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(54) (−)-EPIGALLOCATECHIN GALLATE DERIVATIVES FOR INHIBITING PROTEASOME

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C07D 311/82 (2006.01)
A61K 31/35 (2006.01)

(52) **U.S. Cl.** **549/399**; 514/456

(58) **Field of Classification Search** 549/399; 514/456

See application file for complete search history.

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

JP	10-254087 A	9/1998
WO	WO 99/22728	5/1999

OTHER PUBLICATIONS

Kuhn, D et al Synthetic peracetate Tea Polyphenols as Potent Proteasome Inhibitirs and Apoptosis Inducers in Human Cancer Cells Frontiers in Bioscience 10, 1010-23, May 1, 2005.*

Kohri, T et al Synthesis of (−)-[4-H]Epigallocatechin Gallate and its Metabic Fate in Rates after Intravenous Administration J.Agric. Food Chem, 2001,49,1042-1048.*

Hiipakka, RA et al Sturcture-activity relationships for inhibition of human 5£-reductases by polyphenols Biochemical Pharmacology 63 (2002) 1165-1176.*

Kreimeyer, J et al 'Separations of flavan-3-ols and dimeric proanthocyanidins by capillary electrophoresis' CA 128:221701 (1998).*

Andreas, D et al 'Flavan-3-ols, prodelphinidins and further polyphenols from cistus salvifolius' CA 122:5467 (1995).*

Smith, DM et al Synthetic Analogs of Green Tea Polyphenols as Proteasome Inhibitiors Molecular Medicine 8(7), 382-392 (2002).*

Smith, Docking studies and model development of tea polyphenol proteasome inhibitors: applications to rational drug design, Proteins: Structure, Function and Bioinformatics 54:58-70 (2004).*

Nam, Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo, J. Biol. Chem (2001) 276(16) 13322-13330.*

Kazi, Potential molecular targets of tea polyphenols in human tumor cells: significance in cancer proliferation. PMID: 12494882 (2002).*

Jung et al, Inhibition of tumor invasion and angiogenesis by epigalocatechin gallage (EGCG), a major component of green tea,International Journal of Experiental Pathology, 52,309-316 (2001).*

U.S. Appl. No. 11/600,513, filed Feb. 2007, Chan.*

Danne et al, *Phytochemistry*, "Flavan-3-ols, Prodelphinidins and Further Polyphenols from *Cistus Salvifolius*," vol. 37, No. 2 (1994), pp. 533-538.

Wang et al, *Natural Product Research and Development*, "The synthesis of liposoluble tea polyphenols (LTP) and its resistance to autoxidation of oil," vol. 13, No. 4 (2001), pp. 12-15 (with abstract).

Wang et al, *Fine Chemicals*, "Synthesis of liposoluble tea polyphenols with varying aliphatic groups and their antioxidation activity," vol. 19, No. 2 (2002), pp. 86-89 (with abstract).

De Groot et al, *Bulletin of Magnetic Resonance*, "Advantages of Long-Range-Inept Measurements for Structure Determination of Catechin Esters," vol. 17, No. 1-4 (1995), pp. 242-243.

Lam et al, *Bioorganic & Medicinal Chemistry*, "A potential prodrug for a green tea polyphenol proteasome inhibitor: evaluation of the peracetate ester of (−)-epigallocatechin gallate [(-)-EGCG]," vol. 12, No. 21 (2004), pp. 5587-5593.

* cited by examiner

Primary Examiner—D. Margaret Seaman
(74) *Attorney, Agent, or Firm*—Buchanan Ingersoll & Rooney PC

(57) **ABSTRACT**

(−)-EGCG, the most abundant catechin, was found to be chemopreventive and anticancer agent. However, (−)-EGCG has at least one limitation: it gives poor bioavailability. This invention provides compounds of generally formula 10, wherein R_1 is selected from the group of —H and C_1 to C_6 acyl group; R_2, R_3, and R_4 are each independently selected from the group of —H, —OH, and C_1 to C_6 acyloxyl group; and at least one of R_2, R_3, or R_4 is —H. The derivatives of (−)-EGCG that is at least as potent as (−)-EGCG. The carboxylate protected forms of (−)-EGCG and its derivatives are found to be more stable than the unprotected forms, which can be used as proteasome inhibitors to reduce tumor cell growth.

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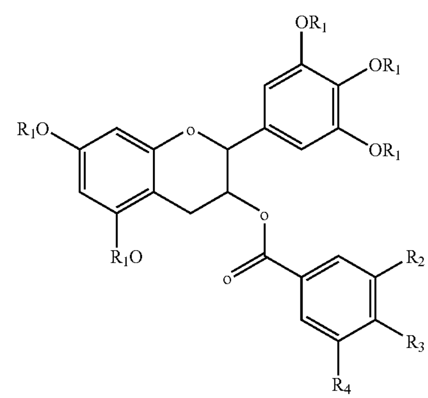


