A Novel Prodrug of the Green Tea Polyphenol (−)-Epigallocatechin-3-Gallate as a Potential Anticancer Agent

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Abstract

The most abundant and biologically active green tea catechin, (−)-epigallocatechin-3-gallate or (−)-EGCG, has been shown to act as a proteasome inhibitor and tumor cell death inducer. However, (−)-EGCG is unstable under physiologic conditions and has poor bioavailability. Previously, in an attempt to increase the stability of (−)-EGCG, we introduced peracetate protections to its reactive hydroxyl groups and showed that this peracetate-protected (−)-EGCG [Pro-EGCG (1); formerly named compound 1] could be converted into (−)-EGCG under cell-free conditions. In the current study, we provide evidence that when cultured human breast cancer MDA-MB-231 cells were treated with Pro-EGCG (1), (−)-EGCG was not only converted but also accumulated, accompanied by enhanced levels of proteasome inhibition, growth suppression, and apoptosis induction, compared with cells treated with natural (−)-EGCG. To investigate the potential use of Pro-EGCG (1) as a novel prodrug that converts to a cellular proteasome inhibitor and anticancer agent *in vivo*, MDA-MB-231 tumors were induced in nude mice, followed by treatment with Pro-EGCG (1) or (−)-EGCG for 31 days. Results of this *in vivo* study showed a significant inhibition of breast tumor growth by Pro-EGCG (1), compared with (−)-EGCG, associated with increased proteasome inhibition and apoptosis induction in tumor tissues. In conclusion, we have shown that Pro-EGCG (1) increases the bioavailability, stability, and proteasome-inhibitory and anticancer activities of (−)-EGCG in human breast cancer cells and tumors, suggesting its potential use for cancer prevention and treatment. [Cancer Res 2007;67(9):4303–10]

Introduction

Second only to water, tea is the most widely consumed beverage in the world. Epidemiologic studies indicate that green tea consumption is associated with cancer-preventative effects (1–3). Biologically active components of green tea include polyphenolic catechins, (−)-epicatechin [(−)-EC], (−)-epicatechin-3-gallate [(−)-ECG], (−)-epigallocatechin [(−)-EGC], and (−)-epigallocatechin-3-gallate [(−)-EGCG; ref. 4]. Several studies indicate that (−)-EGCG is the most abundant and biologically active catechin with respect to anticancer activity in several human cancers (5–7). Various potential mechanisms contributing to the anticancer effects of (−)-EGCG have been described, including suppression of Wnt signaling (8), blockade of methylation (9), and inhibition of matrix metalloproteinase (10) and the proteasome (11–16).

The eukaryotic proteasome is a large multi-catalytic, multi-subunit protease complex possessing at least three distinct activities, which are associated with three different β subunits: chymotrypsin-like (with β5 subunit), trypsin-like (with β2 subunit), and peptidyl-glutamyl peptide-hydrolyzing–like (PGPH- or caspase-like, with β1 subunit; ref. 17). Inhibition of the chymotrypsin-like but not the trypsin-like activity of the proteasome has been found to be associated with induction of tumor cell apoptosis (18, 19). By examining a broad range of cell culture models, it has been found that proteasome inhibitors rapidly induce tumor cell apoptosis, selectively activate the cell death program in cancer or oncogene-transformed but not normal or untransformed cells, and are able to trigger apoptotic death in human cancer cells that are resistant to various anticancer agents (20–24).

The proteasome inhibitor bortezomib (Velcade, PS-341; Millenium Pharmaceuticals, Inc. and Johnson Pharmaceutical Research and Development, L.L.C.) is the first of its class to receive regular approval from the U.S. Food and Drug Administration for the treatment of cancer. However, some toxicity from bortezomib treatment was observed (20, 21), suggesting a need to discover new proteasome inhibitors with no or less toxicity. By reducing or eliminating noxious substances in the treatment of cancer, the recovery period could be lessened and the efficacy of the treatment potentially promoted.

Our previous studies have indicated that the ester bond–containing green tea polyphenol (−)-EGCG (Fig. 1*A*) potently inhibits the proteasomal chymotrypsin-like activity *in vitro* and in cultured tumor cells (15, 16). Proteasome inhibition by natural and synthetic (−)-EGCG analogues leads to accumulation of proteasome target proteins (such as IκBα, Bax, and p27) and subsequent induction of apoptosis in human cancer cell lines, as measured by activation of caspases and cleavage of poly(ADP-ribose) polymerase (PARP; refs. 11–13, 15, 16, 25). However, (−)-EGCG is relatively unstable under neutral or alkaline conditions and could be rapidly degraded, involving deprotonation of hydroxyl groups on the phenol rings (26–29). Furthermore, the hydroxyl groups of (−)-EGCG could be modified through biotransformation reactions, including methylation, glucuronidation, and sulfate formation, resulting in reduced biological activities *in vivo* (26–29).

