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A potential prodrug for a green tea polyphenol proteasome inhibitor: evaluation of the peracetate ester of (−)-epigallocatechin gallate [(−)-EGCG]

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Abstract—Green tea has been shown to have many biological effects, including effects on metabolism, angiogenesis, oxidation, and cell proliferation. Unfortunately, the most abundant green tea polyphenol (−)-epigallocatechin gallate or (−)-EGCG is very unstable in neutral or alkaline medium. This instability leads to a low bioavailability. In an attempt to enhance the stability of (−)-EGCG, we introduced peracetate protection groups on the reactive hydroxyls of (−)-EGCG (noted in text as **1**). HPLC analysis shows that the protected (−)-EGCG analog is six times more stable than natural (−)-EGCG under slightly alkaline conditions. A series of bioassays show that **1** has no inhibitory activity against a purified 20S proteasome in vitro, but exhibits increased proteasome-inhibitory activity in intact leukemic cells over natural (−)-EGCG, indicating an intercellular conversion. Inhibition of cellular proteasome activity by **1** is associated with induction of cell death. Therefore, our results indicate that the protected analog **1** may function as a prodrug of the green tea polyphenol proteasome inhibitor (−)-EGCG.

1. Introduction

Besides water, tea is the most widely consumed beverage in the world. Among the many beneficial biological activities attributed to tea, the cancer-preventive potential of green tea has attracted considerable attention.[1] Tea leaves contain many constituents.[2] Among these constituents are the polyphenolic catechins, which are thought to contribute to the biological effects of tea. The polyphenols found in green tea extracts are (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG).[3] In particular, (−)-EGCG, the most abundant catechin, was found to be the strongest chemopreventive and anticancer agent among the green tea catechins (GTCs).[4,5] However, (−)-EGCG has at least one limitation: it gives poor bioavailability. Previous studies have shown that GTCs are partially absorbed into the blood plasma of both rats and humans.[6–8] A study by Nakagawa and Miyazawa showed that only 0.012% of (−)-EGCG could be absorbed in rats given 56 mg of (−)-EGCG orally.[6] This low absorption was thought to be due to the poor stability of (−)-EGCG in neutral or alkaline solutions, as it has been found that the stability of GTCs is pH-dependent.[9] At high pH values, the basic environment can easily attack the proton of the phenol group, leading to generation of the phenoxide anion. The anion is much more reactive toward electrophilic agents in the body (e.g., free radicals), and also forms the semiquinone radical, which can undergo further dimerization or other reactions. Thus, GTCs are more stable at low pH values. This is in agreement with the reports by Suematsu et al.[10] and Komatsu et al.,[11] who examined the stability of GTCs in canned tea drinks with varying pH values. As pH value of the intestine and body fluid is neutral or slightly alkaline, GTCs will be unstable inside the human body, thus leading to reduced bioavailability.

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The proteasome is a large protein complex with multicatalytic activities that are responsible for the degradation of not only obsolete and misfolded proteins, but also regulatory proteins involved in cell cycle and apoptosis.[12] In proteasome-dependent proteolysis, ubiquitin is first conjugated to the substrate, followed by degradation of the substrate and recycling of the amino acids and ubiquitin.[13] The ubiquitin/proteasome-dependent degradation pathway plays an essential role in up-regulation of cell proliferation, down-regulation of cell death, and development of drug resistance in human tumor cells. Therefore, proteasome inhibitors show great potential as novel anticancer drugs.[12] The 20S proteasome constitutes the proteolytic component of the 26S proteasome.[14–18] It contains at least three major proteasomal activities: chymotrypsin-like, trypsin-like, and caspase-like.[19] Inhibition of the chymotrypsin-like, but not the trypsin-like, activity of the proteasome has been associated with induction of tumor cell apoptosis.[12,20,21] We have previously shown that natural (−)-EGCG and synthetically derived (+)-EGCG are potent inhibitors of the proteasomal chymotrypsin activity, leading to growth arrest and/or apoptosis.[22]