34 Claims, 9 Drawing Sheets

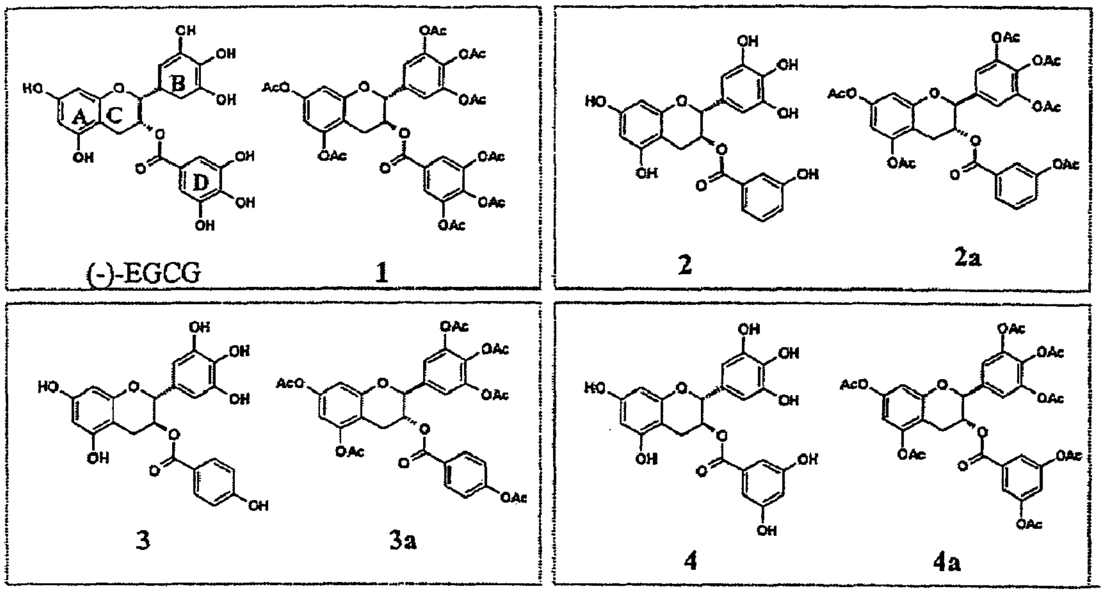


Figure 1

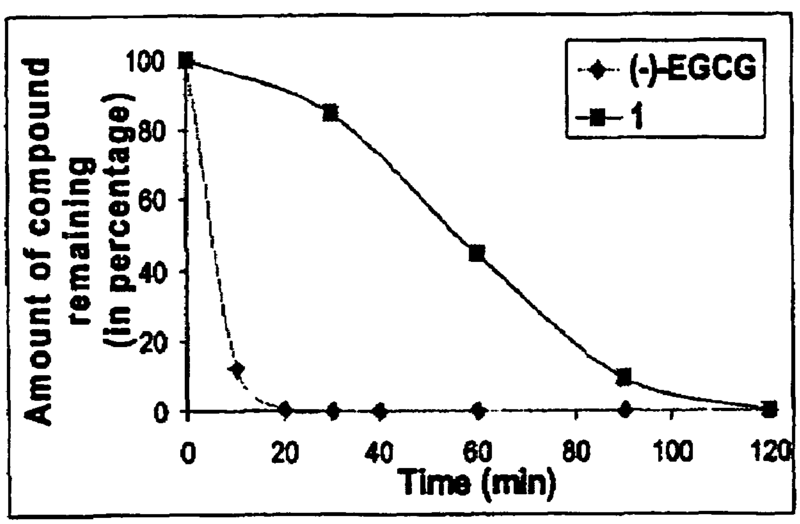


Figure 2

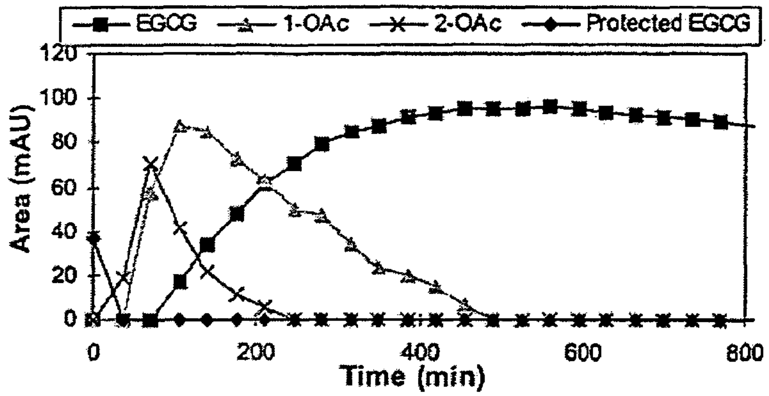


Figure 3

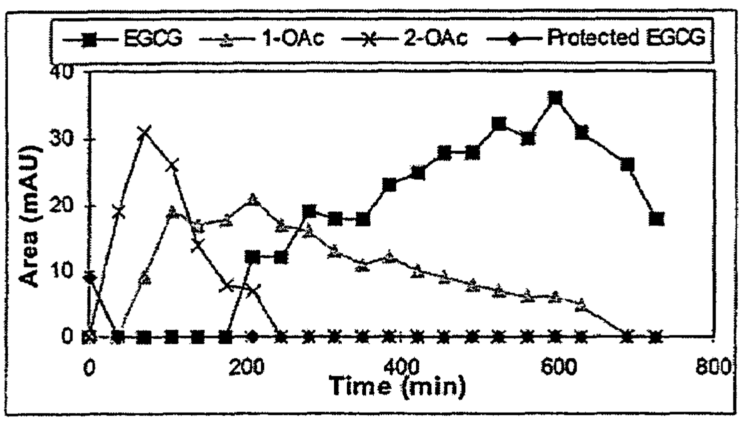


Figure 4

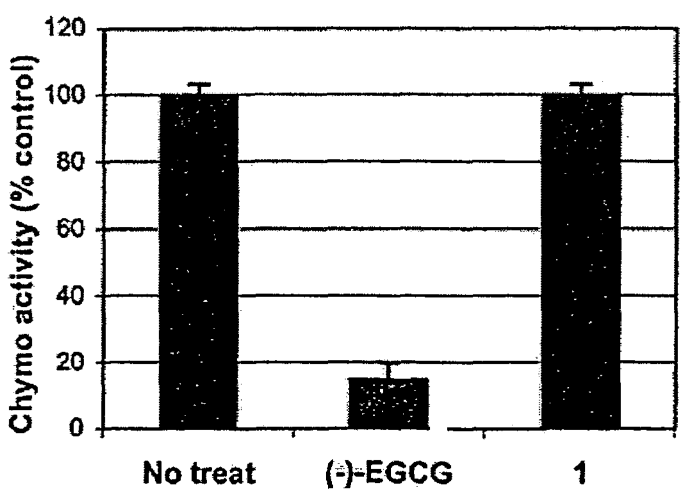
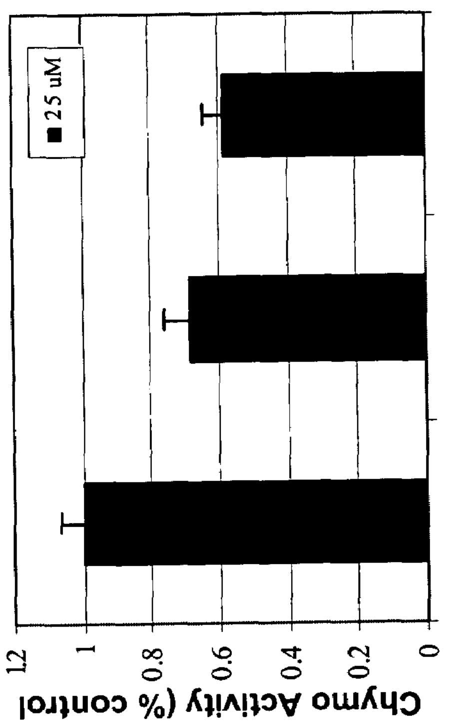


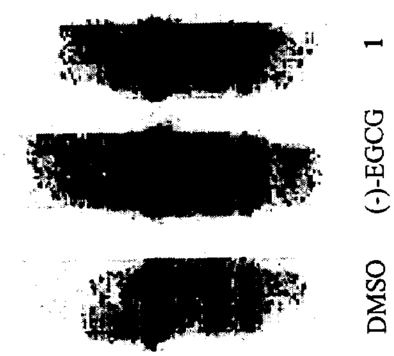
Figure 5

Fig. 6

a.


Chymo Activity (% control)
1.2
1
0.8
0.6
0.4
0.2
0
No treat
EGCG
1
25 uM

b.