Previously, in an attempt to enhance the stability of (−)-EGCG, we introduced peracetate-protecting groups to the reactive hydroxyls of (−)-EGCG [Pro-EGCG (1); Fig. 1*A*] and found that it was converted to the parent (−)-EGCG under cell-free conditions (11). In the current study, we provide additional evidence that in intact human breast cancer MDA-MB-231 cells, Pro-EGCG (1) is

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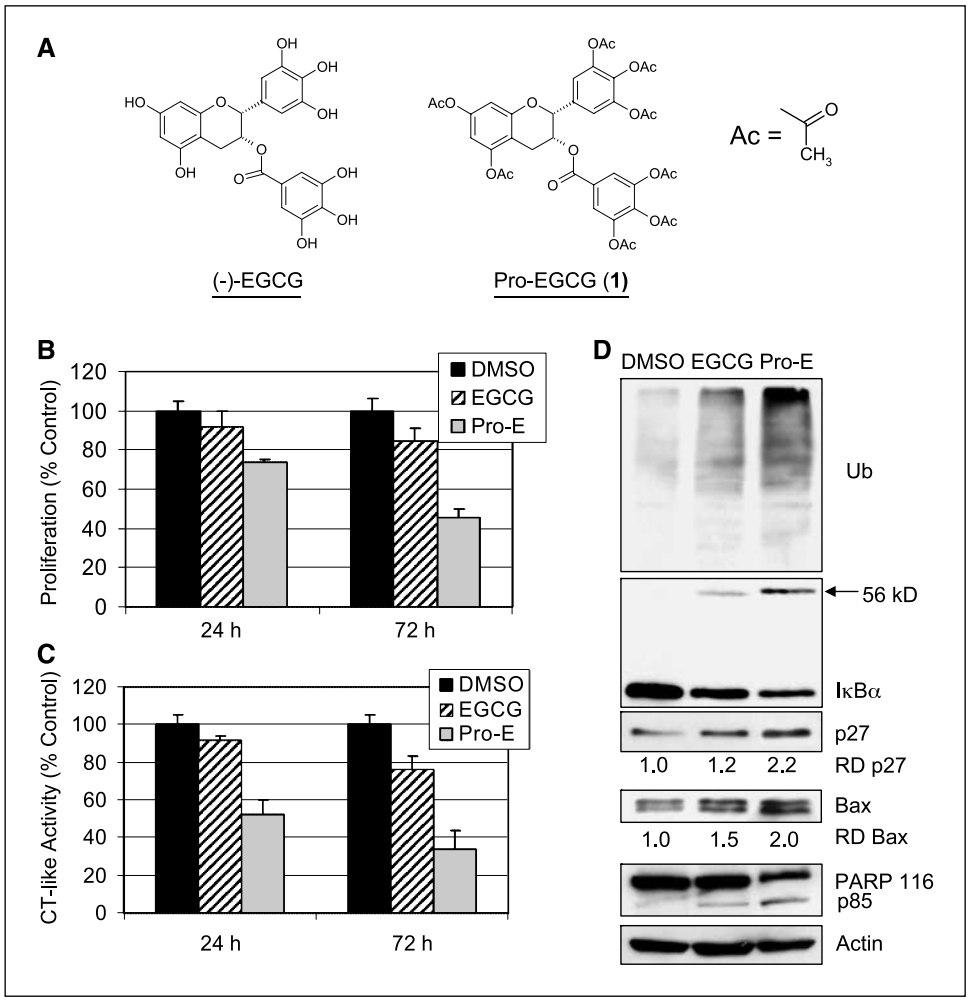

Figure 1. Antiproliferative and proteasomal chymotrypsin-inhibitory activities of Pro-EGCG (1). *A,* chemical structures of (−)-EGCG and Pro-EGCG (1). *B,* proliferation-inhibitory effect of Pro-EGCG (1) (Pro-E) on MDA-MB-231 breast cancer cell line. MDA-MB-231 cells were treated every 24 h for 72 h with 50 μmol/L of (−)-EGCG or Pro-EGCG (1), using DMSO as a control. After 24 or 72 h, medium was removed and cells were treated with MTT solution as described in Materials and Methods. *C,* inhibition of proteasomal chymotrypsin-like activity in MDA-MB-231 cells. Cells were treated with 50 μmol/L of the indicated compound for 24 or 72 h, harvested, and analyzed for the chymotrypsin-like activity, as described in Materials and Methods. *D,* Western blot analysis for accumulation of ubiquitinated proteins, IκB-α, ubiquitinated IκB-α (p56), p27, Bax, and PARP in the above prepared cell extracts after 72-h treatment. Relative density (*RD*) is compared with the control.

also converted to its parent compound, (−)-EGCG, and the converted (−)-EGCG is then accumulated. Consistently, increased levels of proteasome inhibition, growth suppression, and apoptosis induction were observed in these breast cancer cells treated with Pro-EGCG (1), compared with (−)-EGCG. Furthermore, treatment of breast cancer–bearing nude mice with Pro-EGCG (1) resulted in tumor growth inhibition and massive apoptosis induction, which are associated with proteasome inhibition *in vivo*. The data suggest that under physiologic conditions, Pro-EGCG (1) acts as a prodrug of (−)-EGCG, which imparts, after its conversion, more efficient proteasome inhibition than natural (−)-EGCG, and is indicative of a great potential for cancer prevention and treatment.

Materials and Methods

Materials. Bisbenzimide Hoechst no. 33258 stain, 3-[4,5-dimethyltiazol-2-yl]-2.5-diphenyl-tetrazolium bromide (MTT), DMSO, cremophor, and other chemicals were purchased from Sigma-Aldrich. RPMI 1640, penicillin, and streptomycin were purchased from Invitrogen. The fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like) was from Calbiochem. Mouse monoclonal antibody against human PARP was purchased from BIOMOL International LP. Mouse monoclonal antibodies against Bax (B-9), p27 (F-8), and ubiquitin (P4D1); goat polyclonal antibodies against actin (C-11) and IκB-α (C15); and secondary antibodies were from Santa Cruz Biotechnology, Inc.

Synthesis of Pro-EGCG (1). Synthesis of Pro-EGCG (1) from (−)-EGCG was done as described (11, 30).

Cell culture and cell extract preparation. MDA-MB-231 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown at 37°C in a humidified incubator with an atmosphere of 5% CO_2. A whole-cell extract was prepared as previously described (31).