The P13K/Akt signaling is a widely known tumor cell survival pathway.[23] Blocking this pathway is considered as an important mechanism for inhibiting tumor growth. Phosphorylated Akt (p-Akt) is the activated form of Akt. Once Akt is activated, it can mediate cell cycle progression by phosphorylation and consequent inhibition of the cyclin-dependent kinase inhibitor p27.[24] Recently, (−)-EGCG has been found to inhibit the Akt kinase activity via reducing the phosphatidylinositol 3-kinase signals in MMTV-Her-2/neu mouse mammary tumor NF639 cells, leading to reduced tumor cell growth.[25]

We hypothesized that synthesis of a prodrug form of (−)-EGCG would improve its bioavailability. The prodrug should exhibit: [i] superior stability in physiological conditions at a neutral pH; [ii] remain biologically inactive until enzymatic hydrolysis in vivo, leading to the release of the parent drug; [iii] and lastly, the promoiety groups should possess low systemic toxicity. Here, we report for the first time that peracetate (−)-EGCG, **1** (Fig. 1)[26] was found to be more stable than (−)-EGCG. The prodrug was biologically inactive against a purified 20S proteasome activity, but potently inhibited the proteasome in intact tumor cells. Furthermore, administration of the prodrug, but not its parent compound, to intact tumor cells resulted in the loss of phosphorlyated Akt (p-Akt), indicating inactivation of this cancer-associated kinase.[23] Finally, treatment of leukemia Jurkat T cells with **1** induced cell death.

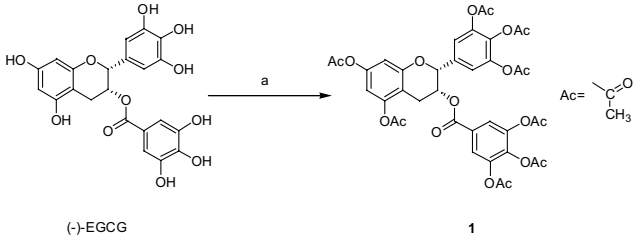


Figure 1. Synthesis of **1** from natural (−)-EGCG. (a) Ac_2O, pyridine, rt, overnight.

2. Materials and methods

2.1. Materials

Highly purified (−)-EGCG, fetal bovine serum (Tissue Culture Biologicals), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), acetonitrile, and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich. Purified 20S proteasome (rabbit) was purchased from Boston Biochem. Fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) was obtained from Calbiochem. Polyclonal antibody to ubiquitin and monoclonal antibody to Actin were obtained from Santa Cruz Biotechnology Inc. Western blot analysis of the AKT2 was performed and detected with antiphospho-Akt-Ser473 (New England Biolabs). RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), penicillin and streptomycin were purchased from invitrogen. Compound **1** was prepared from (−)-EGCG according to the literature procedures.[26]

2.2. Stability tests of (−)-EGCG and 1

(−)-EGCG or **1** (0.1 mM) was incubated with RPMI 1640 culture medium at 37 °C. At different time points, 15 μL of the medium was injected into an HPLC equipped with a C-18 reverse phase column (CAPCELL PAK C18 UG 120, Shiseido Co., Ltd, 4.6 mm i.d. × 250 mm); flow rate, 1 mL/min; detection, UV 280 nm; for (−)-EGCG, time points were 0, 10, 20, 40, 60, 90, 120 min and the mobile phase, 20% aqueous acetonitrile and 0.01% TFA; for prodrug **1**, time points were 0, 30, 60, 90, 120 min and mobile phase, 50% aqueous acetonitrile and 0.01% TFA.

2.3. Enzymatic hydrolysis of 1

Lysis buffer (pH 5) (0.25 mL) was added to 2×10^6 Jurkat T cells. This could break the cell membrane of the cells and release the cytoplasmic enzymes. PBS (0.75 mL) was added which neutralized the medium to the optimum pH value (pH 7) for the enzymes. Prodrug **1** (0.25 mM) was added into the reaction mixture and incubated at 37 °C. At different time points (0, 30, 60, 90, 120, 150, 180, 210, 240, 300, and 360 min), an aliquot (0.06 mL) of the reaction mixture was taken out, filtered, and injected into the HPLC and analyzed as outlined above.

2.4. Hydrolysis of 1 in the presence of vitamin C in culture medium with or without lysates

Compound **1** (35 μM) was incubated with dulbecco's modified eagle medium (DMEM) (1 mL containing 1.67 mg/mL vitamin C) at 37 °C. At different time points, 10 μL of the solution was injected into an HPLC

equipped with a C-18 reverse phase column; flow rate, 1 mL/min; detection, UV 280 nm; mobile phase, 0–8 min (20% aqueous acetonitrile and 0.016% TFA), 8–13 min (varying from 20% aqueous acetonitrile with 0.016% TFA to 60% aqueous acetonitrile with 0.008% TFA).