DMSO
(-)-EGCG
1

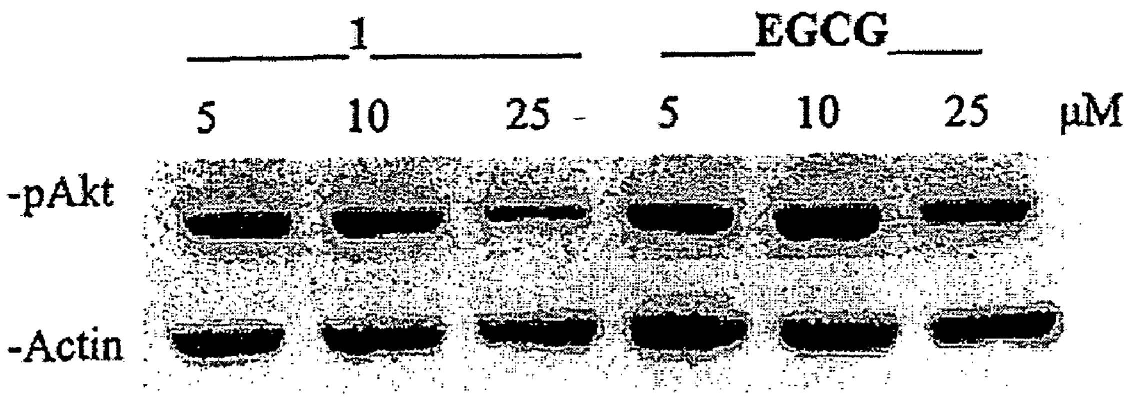


Figure 7

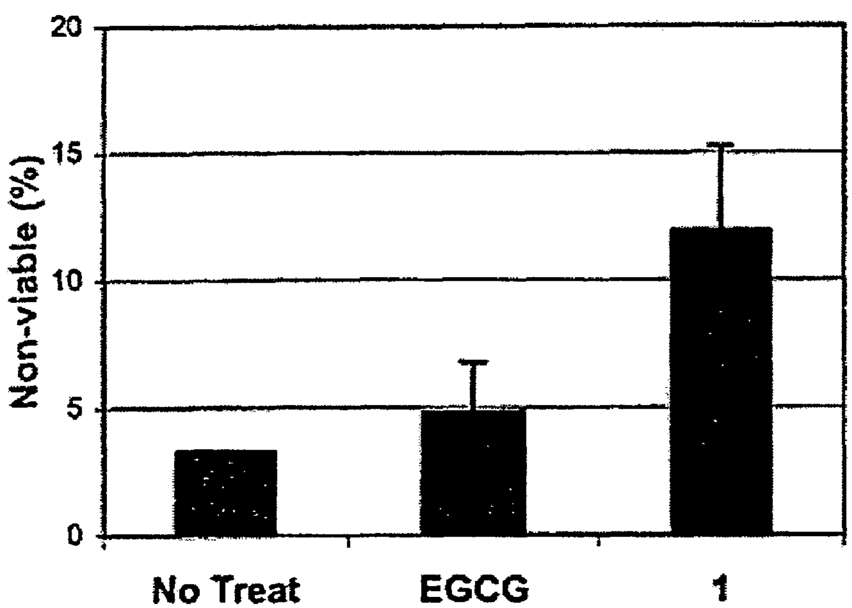


Figure 8

Figure 9

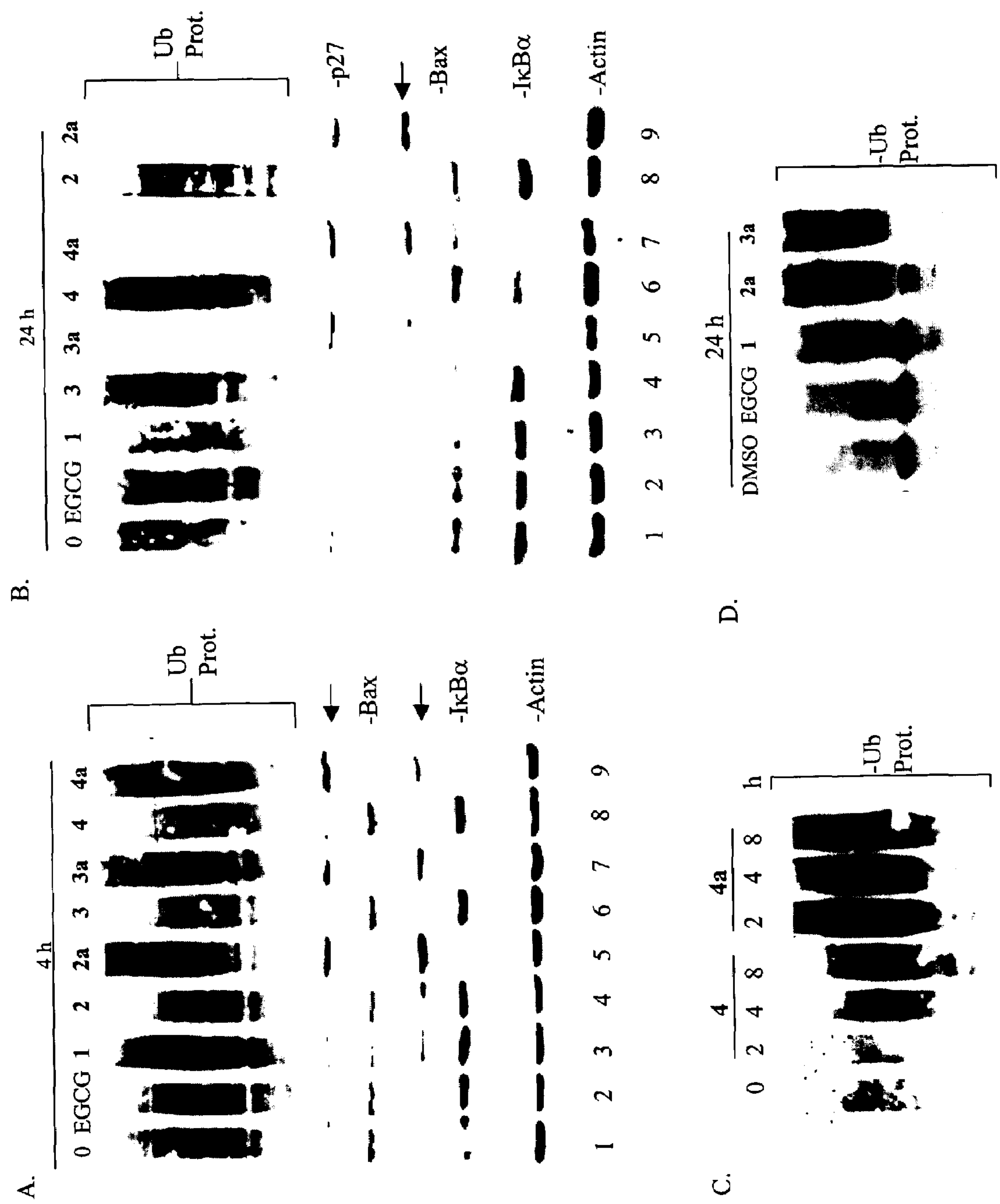
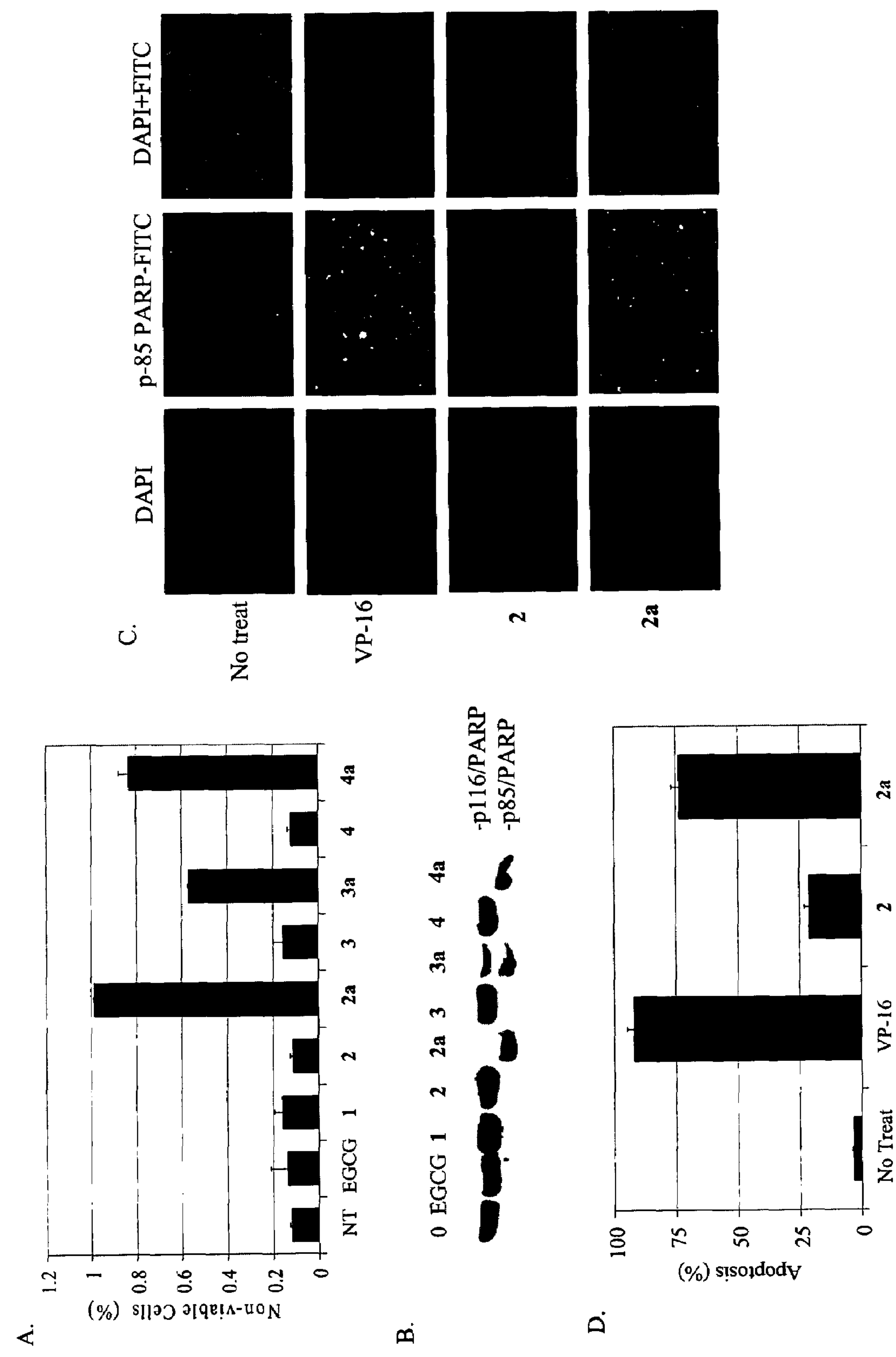

A.
4 h
0 EGCG 1 2 2a 3 3a 4 4a
Ub Prot.
-Bax
-IκBα
-Actin
1 2 3 4 5 6 7 8 9
B.
24 h
0 EGCG 1 3 3a 4 4a 2 2a
Ub Prot.
-p27
-Bax
-IκBα
-Actin
1 2 3 4 5 6 7 8 9
C.
0 2 4 8 2 4 8 h
4
4a
-Ub Prot.
D.
24 h
DMSO EGCG 1 2a 3a
-Ub Prot.

Figure 10


A.
Non-viable Cells (%)
1.2
1
0.8
0.6
0.4
0.2
0
NT
EGCG
1
2
2a
3
3a
4
4a
B.
0 EGCG 1 2 2a 3 3a 4 4a
-p116/PARP
-p85/PARP
C.
DAPI
p-85 PARP-FITC
DAPI+FITC
No treat
VP-16
2
2a
D.
Apoptosis (%)
100
75
50
25
0
No Treat
VP-16
2
2a

