, would attack the ester carbon of (-)-EGCG, leading to an irreversible acylation of the side chain hydroxyl group of the *N*-terminal threonine on β5 and thereby inhibiting the catalytic activity (22 , 24). Surface analysis of the peracetate protected tea polyphenols revealed a significant reduction in nucleophilic susceptibility at the gallate ester carbon (Figure 1B) and, in some cases, a shift of the nucleophilic site to other positions on the molecule (data not shown). Additionally, the size of these compounds would sterically hinder interaction with the chymotrypsin active site. Consistent with these analyses, we found that the protected compounds are not active proteasome inhibitors *in vitro* (Table 1).

Most interestingly, the protected compounds

inhibit the proteasome activity in intact tumor cells (Figure 2), as shown by the following evidence. First, the levels of polyubiquitinated proteins were significantly increased after treatment of Jurkat T or LNCaP cells with a protected analog (Figure 2A, C and D). Second, some putative ubiquitinated form of Bax and IκBα appeared, accompanied with the disappearance of the unmodified proteins in these treated cells (Figure 2A and B). Third, the levels of the proteasome target p27 protein were significantly increased in Jurkat T cells treated with several protected analogs (Figure 2B). Finally, the levels of the proteasomal chymotrypsin-like activity were also greatly inhibited in VA-13 cells treated with protected compounds (Figure 5A). The fact that these protected compounds are inactive to a purified proteasome (Table 1) but are very potent proteasome inhibitors in cultured tumor cells (Figure 2) supports our hypothesis that these acetylated EGCG analogs are prodrugs of polyphenol proteasome inhibitors. The order of the proteasome-inhibitory activity of the protected analogs in intact tumor cells was found to be: **2a**, **4a** > **3a** > **1** > (-)-EGCG.
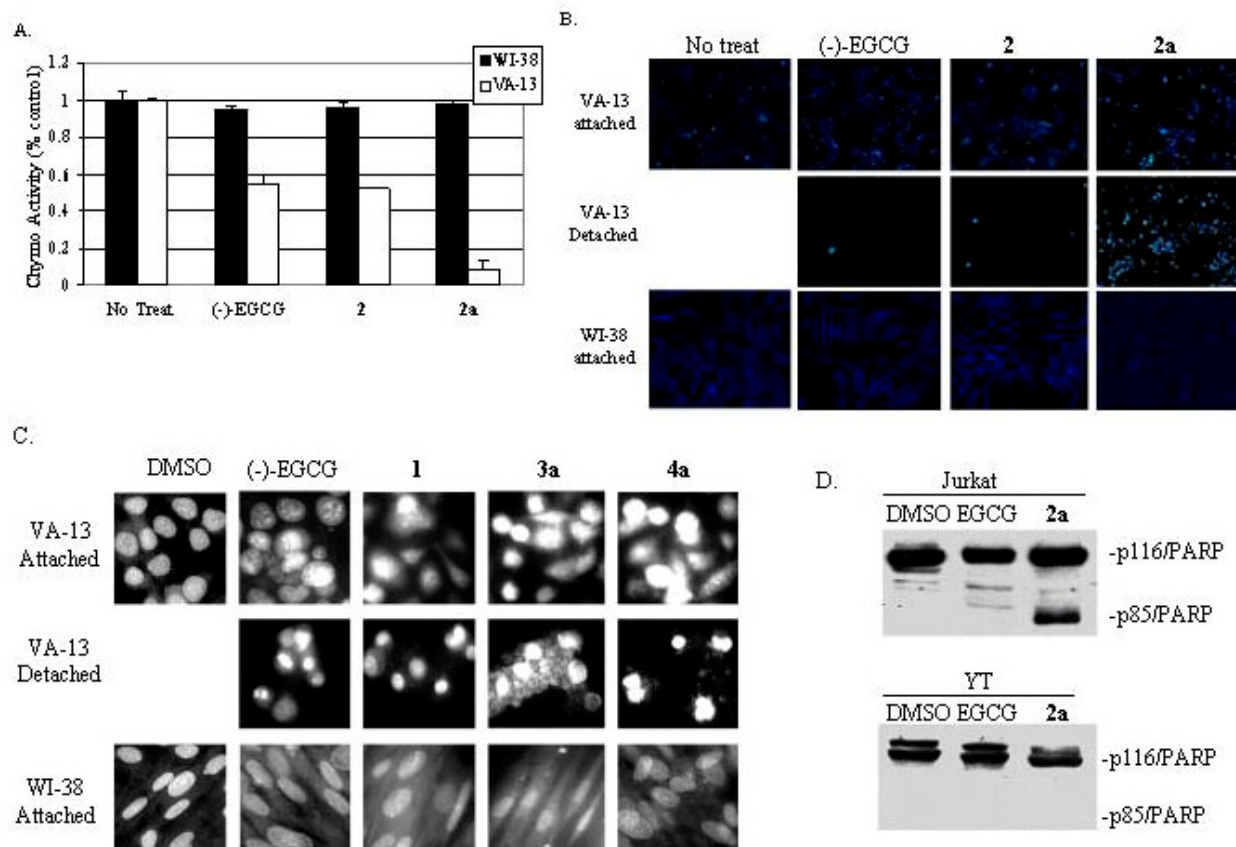
Figure 5. Protected tea analogs inhibit the proteasome activity and induce apoptosis selectively in transformed and cancer cells. Normal WI-38 and SV40-transformed VA-13 cells were treated with 25 µM of indicated compounds for 24 h (A and B) or 36 h (C), or leukemic Jurkat T and non-transformed YT cells were treated with each compound at 25 µM for 24 h (D). A, Chymotrypsin-like activity of the proteasome in intact cells. B, C, Nuclear staining for apoptotic morphology of both detached and attached cells at 10X (B) or 40X (C) magnification. Missing panels indicate that no detachment of cells occurred. D, Western blot analysis using specific antibody to PARP.