For the investigation of hydrolysis of **1** in the presence of lysates, same concentration of **1** was incubated with DMEM (2 mL containing 1.67 mg/mL vitamin C) in the presence of the lysates (5×10^5 breast cancer cells with 0.15 mL lysis buffer). At different time points, an aliquot (0.06 mL) of the reaction mixture was taken out, filtered, injected into the HPLC and analyzed as outlined above.

2.5. Cell culture, extract preparation, and Western blot assay

Human Jurkat T cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2.

A whole-cell extract was prepared as described previously.[20] Briefly, cells were harvested, washed with PBS, and homogenized in a lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4 °C. Afterwards, the lysates were centrifuged at 12,000*g* for 15 min at 4 °C and the supernatants collected as whole-cell extracts. Equal amounts of protein extract (60 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher and Schuell) using a semi-dry transfer system (Bio-Rad). The enhanced chemiluminescence Western blot analysis was then performed using indicated specific antibodies.

2.6. Inhibition of purified 20S proteasome activity by prodrug 1 and (−)-EGCG

The chymotrypsin-like activity of purified 20S proteasome was measured as follows: 0.5 μg of purified 20S proteasome (Methanosarcina thermophile, Recombinant, *Escherichia coli*) was incubated with 20 μM fluorogenic peptide substrate, Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) (Bachem), for 30 min at 37 °C in 100 μL of assay buffer (20 mM Tris–HCl, pH 8.0) with or without a tested compound in a 96-well plate. After incubation, the plate was subjected to direct measurement of the hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups using a Wallac Victor^3(TM) Fluorometer with an excitation filter of 380 nm and an emission filter of 460 nm.

2.7. Inhibition of the proteasome activity in intact cells by tea polyphenols

To measure the inhibition of proteasome activity in situ, Jurkat T (1×10^5 cells/mL/well) were cultured in a 24-well plate. Cells were incubated for 24 h in the presence or absence of (−)-EGCG or **1**, followed by a 3 h incubation with the fluorogenic peptide substrate (Suc-Leu-Leu-Val-Tyr-AMC) for the chymotrypsin-like activity. Afterwards 100 μL was transferred to a 96-well plate and free AMCs were measured as described above.

2.8. Trypan blue assay

The trypan blue dye exclusion assay was performed by mixing 20 μL of cell suspension with 20 μL of 0.4% trypan blue dye before injecting into a hemocytometer and counting. The number of cells that absorbed the dye and those that exclude the dye were counted, from which the percentage of nonviable cell number over total cell number was calculated.

3. Results and discussion

3.1. Synthesis of peracetate protected (−)-EGCG

For the synthesis of **1**, commercially available (−)-EGCG was used as a starting material.[26] Treating the (−)-EGCG with acetic anhydride and pyridine overnight yielded the desired product **1** in 82% yield (Fig. 1). The structure of **1** was confirmed by ^{1}H and ^{13}C NMR, LRMS, and HRMS.

3.2. Chemical stability and enzymatic hydrolysis of peracetate (−)-EGCG, 1

In the first part of our study, we compared the stability of **1** with (−)-EGCG in a culture medium (RMPI), which mimics the body fluid with a pH value around 8. (−)-EGCG or **1** at 0.1 mM was incubated in 1 mL RMPI at 37 °C at indicated times. At different time points, the medium was analyzed by HPLC for the amount of tested compound remaining. Degradation curves are shown in Figure 2.