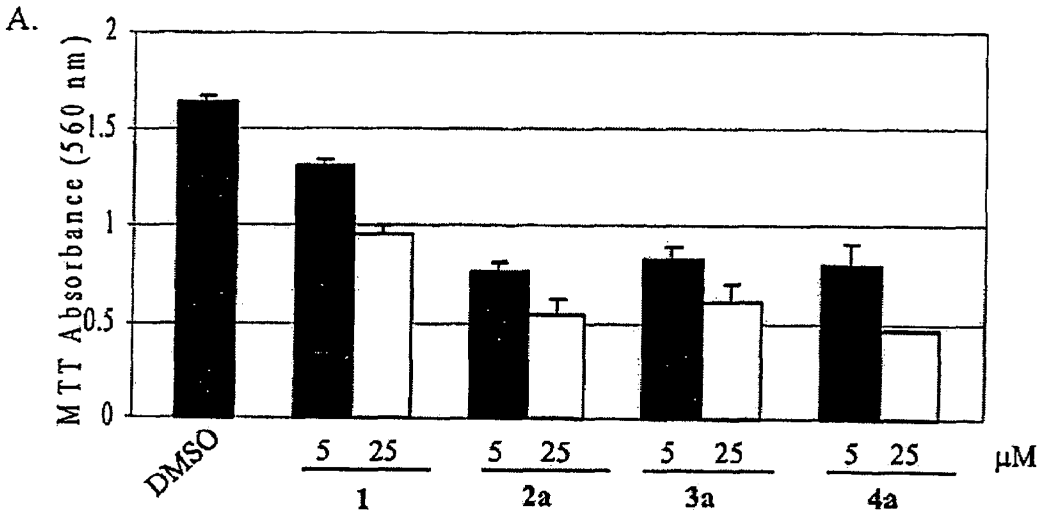


Figure 11a

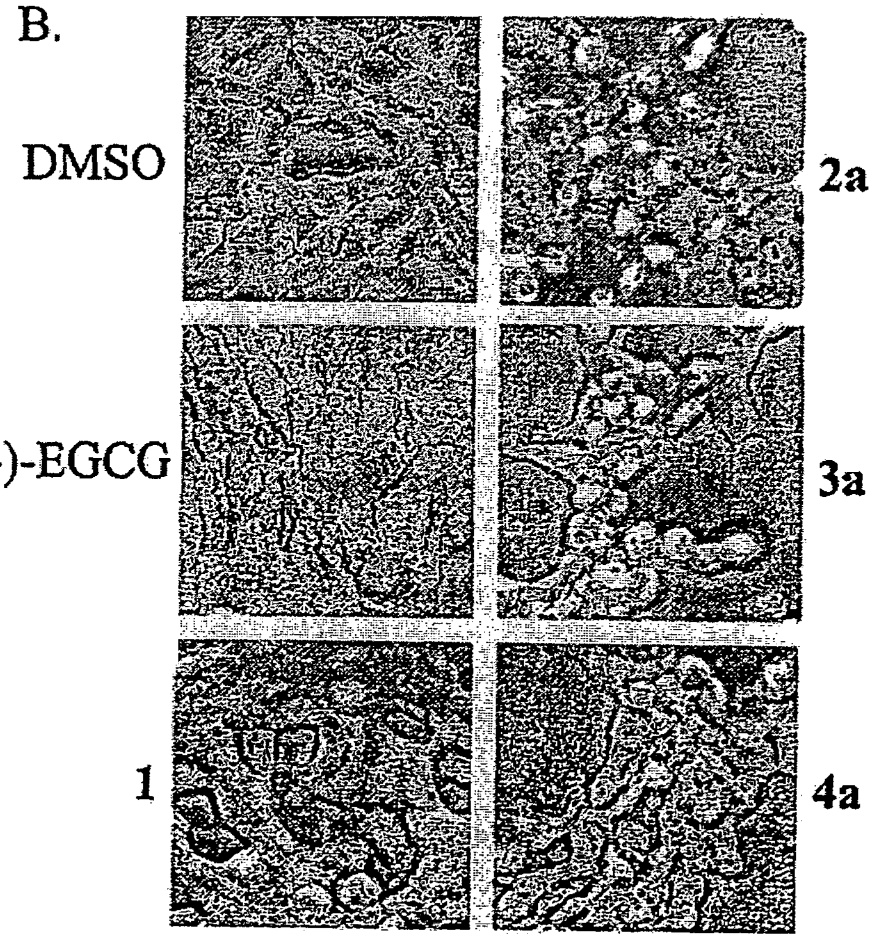


Figure 11b

C.

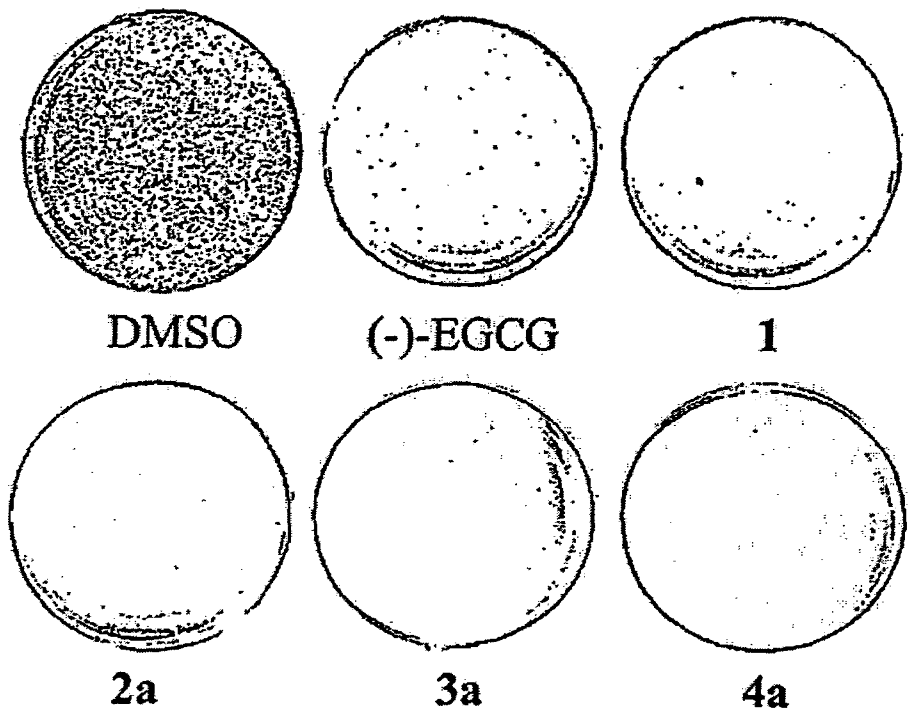


Figure 11c

D.

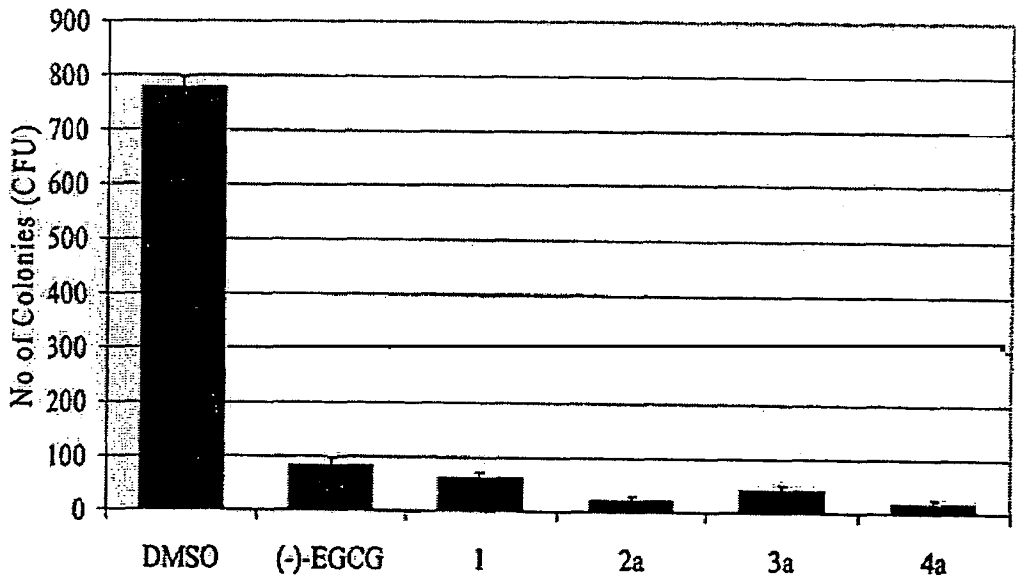
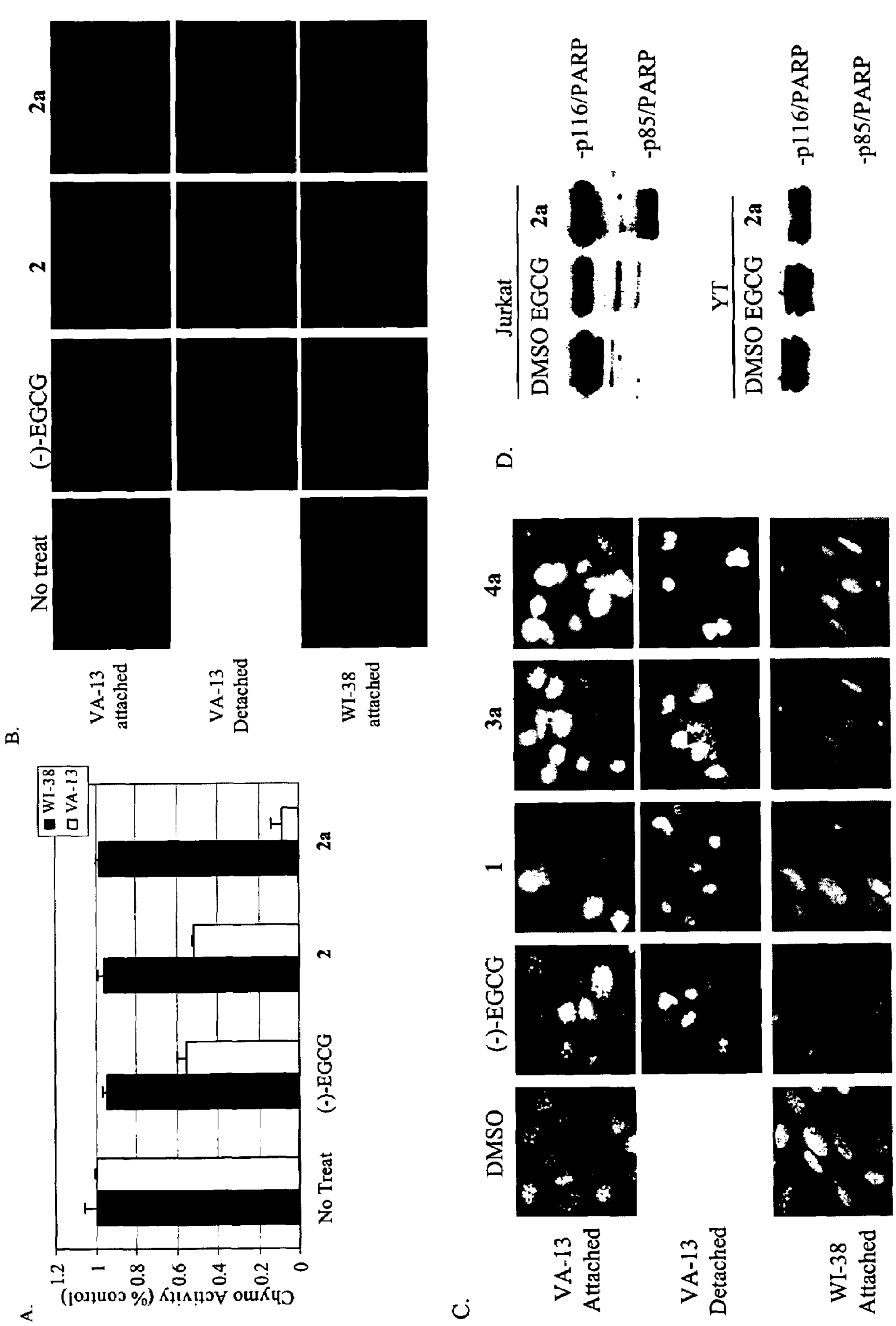