High-performance liquid chromatography analysis. Using a solid phase (Xterra) with unique selectivity properties for polyphenols, gradient elution conditions were identified that separated highly polar compounds [(−)-EGCG] from nonpolar substances [Pro-EGCG (1)] within 25 min. Specificity studies using MDA-MB-231 cell extracts that were added to various concentrations of the various analytes proved that the cell extract did not contain any endogenous substances that would interfere with peak identification as low as 50 ng/mL. The cells were extracted in a cold aqueous formic acid to simultaneously solubilize the cells and stabilize the polyphenol analytes. A general purpose high-performance liquid chromatography (HPLC) method was developed using a Waters Xterra PR18 5-μm (3.9 × 150 mm) reverse-phase column with gradient elution and acetonitrile/methanol under acidic conditions to stabilize the analytes against degradation and to readily extract them from cell pellets. Reference standards used to confirm peak identity and to quantify each analyte in the particular biomatrix of interest were prepared in methanol. Cells were washed with medium and used to prepare cell extracts by adding 1 mL of 0.05% formic acid containing the internal standard to each tube to 10 μg/mL and vortexing to create a slurry. The cell slurry (250 μL) was transferred to an Eppendorf tube containing 500 μL methanol, vortexed,

sonicated for 15 s, and microfuged at 14,000 rpm for 5 min to pellet the macromolecules. Finally, 100 μL were transferred and inserted in the HPLC autosampler and 20 μL were injected for analysis.

Cell proliferation assay. The MTT assay was used to determine the effects of various compounds on proliferation of MDA-MB-231 breast cancer cells. Cells were plated in a 96-well plate and grown to either 70% to 80% (for the 24-h time point) or 30% to 40% (for the 72-h time point) confluency, followed by addition of 50 μmol/L of each compound and 24 or 72 h of incubation at 37°C, as indicated. Inhibition of cell proliferation was measured as previously described (32).

Proteasome activity assay. MDA-MB-231 breast cancer cells were grown to 30% to 40% confluency, treated daily with 50 μmol/L of the indicated compound for 3 days, harvested, and used for whole-cell extract preparation. Whole-cell extracts (10 μg) were incubated with Suc-Leu-Leu-Val-Tyr-AMC (40 μmol/L) fluorogenic substrate at 37°C in 100 μL of assay buffer (50 mmol/L Tris-HCl, pH 8) for 2.5 h. After incubation, production of hydrolyzed 7-amino-4-methylcoumarin (AMC) groups was measured using a Victor3 Multilabel Counter with an excitation filter of 380 nm and an emission filter of 460 nm (Perkin-Elmer).

Cellular and nuclear morphology analysis. A Zeiss Axiovert 25 microscope was used for all microscopic imaging with either phase contrast for cellular morphology or fluorescence for nuclear morphology with Hoechst staining, as previously described (32).

Caspase-3 activity assay. Cells were treated with 50 μmol/L of each compound, harvested, and lysed as previously described (16). Ac-DEVD-AMC (40 μmol/L) was then incubated with the prepared cell lysates for 2.5 h and the caspase-3 activity was measured as previously described (18).

Western blot analysis. MDA-MB-231 cells were treated, harvested, and lysed. Cell lysates (50 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, followed by visualization using the enhanced chemiluminescence kit (Amersham Biosciences) as previously described (33).

Human breast tumor xenograft experiments. Female athymic nude mice, ages 5 weeks, were purchased from Taconic Research Animal Services and housed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of Wayne State University. Human breast cancer MDA-MB-231 cells (5×10^6) suspended in 0.1 mL of serum-free RPMI 1640 were inoculated s.c. in both flanks of each mouse (four mice per group). When tumors reached a size of ~ 150 mm^3, the mice were randomly grouped and treated by daily s.c. injection with 50 mg/kg of (−)-EGCG, Pro-EGCG (1), or vehicle [20% DMSO and 80% cremophor/ethanol (3:1)]. Tumor size was measured every other day using calipers and their volumes were calculated according to a standard formula: width^2 × length / 2. Mice were sacrificed after 31 days of treatment when control tumors reached $\sim 1{,}500$ mm^3. The tumors were collected and photographed, and the tumor tissues were used for different assays for measuring proteasome inhibition and cell death.

Terminal nucleotidyl transferase–mediated nick end labeling, immunostaining, and H&E assays using tumor tissues. Terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay using *in situ* apoptosis detection kit and immunostaining of p27 were done as previously described (34). H&E staining in tumor tissues was done following the manufacturer's protocols. The proteasomal activity assays, caspase-3/caspase-7 activity assays, and Western blot analyses using animal tumor samples were done as described above using cultured breast cancer cells.

Statistical analysis. To evaluate the difference between treated and control animal groups with respect to tumor growth, the Student t test was applied. The level of significance was set at $P < 0.05$.

Results

HPLC analysis indicates conversion of Pro-EGCG (1) to (−)-EGCG in human breast cancer cells. Previously, we showed that under cell-free conditions, Pro-EGCG (1), the peracetate-protected form of (−)-EGCG, could be converted into the parent, unprotected (−)-EGCG (11). To determine whether, within the cellular environment, Pro-EGCG (1) could also be converted to (−)-EGCG, highly metastatic and invasive human breast cancer MDA-MB-231 cells were treated with 50 μmol/L of either Pro-EGCG (1) or (−)-EGCG every 24 h for 72 h, followed by extraction of intracellular polyphenols for HPLC analysis.