When (−)-EGCG was dissolved in the culture medium, it was found to be degraded rapidly within 20 min, demonstrating the low stability of (−)-EGCG in the medium. The result is in agreement with Hong et al.,[27] who examined the stability of (−)-EGCG in McCoy's

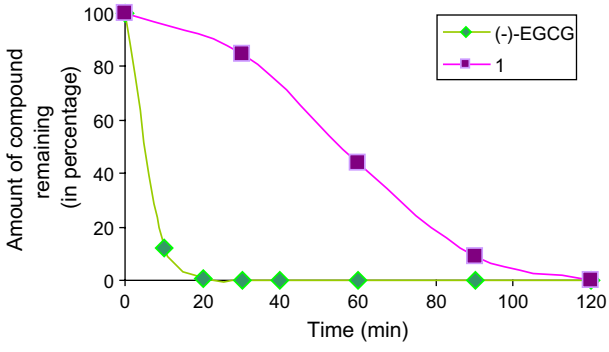


Figure 2. Degradation curve of (−)-EGCG and **1**. Both (−)-EGCG and **1** were incubated in RMPI medium for indicated minutes followed by HPLC analysis. The levels of the compounds remaining in the medium were measured and plotted.

5A medium and showed that more than 90% of (−)-EGCG disappeared within 1 h. In Hong's experiment, they added vitamin C to the medium as well, which slowed down the degradation. Although **1** was also degraded in the medium, as seen in Figure 2, the rate of its degradation was much slower when compared with (−)-EGCG. Compound **1** disappeared completely after 2 h, indicating that it is 6 times more stable than (−)-EGCG in this medium. Therefore, peracetate protection of the phenol groups of (−)-EGCG aids in stabilizing **1** in culture (presumably physiological) conditions.

In order to determine if **1** was hydrolyzed to EGCG under the culture medium conditions, we repeated the experiment but now with added vitamin C (at 1.67 mg/mL) to prevent the rapid degradation of the generated EGCG. As **1** disappeared, a new peak **A** was observed by HPLC to increase and then decline in intensity with time. This was followed by the appearance of another peak **B** in the HPLC, which also eventually declined. Finally, a peak in the HPLC identical in retention time to EGCG was observed to be formed. The time course results of these components were shown in Figure 3. The identity of EGCG was confirmed by UV spectroscopy as well as mass spectrometry. Furthermore, mass spectrometric analyses of peaks **A** and **B** showed that they were the di-acetate and mono-acetate of EGCG, respectively. These results suggested that compound **1** was first hydrolyzed to the di-acetate, then mono-acetate and eventually EGCG under the culture medium conditions.

Compound **1** is presumed to undergo enzymatic hydrolysis to release (−)-EGCG inside the cells. If so, we should be able to detect production of (−)-EGCG as well. In order to investigate these events, 0.1 mM of **1** was first incubated with an extract of leukemia Jurkat T cells, followed by HPLC analysis. After 4 h of incubation, no trace of **1** was observed, and a small peak appeared with a retention time similar to that of (−)-EGCG (Fig. 4b and e). The low level of production of (−)-EGCG from **1** may be due to the fact that (−)-EGCG itself can be easily degraded under physiological conditions. Consistently, a gallic acid-like peak was produced from **1**, which was increased during longer incubation (Fig. 4b–c, and d).

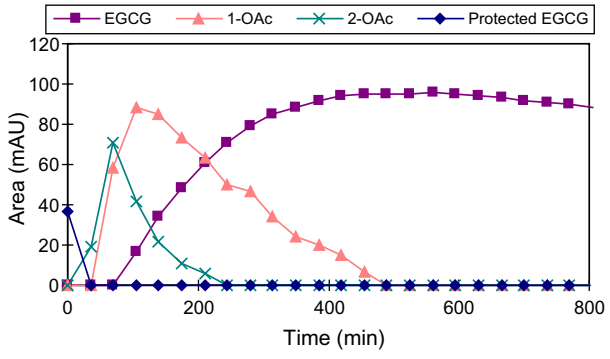


Figure 3. Time-course results of peracetate EGCG (**1**) in culture medium with the presence of vitamin C (area vs time). Compound **1**: ◆; compound **A** (di-acetate): ×M ; compound **B** (mono-acetate): ▲; EGCG: ■.