Figure 11d

Figure 12


A.
Chymo Activity (% control)
1.2
1
0.8
0.6
0.4
0.2
0
WI-38
VA-13
No Treat
(-)-EGCG
2
2a
B.
No treat
(-)-EGCG
2
2a
VA-13 attached
VA-13 Detached
WI-38 attached
C.
DMSO
(-)-EGCG
1
3a
4a
VA-13 Attached
VA-13 Detached
WI-38 Attached
D.
Jurkat
DMSO EGCG 2a
-p116/PARP
-p85/PARP
YT
DMSO EGCG 2a
-p116/PARP
-p85/PARP

(−)-EPIGALLOCATECHIN GALLATE DERIVATIVES FOR INHIBITING PROTEASOME

FIELD OF THE INVENTION

This invention relates to derivates of (−)-epigallocatechin gallate, particularly for use as proteasome inhibitors.

BACKGROUND OF THE INVENTION

The polyphenols found in green tea extracts are (−)-epicatechin (EC), (−) -epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG) and (−)-epigallocatechin-3-gallate (EGCG). In particular, (−)-EGCG, the most abundant catechin, was found to be chemopreventive and anticancer agent among the green tea catechins (GTCs) (4. Fujiki, H. *J Cancer Res Clin Oncol.* 1999, 125, 589-97).

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. 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. 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 (Dou, Q. P.; Li, *B. Drug Resist Update* 1999, 2, 215-23). It has been shown that natural (−)-EGCG and synthetically derived (+)-EGCG are potent inhibitors of the proteasomal chymotrypsin activity, leading to growth arrest and/or apoptosis (Smith, D. M.; Wang, Z.; Kazi, A.; Li, L.; Chan, T. H.; Dou, Q. *P. Mol Med* 2002, 8: 382-92.). US patent publication no. 20040110790 (Zaveri et al.) describes synthetic analogs of green tea polyphenols as chemotherapeutic and chemopreventive agents, but the synthesis provided only racemic compounds, and do not use natural occurring catechins derived from green tea.

The P13K/Akt signaling is a widely known tumor cell survival pathway (Vanhaesebroeck, B.; Alessi, D. R. *Biochem J* 2000, 346, 561-76). 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 cyclindependent kinase inhibitor p27.24 Recently, (−)-EGCG has been found to inhibit the Akt kinase activity via reducing the phosphatidylinositol 3-kinase signals in M MTV-Her-2/neu mouse mammary tumor NF639 cells, leading to reduced tumor cell growth (Pianetti, S.; Guo, S.; Kavanagh, K. T.; Sonenshein, G. E. *Cancer Res* 2002, 62, 652-5).

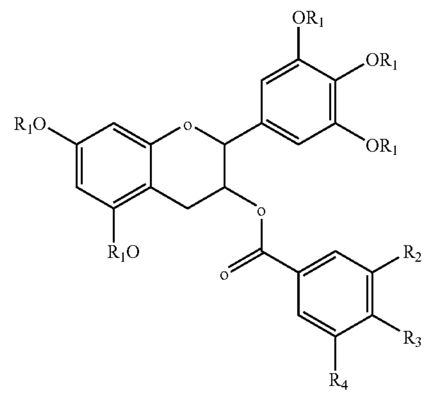
However, (−)-EGCG has at least one limitation: it gives poor bioavailability. A study by Nakagawa et al. showed that only 0.012% of (−)-EGCG could be absorbed in rats given 56 mg of (−)-EGCG orally (Nakagawa, K.; Miyazawa, T. *Anal Biochem.* 1997, 248, 41-9). This low absorption was thought to be due to the poor stability of (−)-EGCG in neutral or alkaline solutions. 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.

OBJECTS OF THE INVENTION

Therefore, it is an object of this invention to provide a (−)-EGCG derivative that is able to resolve at least one or more of the problems as set forth in the prior art. As a minimum, it is an object of this invention to provide the public with a useful choice.

SUMMARY OF THE INVENTION

Accordingly, this invention provides a compound for inhibiting proteasome having the formula:



wherein

R_1 is selected from the group consisting of —H and C_1 to C_6 acyl group;

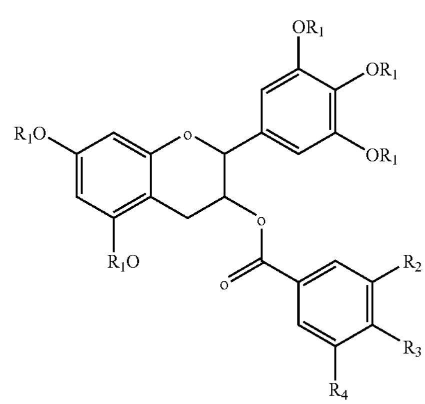
R_2, R_3, and R_4 are each independently selected from the group consisting of —H, —OH, and C_1 to C_6 acyloxyl group; and

at least one of R_2, R_3, and R_4 is —H.

Preferably, R_1 may be —(CO)—CH_3. More preferably, R_2 can be —O—(CO)—CH_3, and R_3=R_4=—H. Optionally, R_3 can be —O—(CO)—CH_3, and R_2=R_4=—H; or R_3 can be —H, and R_2=R_4=—O—(CO)—CH_3.

Additionally, R_1 can be —H. More preferably, R_2 can be —OH, and R_3=R_4=—H. Optionally, R_3 may be —OH, and R_2=R_4=—H; or R_3 may be —H, and R_2=R_4=—OH.

This invention also provides a method of reducing tumor cell growth including the step of administering an effective amount of a compound having the formula:



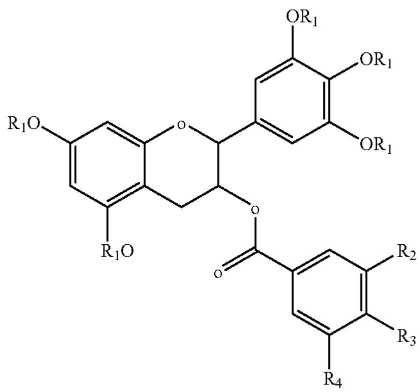
wherein

R_1 is selected from the group consisting of —H and C_1 to C_6 acyl group; and

R_2, R_3, and R_4 are each independently selected from the group consisting of —H, —OH, and C_1 to C_6 acyloxyl group; and

if R_1═H and R_2═R_3═R_4, then R_2 is not —OH.