However, conversion of the prodrugs to the parental (-)-EGCG analogs in cells does not seem to be the only responsible mechanism, due to the dramatic increase of the proteasome-inhibitory activity of the protected compounds **2a, 3a, and 4a** over their unprotected partners (Figure 2). Compound **1** seems to mimic natural (-)-EGCG in the effect that it is good proteasome inhibitor (Figure 2A), but had little cell death-inducing activity (Figures 3B and 4B). It is very possible that some of the protected compounds are changed intracellularly into entirely new compounds with unique chemical structure that have potent proteasome-inhibitory activity. This possibility will be investigated in the near future by analyzing levels of cellular polyphenols produced. Others have shown that (-)-EGCG supplement has a maximal concentration of 4.4 µM in human plasma (38 , 39). We have previously shown that (-)-EGCG has proteasome-inhibitory effects in cultured cells at concentrations as low as 1 µM (40). For this study we used concentrations up to 25 µM to contrast the differences between the unprotected and the protected tea analogs (*i.e.*, minimal effects with unprotected analog treatment even at concentrations of 25 µM *versus* the very potent acetate-protected analogs). Additionally, we have shown that the protected compounds at concentrations as

low as 5 µM have anti-proliferative effects (Figure 4A).

We previously reported that natural (-)-EGCG and its synthetic analogs are potent inhibitors of the proteasome, leading to cell cycle arrest in G_1 and/or induction of apoptosis in cancer cell lines (22-24). We then hypothesized that the protected compounds that potently inhibited tumor cellular proteasome activity (Figure 2) would induce greater growth inhibition and/or more cell death than the unprotected compounds. Indeed, the protected analogs were much more potent inducers of apoptotic cell death than their unprotected partners, when tested in leukemic (Jurkat T), solid tumor, and transformed cell lines, as shown by trypan blue incorporation, PARP cleavage by both Western and immunostaining, caspase activation, and apoptotic morphological changes (Figures 3, 4B, and data not shown). Furthermore, these protected compounds were also capable of inhibiting cell proliferation and colony formation of human breast and prostate tumor cell lines (Figure 4). Therefore, if some new compound(s) with potent proteasome-inhibitory activity were formed intracellularly from the protected analogs (Figure 2), they must be very potent tumor apoptosis inducers in cultured cells. We would anticipate that the

unprotected analogs **2**, **3**, and **4** would also have an effect on proliferation and transformation activity, however we would anticipate that these effects would be low compared to (-)-EGCG based on their IC_{50}'s. Most importantly, an identical order of the potencies of these protected compounds were found to inhibit tumor cellular proteasome activity and to induce apoptosis: **2a**, **4a** > **3a** > **1** > (-)-EGCG.