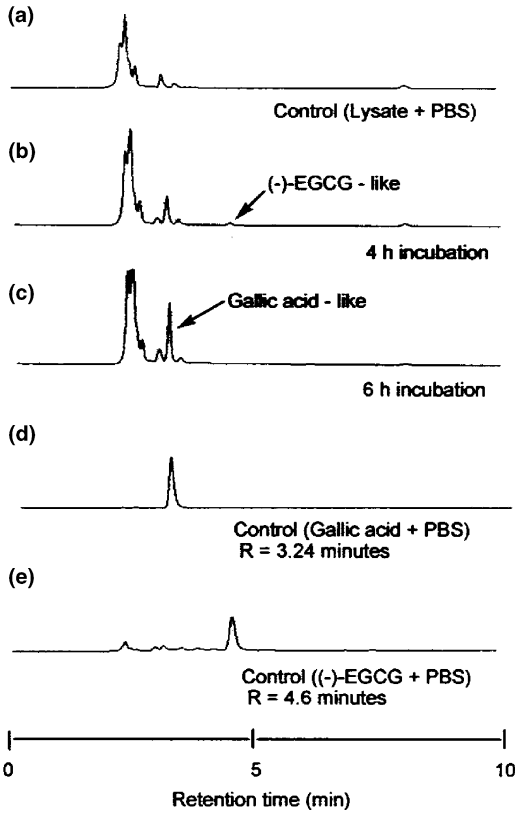


Figure 4. HPLC chromatograms of **1** after incubation with lysate in PBS (formation of the gallic acid-like peak and (−)-EGCG-like peak). Compound **1** was incubated with lysate in PBS for the indicated hours and followed by HPLC analysis. The retention times of reaction products were confirmed by using natural (−)-EGCG and gallic acid standards [controls: (d) and (e)].

The generation of (−)-EGCG from compound **1** under cellular conditions could be more clearly demonstrated by the addition of vitamin C to prevent the rapid disappearance of (−)-EGCG. In this case, we performed the experiment in medium with breast cancer cell lysate. HPLC analyses showed the disappearance of **1**, together with the transient formation of **A** (the di-acetate), **B** (the mono-acetate) and then (−)-EGCG in a time-course results (Fig. 5) similar to Figure 3. The results summarized in Figures 4 and 5 suggested that in medium with the addition of lysate, compound **1** underwent hydrolysis forming the di-acetate of EGCG, then the mono-acetate, then EGCG, and eventually gallic acid (Scheme 1).

3.3. Inhibition of the proteasomal activity in vitro and in vivo by 1 and (−)-EGCG

(−)-EGCG has been found to potently and selectively inhibit the chymotrypsin-like activity of proteasomes in vitro and in vivo.[28] If **1** is to function as the prodrug of (−)-EGCG, it should remain biologically inactive until de-acetylation inside the cell where it is converted into its parent compound. In order to test this hypothesis, proteasome activity was tested both in vitro and in

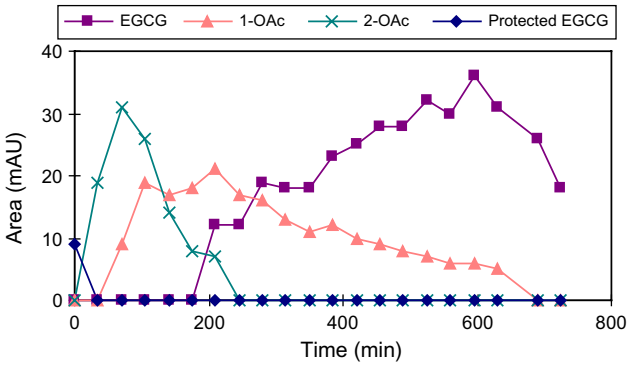
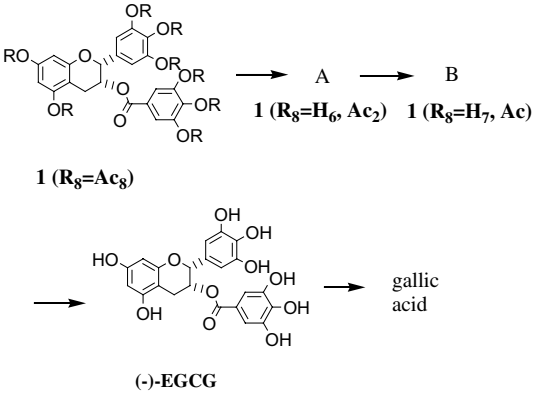


Figure 5. Time-course results of peracetate EGCG (**1**) in culture medium with the presence of vitamin C with the addition of lysate (area vs time). Compound **1**: ◆; compound **A** (di-acetate): ×; compound **B** (mono-acetate): ▲; EGCG: ■.