The HPLC results indicated that in Pro-EGCG (1)–treated MDA-MB-231 cells, 1,810 pmol Pro-EGCG (1)/mg protein were recovered (Table 1). Additionally, 530 pmol (−)-EGCG/mg protein were recovered in the same cells (Table 1), showing that Pro-EGCG (1) was absorbed and converted to (−)-EGCG in these cells. In comparison, in MDA-MB-231 cells treated with (−)-EGCG, only 222 pmol of (−)-EGCG/mg protein was recovered, 2.4-fold less than that recovered from the Pro-EGCG (1)–treated cells (Table 1). Therefore, Pro-EGCG (1) seems to be better absorbed into the cells, converted into (−)-EGCG, and accumulated in greater quantity than natural (−)-EGCG does under the same conditions. This finding provides further evidence for our previous hypothesis that Pro-EGCG (1) behaves as a prodrug form of (−)-EGCG (11). It should be noted that similar levels of (−)-EGCG (50–150 pmol/mg protein) were also recovered from other cultured cell lines after (−)-EGCG treatment (26–29) and that Pro-EGCG (1) has superior bioavalability over (−)-EGCG (35).

Pro-EGCG (1) inhibits proliferation of MDA-MB-231 breast cancer cells. We then investigated the effects of Pro-EGCG (1) and (−)-EGCG on proliferation of MDA-MB-231 cells. A 24-h treatment of MDA-MB-231 cells with Pro-EGCG (1) or (−)-EGCG at 50 μmol/L revealed only 25% and 10% inhibition, respectively (Fig. 1*B*). Therefore, we lengthened the treatment period by treating these cells with 50 μmol/L of each compound every 24 h for 72 h, followed by an MTT assay. At the end of the experiment, Pro-EGCG (1) inhibited cell proliferation by $\sim 55\%$ whereas (−)-EGCG inhibited only by 16% (Fig. 1*B*). This result suggests that Pro-EGCG (1) is ~ 3.5-fold more potent than (−)-EGCG in inhibiting breast cancer cell proliferation.

Inhibition of the proteasome chymotrypsin-like activity by Pro-EGCG (1) in MDA-MB-231 breast cancer cells. We hypothesized that Pro-EGCG (1) could target the tumor cellular proteasome in MDA-MB-231 cells. To explore whether Pro-EGCG (1) has greater proteasomal chymotrypsin-like inhibitory activity than (−)-EGCG, MDA-MB-231 cells were treated with 50 μmol/L of each compound for 24 h. After each treatment, proteins were extracted and used for measuring proteasome inhibition by the proteasomal chymotrypsin-like activity assay. The 24-h treatment revealed that the proteasomal chymotrypsin-like activity was inhibited by 48% and 8% in the Pro-EGCG (1)– and (−)-EGCG–treated cells,

Table 1. Amount of recovered (−)-EGCG in MDA-MB-231 cells after treatment with 50 μmol/L of (−)-EGCG or Pro-EGCG (1) for 72 h

Treatment	Recovered (−)-EGCG, pmol/mg protein	Recovered Pro-EGCG (1), pmol/mg protein
(−)-EGCG	222 ± 26	n.d.
Pro-EGCG (1)	530 ± 141	1,810 ± 126

Abbreviation: n.d., none detected.

respectively (Fig. 1*C*). When the treatment period was lengthened to every 24 h for 72 h, we found that Pro-EGCG (1) significantly inhibited the proteasomal chymotrypsin-like activity by 66% whereas (−)-EGCG was considerably less effective, inducing only ~24% inhibition (Fig. 1*C*). Therefore, Pro-EGCG (1) treatment exhibited ~3-fold greater inhibition of chymotrypsin-like activity compared with (−)-EGCG treatment.

Inhibition of proteasomal activity should cause accumulation of ubiquitinated proteins and natural proteasome targets (such as IκB-α; refs. 18, 25, 36). Accumulation of IκB-α protein prevents the activation of antiapoptotic nuclear factor κB (37), resulting in apoptosis. A significant amount of ubiquitinated proteins were indeed detected in cells treated with Pro-EGCG (1) after 72-h treatment (Fig. 1*D*). We have reported a ubiquitinated form of IκB-α protein with molecular weight of ~56 kDa (33). A similar p56 band appeared after Pro-EGCG (1) treatment, detectable by the specific antibody to IκB-α (Fig. 1*D, arrow*). Levels of p27 and Bax, two well-known target proteins of the proteasome (25, 36), were also increased in cells treated with Pro-EGCG (1) (2.2- and 2.0-fold, respectively, compared with the control; Fig. 1*D*). Comparatively, (−)-EGCG treatment under the same conditions induced limited proteasome inhibition and accumulation of proteasome target proteins (Fig. 1*C* and *D*).

Induction of apoptosis by Pro-EGCG (1) in MDA-MB-231 breast cancer cells. It has been shown that inhibition of the proteasomal chymotrypsin-like but not trypsin-like activity is associated with apoptosis induction in cancer cells (18, 19). To investigate whether the proteasomal inhibition by Pro-EGCG (1) treatment is associated with apoptosis induction, both morphologic changes and apoptosis-specific PARP cleavage were investigated in MDA-MB-231 cells treated with Pro-EGCG (1) or (−)-EGCG after 72 h. Changes in cell morphology were observed after 72 h in both Pro-EGCG (1)– and (−)-EGCG–treated cells (Fig. 2*A*). However, the Pro-EGCG (1)–treated cells exhibited the most extreme morphologic changes with cell membranes that appeared irregular and jagged compared with the control (Fig. 2*A*). Morphologies after 24-h treatment were not significantly changed (data not shown). Consistent with apoptosis induction, the appearance of punctate, brightly stained apoptotic nuclei was observed in the Pro-EGCG (1)–treated cells whereas very few appeared in the (−)-EGCG–treated cells after Hoechst staining (Fig. 2*B*). Furthermore, treatment with Pro-EGCG (1) induced activation of caspase-3/caspase-7 after 24 and 72 h of treatment (2- and 3-fold, respectively; Fig. 2*C*). Compared with (−)-EGCG treatment, Pro-EGCG (1) was 2.1-fold more potent in the activation of caspase-3/caspase-7 (Fig. 2*C*) and more effectively induced cleavage of the intact PARP protein after 72 h of treatment (Fig. 1*D*). Although the (−)-EGCG–treated cells also displayed apoptotic events in these assays, the Pro-EGCG (1)–treated cells exhibited considerably stronger indices of apoptotic cell death. These results show that Pro-EGCG (1) treatment imparts more potent inhibition of proteasomal chymotrypsin-like activity and induction of apoptosis in human breast cancer MDA-MB-231 cells than (−)-EGCG treatment.