It is another aspect of this invention to provide the use of a compound of having the formula:



wherein

R_1 is selected from the group consisting of —H and C_1 to C_6 acyl group; and

R_2, R_3, and R_4 are each independently selected from the group consisting of —H, —OH, and C_1 to C_6 acyloxyl group; and

if R_1═H and R_2═R_3═R_4, then R_2 is not —OH in the manufacturing of a medicament for reducing tumor cell growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the present invention will now be explained by way of example and with reference to the accompanying drawings in which:

FIG. **1** shows the structures of the (−)-EGCG, and examples of the (−)-EGCG derivatives of this invention;

FIG. **2** shows the degradation curve of (−)-EGCG and 1;

FIG. **3** shows the 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): x; compound B (mono-acetate): ▲; EGCG: ■;

FIG. **4** shows the 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): x; compound B (mono-acetate): ▲; EGCG: ■;

FIG. **5** shows the inhibition of the chymotrypsin-like activity of the purified 20S proteasome by 1 and (−)-EGCG;

FIG. **6** shows (a) the inhibition of Proteasome Actvitiy by 1 and (−)-EGCG in vivo; (b) Western blot assay of ubiquitin after treatment with 1 and (−)-EGCG;

FIG. **7** shows the amount of p-Akt levels with 1 and (−)-EGCG treatment;

FIG. **8** shows the cell viability in Jurkat cells treated with 1 and (−)-EGCG;

FIG. **9** shows the results of treating Jurkat cells with 25 μM of each indicated polyphenol for 4 h (A), up to 8 (C), or 24 h (B), or of LNCaP cells 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;

FIG. **10** shows the results of treating Jurkat T cells (A and B) or VA-13 (C and D) cells 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 by counting the number of apoptotic cells over the total number of cells in the same field. Data are mean of duplicate experiments ±SD;

FIG. **11** shows the 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 40× magnification (Leica, Germany). C, Soft agar assay. LNCaP cells were plated in soft agar with the solvent DMSO or 25 pM 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; and

FIG. **12** shows the results of treating normal WI-38 and SV-40-transformed VA-13 cells 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 and C, Nuclear staining for apoptotic morphology of both detached and attached cells at 10× (B) or 40× (C) magnification. Missing panels indicate that no detachment of cells occurred. D, Western blot analysis using specific antibody to PARP.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

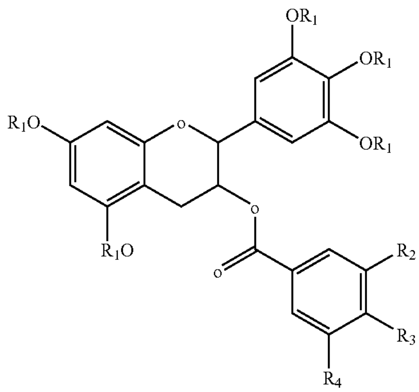
This invention is now described by way of example with reference to the figures in the following paragraphs.

Objects, features, and aspects of the present invention are disclosed in or are obvious from the following description. It is to be understood by one of ordinary skill in the art that the present discussion is a description of exemplary embodiments only, and is not intended as limiting the broader aspects of the present invention, which broader aspects are embodied in the exemplary constructions.

In this invention, a prodrug form of (−)-EGCG is synthesized that improves its bioavailability. The prodrug exhibits: [i] improved 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 possess low systemic toxicity

Further, three derivatives of (−)-EGCG and their prodrug forms are synthesized, and surprisingly,

The general formula of the compounds of this invention has the formula:



wherein

R_1 is selected from the group of —H and C_1 to C_6 acyl group; and

R_2, R_3, and R_4 are each independently selected from the group of —H, —OH, and C_1 to C_6 acyloxyl group.

Of course, when R_2=R_3=R_4, R_2 is —OH, the compound becomes (−)-EGCG, and therefore is not the subject of this invention.

In the definitions of the compound above, collective terms were used which generally represent the following groups:

C_1-C_6 acyl: having the structure —(CO)—R, wherein R is hydrogen or straight-chain or branched alkyl groups having 1 to 5 carbon atoms, such as methyl, ethyl, propyl, 1-methylethyl, butyl, 1-methylpropyl, 2-methylpropyl, 1,1-dimethylethyl, pentyl, 2-methylbutyl. The alkyl group R can be partially or fully halogenated". The term "partially or fully halogenated" is meant to express that in the groups characterized in this manner the hydrogen atoms may be partially or fully replaced by identical or different halogen atoms, for example chloromethyl, dichloromethyl, trichloromethyl, fluoromethyl, difluoromethyl, trifluoromethyl, chlorofluoromethyl, dichlorofluoromethyl, chlorodifluoromethyl, 1-fluoroethyl, 2-fluoroethyl, 2,2-difluoroethyl, 2,2,2-trifluoroethyl, 2-chloro-2-fluoroethyl, 2-chloro-2,2-difluoroethyl, 2,2-dichloro-2-fluoroethyl, 2,2,2-trichloroethyl and pentafluoroethyl;

C_1-C_6 acyloxyl: having the structure —O—(CO)—R, wherein R is hydrogen or straight-chain or branched alkyl groups having 1 to 5 carbon atoms as mentioned above.

According to this invention, peracetate (−)-EGCG, 1 was synthesized (FIG. **1**). 1 was found to be more stable than (−)-EGCG. The prodrug was biologically inactive against a purified 20S proteasome, 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. Finally, treatment of leukemia Jurkat T cells with 1 induced cell death.

In order to evaluate whether other peractete protected tea polyphenols possessed greater bioactivity than their unprotected parent, several synthetic analogs to (−)-EGCG that possess deletions of the hydroxyl groups on the gallate ring were synthesized. Additionally, to enhance the stability of the molecules, the hydroxyl groups were converted to acetate groups to create a prodrug. Surprisingly, the protected analogs were found to be more potent proteasome inhibitors in intact tumor cells than their unprotected counterparts.

The synthesis and characterization of the compounds of this invention will be detailed in the following sections.

MATERIALS AND METHODS

Reagents

Fetal Bovine Serum was purchased from Tissue Culture Biologicals (Tulare, Calif.). Mixture of penicillin-streptomycin-1-Glutaxnine, RPMI, and DMEM are from Invitrogen (Carlsbad, Calif.). Dimethyl sulfoxide (DMSO), N-acetyl-$_L$-cysteine (NAC), Hoechst 33342, 3-((4,5)-dimethylthiazol-2-yl)-2,5-diphenylteolium bromide (MTT), bovine serum albumin (BSA), and (−)-EGCG were purchased from Sigma (St. Louis, Mo.). Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chyrnotrypsin-like activity) was obtained from Biomol (Plymouth Meeting, Pa.). Purified 20s proteasome from rabbit was acquired from Boston Biochem (Cambridge, Mass.). Arnplex Red H_2O_2 assay kit was purchased from Molecular Probes (Eugene, Oreg.). Monoclonal antibodies to Bax (H280) and Ubiquitin (P4D1), and polyclonal antibodies to IKB-a (C15), and Actin (C11) as well as anti-goat, anti-rabbit, and anti-mouse IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Monoclonal antibody to p27 (554069) was purchased from BD Biosciences (San Diego, Calif.). Vectashield Mounting Medium with DAPI was purchased from Vector Laboratories, Inc. (Burlingame, Calif.). The Polyclonal antibody, specific to the PARP cleavage site and FITC-conjugated, was acquired from Biosource (Camarillo, Calif.). CaspACE FITC-VAD-FMK marker was purchased from Promega (Madison, Wis.).

Synthesis of Synthetic Tea Polyphenol Analogs. Synthesis of 1, 2, 2a, 3, 3a, 4, and 4a (FIG. **1**)

1 was prepared according to literature procedures (Kohri, T.; Nanjo, F.; Suzuki, M.; Seto, R.; Matsumoto, N.; Yamakawa, M.; Hojo, H.; Hara, Y.; Desai, D.; Amin, S.; Conaway, C. C.; Chung, F. L. *J Agric Food Chem* 2001, 49: 1042-8), but the synthesis of 1 will be iluustrated here. For the synthesis of 1, commercially available (−)-EGCG was used as a starting material. 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 1H and 13C NMR, LRMS and HRMS.

Mp 157.1° C. LRMS m/z (ESI) 817 (MNa^+) HRMS found, 817.1544; $C_{38}H_{34}O_{19}Na$ requires 817.1592. ^1H NMR ($CDCl_3$, 500 MHz) δ 7.62 (s, 2H), 7.24 (s, 2H), 6.73 (s, 1H), 6.61 (s, 1H), 5.64 (br s, 1H), 5.18 (s, 1H), 3.02 (m, 2H), 2.29 (s, 3H), 2.28 (s, 9H), 2.27 (s, 3H), 2.24 (s, 3H), 2.23 (s, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.89, 168.40, 167.59, 167.43, 166.72, 166.20, 163.51, 154.71, 149.72, 149.64, 143.38, 143.29, 138.93, 135.06, 134.34, 127.41, 122.34, 118.79, 109.42, 109.00, 108.06, 76.46, 67.98, 25.85, 21.06, 20.75, 20.54, 20.11.

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

This compound was synthesized according to literature procedures (Li, L. H.; Chan, T. H. *Org. Lett.* 2001, 3, 739-741).

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

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 (10 mL). The mixture was refluxed for 2 hours. After which the excess $(COCl)_2$ and the solvent were removed by distillation and the resulting residue was dried under vacuum overnight. The residue was redissolved in CH_2Cl_2 (5 mL) and added to a solution of 5 (0.20 g, 0.26 mmol) and DMAP (0.08 g, 0.64 mmol) in CH_2Cl_2 (10 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 2b as a white solid (0.22 g, 88%).