Scheme 1.

intact Jurkat T cells with either **1** or (−)-EGCG (as a positive control). First, **1** and commercial (−)-EGCG were dissolved in DMSO and their effects on the chymotrypsin-like activity of purified 20S proteasomes were measured. At 10 μM, **1** was completely inactive in inhibiting the chymotrypsin-like activity of the purified 20S proteasome (Fig. 6). In contrast, (−)-EGCG at 10 μM inhibited 80–90% of the proteasomal chymotrypsin-like activity (Fig. 6). Therefore, as predicted, **1** outside of a cellular system is not a proteasome inhibitor.

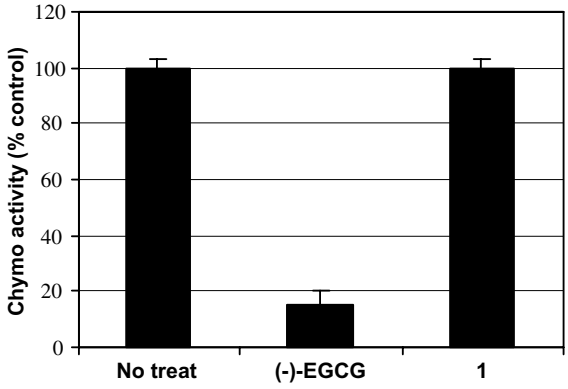


Figure 6. Inhibition of the chymotrypsin-like activity of the purified 20S proteasome by **1** and (−)-EGCG. The purified 20S proteasome was incubated with 10 μM **1** or (−)-EGCG for 30 min. Percentage of chymotrypsin (chymo) activity was then determined as described under Section 2.

If **1** converts to (−)-EGCG inside the cells, we should be able to detect proteasome inhibition in vivo. To examine this possibility, we treated human Jurkat cells with 25 μM of **1** or (−)-EGCG for 12 or 24 h, followed by measurement of proteasome activity by using a chymotrypsin-like specific fluorogenic substrate in intact cells (Fig. 7a) or Western blot for ubiquitinated proteins (Fig. 7b). Treatment of Jurkat T cells with (−)-EGCG for 24 h inhibited proteasome activity by 31% versus 42% inhibition with **1** (Fig. 7a). To analyze the intracellular level of polyubiquitinated proteins, cells were lysed after 12 h incubation and subjected to Western blotting. Compound **1** showed comparable levels of ubiquitinated proteins to that of natural (−)-EGCG (Fig. 7b). Therefore, **1** is equally potent to, if not more potent than, (−)-EGCG in inhibiting the proteasomal activity in intact cells. On the other hand, even though **1** is six times more stable compared with EGCG, the potency of its biological activities in cells did not increase to a similar extent. This is because the amount of EGCG generated from **1**, and thus its biological activity inside the cells depends on a combination of factors: the relative permeability of **1** into the cells, the amount of esterase enzymes and

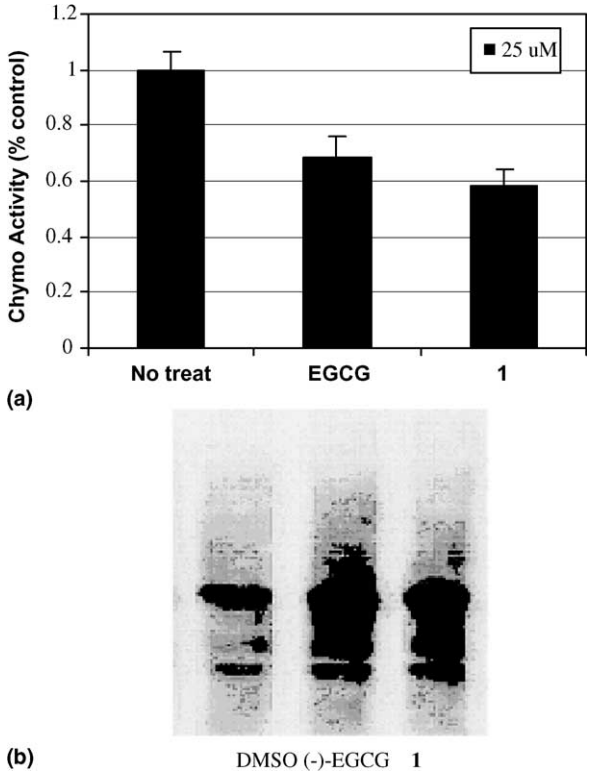


Figure 7. Inhibition of proteasome actvitiy by **1** and (−)-EGCG in vivo. Jurkat cells were treated with 25 μM with of **1** or (−)-EGCG. (a) Proteasome activity was measured after 24 h by adding a chymotrypsin-like specific substrate to intact cells and the level of fluorescence was measured on a multilabel plate reader, or (b) Western blot assay using a specific antibody to ubiquitin after 12 h treatment with drug.