Pro-EGCG (1) inhibits the growth of human breast cancer xenografts, associated with proteasome inhibition and apoptosis induction *in vivo*. Because Pro-EGCG (1) treatment showed the improved proteasome-inhibitory and apoptosis-inducing activities in cultured breast cancer cells compared with (−)-EGCG (Figs. 1 and 2), we then investigated whether Pro-EGCG (1) could do so *in vivo* and exert improved antitumor activity in a mouse model. MDA-MB-231 cells (5×10^6) were implanted s.c. in 5-week-old female athymic nude mice. On reaching a palpable size (~150 mm^3), the mice were treated s.c. daily with either the vehicle control or 50.0 mg/kg of Pro-EGCG (1) or (−)-EGCG (as a comparison). Significant inhibition of tumor growth by Pro-EGCG (1) was observed as early as 5 days after treatment and persisted after 31 days of treatment, showing that Pro-EGCG (1) has antitumor activity (Fig. 3*A*). By comparison, EGCG had very little effect on tumor growth for the first 21 days relative to control and showed much weaker inhibition of tumor growth relative to Pro-EGCG (1) (Fig. 3*A*). At the end of 31 days, control tumors grew to an average size of 1,582 ± 29 mm^3 whereas (−)-EGCG–treated tumors grew to 1,223 ± 21 mm^3, and Pro-EGCG (1)–treated tumors grew to only 723 ± 50 mm^3 (Fig. 3*A*). This corresponds to 54% growth inhibition in the Pro-EGCG (1)–treated tumors ($P < 0.01$; Fig. 3*A*) and 23% growth inhibition in the (−)-EGCG–treated tumors ($P < 0.05$; Fig. 3*A*). Furthermore, these findings indicate that Pro-EGCG (1) was 2.3-fold more effective at growth inhibition *in vivo* than (−)-EGCG in this breast tumor model. A similar study conducted using the prostate cancer cell line PC-3 indicated that Pro-EGCG (1) was an equally effective growth inhibitor (54% growth inhibition) whereas (−)-EGCG again exhibited limited effectiveness (24% growth inhibition; data not shown).

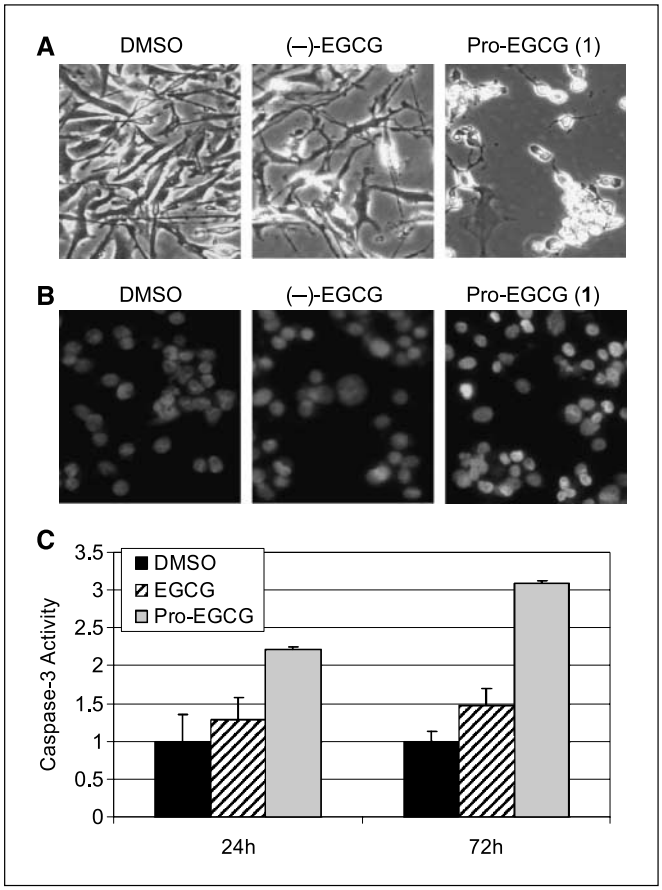

Figure 2. Induction of apoptosis by Pro-EGCG (1) in MDA-MB-231 breast cancer cells. *A* and *B,* MDA-MB-231 cells were treated with 50 μmol/L of the indicated compound every 24 h for 72 h. *A,* examination for apoptotic morphologic changes visualized by phase-contrast imaging (magnification, ×100). *B,* apoptotic nuclear changes. Nuclei that were punctuate or granular and bright were considered apoptotic (magnification, ×100). *C,* caspase-3/caspase-7 activity assay as described in Materials and Methods.