LRMS m/z (ESI) 989 (MNa^+) HRMS found, 989.3657; $C_{64}H_{54}O_9Na$ requires 989.3666. ^1H NMR ($CDCl_3$, 500 MHz) 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 (dd, J=16.5, 6.5 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) 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, 78.38, 74.98, 71.07, 70.02, 69.81, 24.18.

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

Suspension of 2b (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_2Cl_2 2:1) to afford the product 2 as a white solid (80 mg, 79%).

LRMS m/z (ESI) 449 (MNa^+) HRMS found, 449.0887; $C_{22}H_{18}O_9Na$ requires 449.0849. ^1H NMR (CD_3OD, 500 MHz) 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_3OD, 100 MHz) 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.

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

Suspension of 2b (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 (2) 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). 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 5×5 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 2a as a white powder (0.061 g, 85%).

Mp 71.6° C. LRMS m/z (ESI) 701 (MNa^+) HRMS found, 701.1541 $C_{34}H_{30}O_{15}Na$ requires 701.1482. ^1H NMR ($CDCl_3$, 500 MHz) 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 ($CDCl_3$, 100 MHz) 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, 69.17, 23.65, 20.96, 20.92, 20.65, 20.47, 20.10.

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

The title compound was prepared in a similar manner as described for 2b, using 5 (0.08 g, 0.1 mmol) and 4-(benzyloxy)benzoic acid (0.049 g, 0.22 mmol) giving 2b as a white solid (0.087 g, 90%).

LRMS m/z (ESI) 989 (MNa^+) HRMS found, 989.3666 $C_{64}H_{54}O_9Na$ requires 989.3666. ^1H NMR ($CDCl_3$, 500 MHz) 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 ($CDCl_3$, 100 MHz) 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, 74.98, 71.05, 69.95, 69.79, 69.26, 24.01.

(2S*,3R*)-trans-5,7-Bis(hydroxy)-2-13,4,5-tris(hydroxy)phenyl chroman-3-yl 4-(hydroxy)benzoate (3)

The title compound was prepared in a similar manner as described for 2 using 3b (0.24 g, 0.25 mmol) to afford 3 as a white solid (79 mg, 75%).

LRMS m/z (ESI) 449 (MNa^+) HRMS found, 449.0840; $C_{22}H_{18}O_9Na$ requires 449.0849. ^1H NMR (CD_3OD, 500 MHz) δ 7.75 (d, J=8.5 Hz, 2H), 6.77 (d, J=8.5 Hz, 2H), 6.42 (s, 2H), 5.95 (dd, 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_3OD, 100 MHz) 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.

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

The title compound was prepared in a similar manner as described for 2a, using 3b (0.15 g, 0.16 mmol) to afford 3a as a white solid (92.6 mg, 88%).

Mp 189.4° C. LRMS m/z (ESI) 701 (MNa^+) HRMS found, 701.1467; $C_{34}H_{30}O_{15}Na$ requires 701.1482. ^1H NMR ($CDCl_3$, 500 MHz) 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 ($CDCl_3$, 100 MHz) 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, 69.01, 23.64, 20.97, 20.63, 20.46, 20.00.

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

The title compound was prepared in a similar manner as described for 2b, using 5 (0.3 g, 0.4 mmol) and 3,5-bis(benzyloxy)benzoic acid (0.27 g, 0.81 mmol) giving 4b as a white solid (0.36 g, 85%).

LRMS m/z (ESI) 1095 (MNa^+) HRMS found, 1095.4059; $C_{71}H_{60}O_{10}Na$ requires 1095.4084. ^{1}H NMR ($CDCl_3$, 500 MHz) 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 ($CDCl_3$, 100 MHz) 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.

(2S*,3R*)-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 4b (0.17 g, 0.16 mmol) to afford 4 as a white solid (50 mg, 71%).

LRMS m/z (ESI) 465 (MNa^+) HRMS found, 465.0844; $C_{22}H_{18}O_9Na$ requires 465.0798. ^{1}H NMR (CD_3OD, 500 MHz) 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_3OD, 100 MHz) 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.

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

The title compound was prepared in a similar manner as described for 2a using 4b (0.15 g, 0.14 mmol) to afford 4a as a white solid (72 mg, 70%).

Mp 105.5° C. LRMS m/z (ESI) 759 (MNa^+) HRMS found, 759.1530; $C_{36}H_{32}O_{17}Na$ requires 759.1537. ^{1}H NMR ($CDCl_3$, 500 MHz) 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 ($CDCl_3$, 100 MHz) 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, 69.48, 23.77, 20.94, 20.86, 20.64, 20.45, 19.99.

As the carboxylate or —OH groups of the compound 1, 2, 3 and 4 can readily undergo acyl exchange, one skilled in the art shall be able to replace the acyl groups on the carboxylate groups of these compounds.

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 minutes and the mobile phase, 20% aqueous acetonitrile and 0.01% TFA; for prodrug 1, time points were 0, 30, 60, 90, 120 minutes and mobile phase, 50% aqueous acetonitrile and 0.01% TFA.

Enzymatic hydrolysis of 1

Lysis buffer (pH 5) (0.25 mL) was added to 2×106 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 minutes), an aliquot (0.06 mL) of the reaction mixture was taken out, filtered and injected into the HPLC and analyzed as outlined above.

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 minutes (20% aqueous acetonitrile and 0.016% TFA), 8-13 minutes (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 (5×105 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.

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% (vlv) fetal bovine serum, 100 Ulml 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-transformed (VA-13) human fibroblast cells were grown in Dulbecco's modified Eagle's mediums supplemented with 10% (vlv) fetal bovine serum, 100 U/ml penicillin, 100 pg/ml streptomycin. All cell cultures were maintained in a 5% CO_2 atmosphere at 37° C.

Cell Extract preparation and Western Blotting

Whole cells extracts were prepared as described previously (An B, Goldfarb R H, Siman R, Dou Q P. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not

normal, human fibroblasts. *Cell Death Differ* 1998;5: 1062-75.). Analysis of Bax, IKBa, p27, PAW, and ubiquitinated protein expression were performed using monoclonal or polyclonal antibodies according to previously reported protocols (An B et. al.).

Inhibition of Purified 20s Proteasome Activity by (–)-EGCG or Synthetic Tea Polyphenols

Measurement of the chyrnotrypsin-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 (Nam S et. al.).

Inhibition of Proteasome Activity in Intact Cells by Natural/Synthetic Tea Polyphenols

VA-13 or WI-38 cells were grown in 24 well plates (2 ml/well) to 70-80% confluency, followed by 24 h treatment with 25 pM (–)-EGCG, 2, or 2a. 40 pM Suc-Leu-Leu-Val-Tyr-AMC substrate was then added for 2.5 h at 37° C. and the chyrnotrypsin-like activity was measured as described above.

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 poly(ADP-ribose) polymerase (PARP) and visualized on an Axiovert 2 5 (Zeiss; Thornwood, N.Y.) microscope. Cells were grown to about 80% confluency in 60 mm dishes. VA-13 cells were then treated with VP-16, 2, or 2a (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 between all steps listed below and all washes are 1 min duration with PBS. Cells were then fixed in ice-cold 70% ethanol, permeabilized in 0.1% Triton-X-100 and blocked for 30 min in 1% bovine serum albumin (BSA) at room temperature. Incubation with the primary FITC-conjugated-p85/PARP antibody was for 30 min at 4° C. 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.

Trypan Blue Assay

Trypan blue assay was used to ascertain cell death in Jurkat T cells treated with natural and synthetic polyphenols as indicated. Apoptotic morphology was assessed using phase-contrast microscopy as described previously (Kazi A, Hill R, Long T E, Kuhn D J, Twos E, Dou Q P. Novel N-thiolated beta-lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells. *Biochem Pharmacol* 2004;67:365-74; Kuhn D J, Smith D M, Pross S, Whiteside T L, Dou Q P. Overexpression of interleukin-2 receptor alpha in a human squamous cell carcinoma of the head and neck cell line is associated with increased proliferation, drug resistance, and transforming ability. *J Cell Biochem* 2003;89:824-36).

MTT Assay

MTT was used to determine effects of polyphenols on overall proliferation of tumor cells. Human breast MCF-7 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, the MTT was removed and 100 μl of DMSO was added and colorimetric analysis performed using a multilabel plate reader at 560 nm (Victor3; Perkin Elmer). Absorbance values plotted are the mean from triplicate experiments.

Soft Agar Assay

LNCaP cells (2×10^4) were plated in soft agar on 6 well plates in the presence of (–)-EGCG or protected tea analogs (25 pM) or in DMSO (control) to determine cellular transformation activity as described previously (Kazi A et al).

Nuclear Staining

After each drug treatment, both detached and attached VA-13 and WI-38 cells were stained Hoechst 33342 to assess for apoptosis. Briefly, cells were washed 2× in PBS, fixed for 1 h with 70% ethanol at 4° C., washed 3× in PBS, and stained with 50 pM Hoechst for 30 min in the dark at room temperature. Detached cells were plated on a slide and attached cells were visualized on the plate with a fluorescent microscope at 10× or 40× resolution (Zeiss, Germany). Digital Scientific obtained images with AxioVision 4.1 and adjusted using Adobe Photoshop 6.0.