Many of the currently available chemotherapeutic agents are incapable of selectively targeting cancer cells from normal cells, leading to treatments that are almost as hazardous as the disease itself. Therefore, tumor selectivity is a most important measure for developing new anti-cancer drugs. When the protected EGCG analogs were tested in human normal and non-transformed cells, we found that they had little to no effects. Specifically, the protected compound **2a** [as well as the unprotected **2** and (-)-EGCG with decreased potencies] inhibited the proteasomal chymotrypsin-like activity in the SV40-transformed, but not in normal, human fibroblasts after 24 h treatment (Figure 5A). Accompanied with that, **2a** induced detachment and apoptotic morphological changes in the transformed but not normal cells (Figure 5B). In another experiment at 36 h time point, all the protected tea polyphenol analogs **1**, **3a** and **4a**, as well as natural (-)-EGCG, induce detachment and apoptotic morphological changes in VA-13, but not normal WI-38 cells (Figure 5C). Finally, when leukemia Jurkat T and non-transformed human natural killer cells (YT) were compared, the protected **2a** induced apoptosis-specific PARP cleavage in Jurkat T, but not YT cells (Figure 5D). All these data strongly suggest a great potential for these protected EGCG analogs to be developed into novel anti-cancer agents.

(-)-EGCG has been shown to be absorbed mainly through the small intestine and is subject to many metabolic reactions including *O*-methylation, glucuronidation, and sulfation (41). Additionally, there are conflicting opinions on the oxidative effect of (-)-EGCG in mammalian cells. Many reports show that (-)-EGCG is a powerful antioxidant (34 , 35). (-)-EGCG's antioxidant activity is most likely due to the free radical scavenging activity to form the phenoxy radical or phenoxide ion. Other data suggests that (-)-EGCG administration alone induces reactive oxygen species (ROS) through the formation of H_2O_2 in the presence of metal ions to form free radicals (36 , 37). We evaluated the formation of H_2O_2 caused by the synthetic tea polyphenols to determine if there might be a correlation between ROS production and their effects on the proteasome and apoptotic signaling pathways. While both unprotected and protected analogs induced H_2O_2 production to varying degrees, we found that treatment with NAC, a pharmacological stimulator of glutathione (a powerful antioxidant), was capable of preventing H_2O_2 production by (-)-EGCG, **3**, and **3a** (data not shown). However, NAC had no effect on the ability of **3** or **3a** to inhibit the chymotrypsin-like activity of the proteasome, nor did it affect the amount of apoptosis in Jurkat T cells (data not shown). Therefore, the synthetic protected and unprotected compounds most likely do not rely on generating ROS to inhibit proteasome activity or induce apoptosis.

With greater than 42% of cancer patients dying from the disease, there is much need to develop new chemotherapeutic agents with low toxicities and examine possible cancer preventive substances. It has been reported that dietary intake of green tea is able to produce adequate therapeutic concentrations in the body with no toxic effects observed, but the polyphenols have poor bioavailability (38 , 42 , 43). Addition of acetate, a natural hepatic degradation product, should increase the bioavailability of the polyphenol since polyphenols in their unprotected form rapidly degrade in neutral and alkaline environments. Many believe that (-)-EGCG is the most potent natural tea polyphenol (1-3). However, the synthetic green tea analogs presented here possess a greater ability than (-)-EGCG to inhibit proteasome and induce apoptosis in a tumor/transformed cell-selective manner. Further determination of the metabolites in cell extract of the peracteate-protected analogs is needed to compare with natural tea catechins. In addition, animal studies are also needed to confirm if the protected analogs possess improved stability, proteasome-inhibitory activity and anti-tumor activity at reduced concentrations, compared to that of natural tea polyphenols.

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