The MDA-MB-231 tumors were then collected and used for proteasome activity and apoptosis assays. The proteasomal chymotrypsin-like activity was inhibited by 40% in the tumors treated with Pro-EGCG (1) compared with control [3.2-fold greater inhibition than (−)-EGCG treatment; Fig. 3*B*]. Consistently, Western blot analysis showed that levels of ubiquitinated proteins and IκB-α, p27, and Bax proteins were accumulated in tumors treated with Pro-EGCG (1) compared with the vehicle control (Fig. 3*C*). On average, Pro-EGCG (1)–treated tumors displayed accumulated IκB-α by 2.0-fold, p27 by 1.5-fold, and Bax by 3.3-fold (Fig. 3*C*). Immunohistochemistry confirmed the increased expression of p27 in the tumors treated with Pro-EGCG (1) (Fig. 4*A*). In comparison, (−)-EGCG treatment had much less proteasome-inhibitory effect (Figs. 3 and 4). Therefore, Pro-EGCG (1) treatment inhibited tumor proteasome activity *in vivo* with increased potency compared with (−)-EGCG treatment.

To determine whether apoptosis is responsible for the observed antitumor activity of Pro-EGCG (1), several assays were done using tumor tissue samples. Caspase-3 activity was increased in the Pro-EGCG (1)–treated animal tumors, compared with the control animals, and was 1.7-fold higher than that of (−)-EGCG–treated tumors (Fig. 3*D*). Furthermore, apoptotic cells, as indicated by TUNEL positivity, and apoptotic nuclei, as shown by nuclear condensation using H&E staining, were observed in tumors from animals treated with Pro-EGCG (1) but not with (−)-EGCG (Fig. 4*B* and *C*). Finally, a cell death–associated PARP cleavage fragment was detected to the greatest extent in extracts of tumors treated with Pro-EGCG (1) (Fig. 3*C*). Again, when samples from (−)-EGCG–treated tumors were analyzed in these assays, much less apoptosis was detected (Figs. 3 and 4). During the 31-day period of Pro-EGCG (1) and (−)-EGCG treatment, no overall gross toxicity was observed and animals showed no weight loss, decreased activity, or anorexia.

Discussion

The pervasiveness of drug resistance extends to virtually every known disseminated cancer and, therefore, pursuing molecular targets for cancer therapeutics has become increasingly important. Numerous studies indicate that cancer and transformed cells are significantly more sensitive to proteasome inhibition than normal, nontransformed cells (12, 13, 20–22, 38), providing relevance for proteasome inhibitors as potential novel anticancer drugs. Our previous studies have suggested that polyphenolic compounds from natural plant sources are innocuous toward normal cells whereas they could inhibit the proteasome in cancer cells (15, 16).

We have shown that (−)-EGCG is a natural inhibitor of proteasomal chymotrypsin-like activity (15, 16). However, (−)-EGCG is unstable under physiologic conditions and could be rapidly degraded or metabolized through interactions with the hydroxyl groups on the phenol rings (26–29). Because of these factors, (−)-EGCG is known to have poor bioavailability, an issue which has often been overlooked in translating *in vitro* activities into *in vivo* animal and human studies (39).

To improve the bioavailability of (−)-EGCG, we have previously synthesized Pro-EGCG (1), a protected, seemingly prodrug form of (−)-EGCG, using peracetylation (11). Pro-EGCG (1) most likely requires the presence of esterases within the cellular milieu for its conversion to (−)-EGCG and has been shown to have no effect against a purified 20S proteasome (11). In the current study, we