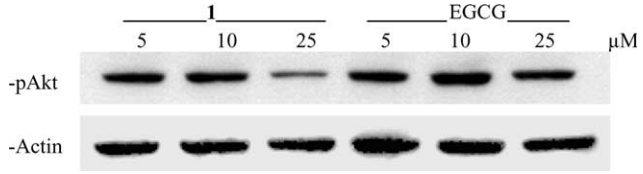


Figure 8. Amount of p-Akt levels with **1** and (−)-EGCG treatment. Jurkat cells were treated with 5, 10, or 25 μM **1** and (−)-EGCG for 24 h. Following treatment, protein expression of p-Akt was determined by immunoblotting with an antiphospho-Akt antibody.

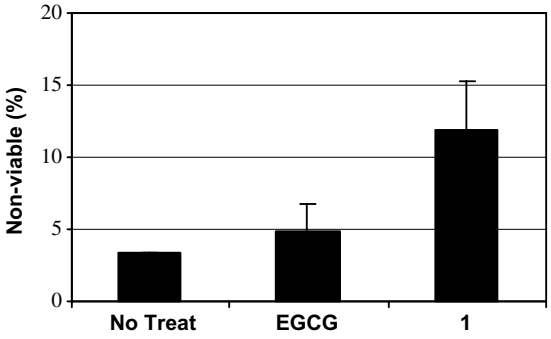


Figure 9. Cell viability in Jurkat cells treated with **1** and (−)-EGCG. Cells were treated with 10 μM for 24 h, followed by trypan blue exclusion assay. Values are the mean of at least three experiments ± SD. The numbers given are percentages of nonviable cells to total cells.

the amount of anti-oxidants that may be present in the cells at any time.

3.4. Dephosphorylation of Akt in Jurkat cells by 1 and (−)-EGCG

(−)-EGCG and **1** were incubated with Jurkat T cells for 24 h at 5, 10, and 25 μM, followed by Western blot analysis using a specific antibody to phosphorylated Akt (Fig. 8). (−)-EGCG at 25 μM was found to reduce the level of p-Akt by 32% compared to treatment with **1**, which lead to a 73% decrease in activated Akt at 25 μM as indicated by densitometric analysis (Fig. 8). Actin was used as a loading control.

3.5. Cell death induced by 1 and (−)-EGCG

We next assessed the abilities of (−)-EGCG and **1** to induce cell death in Jurkat T cells treated with 10 μM for 24 h. We observed that while (−)-EGCG had a minimal effect on cell death (5%), **1** was capable of inducing up to 15% cell death at that concentration (Fig. 9). Therefore, the greater abilities of **1** to inhibit cellular proteasome activity (Fig. 7) and to inactivate Akt (Fig. 8) are associated with its increased cell death-inducing activity (Fig. 9).

4. Conclusion

Based on our present study, we have successfully designed and synthesized an (−)-EGCG prodrug (**1**), which is more stable than (−)-EGCG at neutral pH and shows greater efficacy in proteasome inhibition and cell death induction. It appears that the prodrug inhibits the same target (the proteasome) as its parent compound in intact cells while it is biologically inactive itself. All the findings indicate that **1** meets the requirements for being an (−)-EGCG prodrug and seems to be a promising compound for further studies in investigation of the bioavailability and evaluation of the pharmacological activity.

In conclusion, acetate protection of (−)-EGCG's phenol groups is a useful tool for improving the stability and enhancing the activity of (−)-EGCG. Synthesis of a series of stable (−)-EGCG analogs with and without peracetate protected groups is currently in progress, in the hope of creating more stable and potent polyphenols with the potential of becoming novel anticancer drugs.[29]

Acknowledgements

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