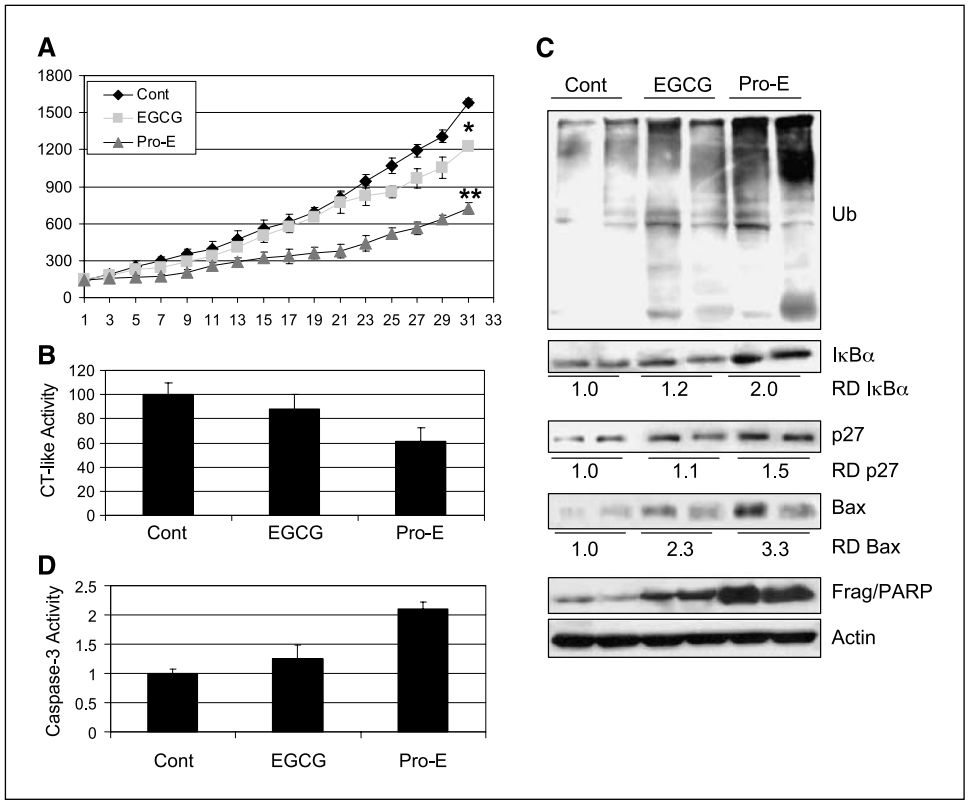

Figure 3. The antitumor activity of Pro-EGCG (1) is associated with inhibition of the proteasomal chymotrypsin-like activity and induction of apoptosis *in vivo*. Female nude mice bearing MDA-MB-231 tumors were treated with the control solvent (*Cont*), (−)-EGCG, or Pro-EGCG (1) (*Pro-E*) at 50 mg/kg/d for 31 d. *A,* inhibition of MDA-MB-231 tumor growth by Pro-EGCG (1). *Points*, mean tumor volume in each experimental group containing 10 mice; *bars,* SD; **, $P < 0.05$; ***, $P < 0.01$. *B* to *D,* effects of Pro-EGCG (1) at the end point of the experiment. Tumors were collected after 31 d of treatment and the prepared tissues were analyzed by the proteasomal chymotrypsin-like activity assay (*B*), Western blotting (*C*), and caspase-3/caspase-7 activity assay (*D*). Inhibition of proteasome activity (*B*) and accumulation of p27, IκB-α, and Bax proteins (*C*) were found in tumors treated with Pro-EGCG (1) (two representative tumor samples are shown), compared with mice treated with the control solvent alone or (−)-EGCG. Caspase-3/caspase-7 activation and a cleaved PARP fragment (~55 kDa; *C*) were also observed. Relative density is represented as an average of two samples compared with the control.

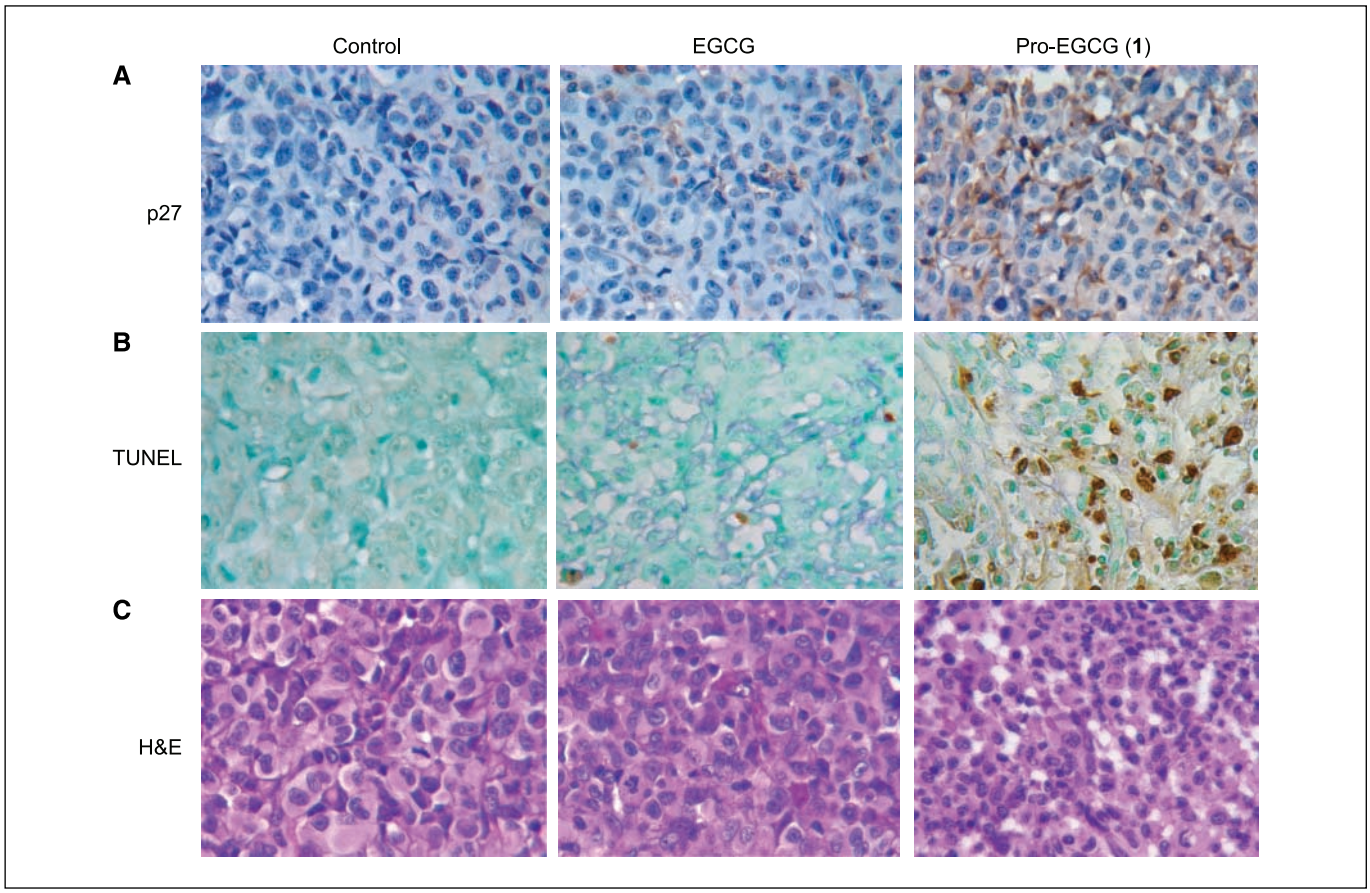

Figure 4. Immunohistochemistry with p27 antibody (*A*), TUNEL assay (*B*), and H&E staining (*C*) using mouse tumor samples (prepared from the same experiment in Fig. 3).

show that Pro-EGCG (1) can indeed be converted to the parent (−)-EGCG in human breast cancer MDA-MB-231 cells (Table 1). Moreover, the amount of recovered (−)-EGCG from the Pro-EGCG (1)–treated cells was ~2.4-fold greater than the amount of (−)-EGCG recovered from the (−)-EGCG–treated cells at 72 h (Table 1). Furthermore, a considerable amount of Pro-EGCG (1) remained inside the cells at 72 h (Table 1), indicating the potential for additional conversion to (−)-EGCG.

Although both enhancing (−)-EGCG entry into cells and preventing its degradation are important for maintaining effective cellular (−)-EGCG concentration, we reasoned that preventing (−)-EGCG degradation is technically more difficult than improving (−)-EGCG entry into cells. Our data have clearly indicated that the peracetate-protected form of (−)-EGCG has improved ability to enter tumor cells. Furthermore, the conversion of (−)-EGCG from Pro-EGCG (1) seems to be stable and accumulation in the breast cancer cells was observed. We have similarly found that in human leukemia Jurkat T cells, Pro-EGCG (1) can also be converted to (−)-EGCG, which was subsequently accumulated.[4] A recent report in which peracetylation increased the bioavailability of (−)-EGCG in esophageal and colon cancer cells (35) substantiates our findings.

Treatment with (−)-EGCG has been shown to induce apoptosis in MDA-MB-231 cells (40) and to accumulate cell cycle regulatory proteins such as p21 and p27 (41). To test our hypothesis that Pro-EGCG (1) increases the bioavailability of (−)-EGCG and subsequent proteasome inhibition over (−)-EGCG alone, MDA-MB-231 breast cancer cells were treated daily for 72 h and examined for their proteasomal activity. After 72 h, Pro-EGCG (1) treatment induced inhibition of chymotrypsin-like proteasome activity in MDA-MB-231 cells, with at least 2-fold greater potency than (−)-EGCG treatment (Fig. 1*C*). The observed proteasome inhibition from Pro-EGCG (1) treatment correlated with the accumulation of ubiquitinated proteins, ubiquitinated IκB-α, and other proteasome target proteins, p27 and Bax (Fig. 1*D*), and induced a 50% reduction in cell proliferation (Fig. 1*B*). Importantly, the 2- to 3-fold more (−)-EGCG recovered from the Pro-EGCG (1)–treated cells (Table 1) also correlates well with the 2- to 3-fold increase in inhibition of proteasomal activity and proliferation (Fig. 1).

To be sure that this apparent proteasome inhibition by Pro-EGCG (1) treatment would lead to induction of apoptosis, we examined morphologic changes (indicative of apoptotic cell death), the presence of apoptotic nuclei, the event of activated caspase-3/caspase-7, and, ultimately, production of the cleaved PARP fragment (Fig. 2). As MDA-MB-231 cells were exposed to Pro-EGCG (1), changes in cell morphology were evident, along with condensed nuclei representative of apoptosis (Fig. 2*A* and *B*). Confirming these findings, Pro-EGCG (1)–treated cells

[4] K.R. Landis-Piwowar, S.B. Wan, R.A. Wiegand, D.J. Kuhn, T.H. Chan, and Q.P. Dou, unpublished data.

displayed caspase-3/caspase-7 activation and cleaved PARP most noticeably after 72-h treatment (Fig. 2*C* and *D*). Again, (−)-EGCG was a less potent inducer of apoptosis under the same conditions (Fig. 2).

Because these *in vitro* findings were so promising, we chose to examine the effects of Pro-EGCG (1) versus (−)-EGCG treatment in a mouse model. Treatment with Pro-EGCG (1) resulted in significant inhibition of proteasomal chymotrypsin-like activity, accumulation of several proteasome target proteins (i.e., p27), and induction of apoptosis in tumors (Figs. 3 and 4). Associated with the observed proteasome inhibition and apoptosis induction, significant tumor growth inhibition (~54%) by Pro-EGCG (1) was observed in this breast tumor model (Fig. 3*A*) and in a prostate tumor system (data not shown). Again, (−)-EGCG treatment caused much less proteasome inhibition, apoptosis induction, and tumor growth suppression (Figs. 3 and 4).

It is important to note that the molecular weight of Pro-EGCG (1) is 1.7-fold greater than (−)-EGCG and, therefore, the amount used for these *in vivo* studies was proportionally lower. Given that 54% growth inhibition was observed in the Pro-EGCG (1)–treated tumor samples, we presume that potentially as much as an additional 1.7-fold greater growth inhibition may have been detected had the tumor samples been treated with an equal number of drug molecules.

The concentrations of (−)-EGCG and Pro-EGCG (1) used to treat cultured breast cancer cells are higher than those found in the blood of tea drinkers. However, the dose for either Pro-EGCG (1) or (−)-EGCG treatment in our mouse model seemed to be well tolerated. During the 31-day treatment, there was no overall gross toxicity observed. However, more detailed microscopic and macroscopic pathologic studies are required to definitively document the lack of toxicity when Pro-EGCG (1) is used at these concentrations.

The findings reported here indicate that Pro-EGCG (1) seems to be a promising, novel anticancer prodrug *in vitro* and *in vivo*, and its mechanism of action involves targeting the tumor cellular proteasome and inhibiting the growth of human tumor cells. Using an innocuous natural product or its prodrug as a chemosensitizer may reduce the toxicity and boost the effectiveness of current chemotherapeutics. Future analysis of Pro-EGCG (1) in a combinational regimen may elucidate increased effectiveness of this novel agent in additional cancer models.

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