Results

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Chemical Stability and Enzymatic Hydrolysis of Peracetate (–)-EGCG, 1

The stability of 1 with (–)-EGCG in a culture medium (RMPI) is compared, 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 FIG. **2**.

When (–)-EGCG was dissolved in the culture medium, it was found to be degraded rapidly within 20 minutes, demonstrating the low stability of (–)-EGCG in the medium. Although 1 was also degraded in the medium, as seen in FIG. **2**, the rate of its degradation was much slower when compared with (–)-EGCG. 1 disappeared completely after 2 hours, 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 hydrolysed to EGCG under the culture medium conditions, the experiment was repeated 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 FIG. **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 diacetate and mono-acetate of EGCG respectively. These results suggested that compound 1 was first hydrolysed to the diacetate, then mono-acetate and eventually EGCG under the culture medium conditions.

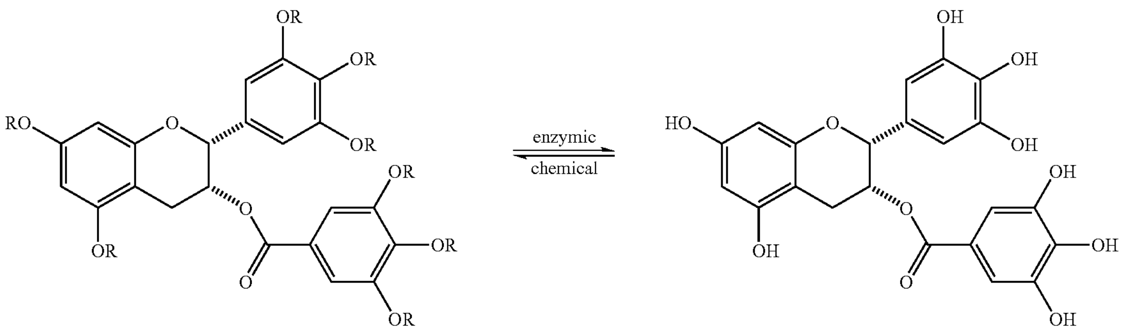
The generation of (–)-EGCG from compound 1 under cellular conditions could be more clearly demonstrated by the addition of vitamin C to prevent the rapid disappearance of (–)-EGCG. In this case, we performed the experiment in medium with breast cancer cell lysate. HPLC analyses

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. **4**) similar to FIG. **3**. Therefore, it is believed that 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).

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

(−)-EGCG and 1 w ere 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. **7**). (−)-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. **7**). Actin was used as a loading control.

Scheme 1



Inhibition of the Proteasomal Activity In Vitro and In Vivo by 1 and (−)-EGCG

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 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. **5**). In contrast, (−)-EGCG at 10 μM inhibited 80-90% of the proteasomal chymotrypsin-like activity. Therefore, as predicted, 1 outside of a cellular system is not a proteasome inhibitor.

If 1 converts to (−)-EGCG inside the cells, proteasome inhibition in vivo should be detected. To examine this possibility, human Jurkat cells was treated with 25 μM of 1 or (−)-EGCG for 12 or 24 h, followed by measurement of proteasome activity by using a chyrnotrypsin-like specific fluorogenic substrate in intact cells (FIG. **6***a*) or Western blot for ubiquinated proteins (FIG. **6***b*). Treatment of Jurkat T cells with (−)-EGCG for 24 h inhibited proteasome activity by 31% versus 42% inhibition with 1 (FIG. **6***a*). To analyze the intracellular level of polyubiquitinated proteins, cells were lysed after 12 hours incubation and subjected to Western blotting. 1 showed comparable levels of ubiquitinated proteins to that of natural (−)-EGCG (FIG. **6***b*). 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. It is possible that 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 the amount of anti-oxidants that may be present in the cells at any time.

Cell death Induced by 1 and (−)-EGCG

The abilities of (−)-EGCG and 1 to induce cell death in Jurkat T cells treated with 10 μM for 24 h are also accessed. While (−)-EGCG had a minimal effect on cell death (5%), 1 was capable of inducing up to 15% cell death at that concentration (FIG. **8**). Therefore, the greater abilities of 1 to inhibit cellular proteasome activity (FIG. **6**) and to inactivate Akt (FIG. **7**) are associated with its increased cell death-inducing activity (FIG. **8**).

Acetylated Synthetic Tea Polyphenols Do Not Inhibit the Purified 20s Proteasome Activity

Up to 25 μM of all protected and unprotected compounds were incubated with a purified 20s proteasome and a fluorogenic substrate for chyrnotrypsin 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, followed by 2 (IC_{50} about 9.9 μM). IC_{50} values of compounds 3 and 4 were found to 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 results above.

Protected Tea Analogs Exhibit Greater Proteasome-inhibitory Potency in Intact Tumor Cells

To determine what effects the synthetic tea analogs had on the proteasome in vivo, Jurkat cells were treated with 25 μM of each synthetic compound for either 4 or 24 h, with (−)-EGCG as a control (FIGS. **9**A and **9**B). After 4 h of treatment, Western b lot analysis shows that the acetate-protected analogs induced a greater amount of ubiquitinated proteins (FIG. **9**A, Lanes 5, 7, and 9), indicating that proteasome activity is abrogated. 1 was used as a control based on the above previous data showing that peracetate-protected (−)-EGCG is more potent than natural (−)-EGCG (FIG. **9**A, Lanes 3 vs. 2). Additionally, Western blots for Bax and IκBα, known as two proteasome targets, revealed the disappearance of these proteins and the appearance of a higher molecular weight band,

which is speculated to be multi-ubiquinated forms of the proteins (FIG. 9A, lanes 3, 5, 7, and 9). However, after 24 h treatment, (−)-EGCG and its unprotected analogs exhibited a greater amount of ubiquitinated proteins (FIG. 9B. Lanes 2, 4, 6, and 8). This is consistent with the idea that protected analogs are potent inhibitors of the proteasome at an earlier time point and that after 24 h of treatment the ubiquitinated proteins are being depleted by deubiquitinating enzymes. Accumulation of p27, another proteasome target, is also found in Jurkat cells treated with the protected analogs 2a, 3a and 4a (FIG. 9B, Lanes 9, 5, 7). The putative ubiquitinated IκBα band is still found in cells treated with 2a, 3a, and 4a (FIG. 9B, Lanes 9, 5, 7). The presumed ubiquinated IκBα band is now absent in the 24 h treatment, possibly due to deubiquitination (FIG. 9B). Actin was used as a loading control in this experiment.

Protected Analogs Induce Greater Cell Death in Leukemic Cells

In a kinetics experiment using a pair of analogs, 4 and 4a, it was found that the unprotected analog 4 induced accumulation of ubiquitinated proteins with highest expression after 8 hours of polyphenol treatment (FIG. 9C). Conversely, the protected 4a showed increased ubiquitinated protein accumulation as early as 2 h and lasting up to 8 h (FIG. 9C). 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 (FIG. 9D).

Protected Analogs are More Potent Apoptosis Inducers

It has been shown that proteasome inhibition can induce apoptosis in a wide variety of cancer cells, but not in normal, non-transformed cells (An B et al). Jurkat T cells are treated with 25 μM of each of the selected polyphenols and their protected analogs for 24 h to investigate their abilities to induce apoptotic cell death. Trypan blue incorporation assay revealed that 2a, 3a and 4a, but not others, induced death in 99, 57, and 83% of Jurkat cells, respectively (FIG. 10A). Similarly, Western blot analysis showed that only 2a, 3a, and 4a induced apoptosis-specific PARP cleavage after 24 h (FIG. 10B). An immunofluorescent stain that detects only the cleavage PARP fiagment (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 (FIGS. 10C and 10D). The unprotected 2 induced much less apoptosis (21%), while 25 μM VP-16, used as a positive control, induced 92% apoptosis (FIGS. 10C and 10D). Counterstain with DAPI, which binds to the minor groove in A-T rich regions of DNA, was decreased drastically in apoptotic cells (FIG. 10C), consistent with DNA fragmentation in late stage apoptosis.

Inhibition of Tumor Cell Proliferation by Protected Polyphenols

Treated breast cancer (MCF-7) cells were then treated with 5 or 25 μM of peracetate-protected analogs for 24 h, followed by MTT analysis to determine their effects on cell proliferation. Compound 1 at 25 μM inhibited cellular proliferation by 40% (FIG. 11A). The protected compounds 2a, 3a, and 4a caused 50% inhibition at 5 μM and 70% at 25 μM, respectively (FIG. 10A).

Human prostate cancer LNCaP cells were then treated for 24 h with each selected tea polyphenol protected analogs 1, 2a, 3a, 4a at 25 μM, followed by determining the apoptotic morphological changes. Again, the protected analogs 2a, 3a, and 4a caused dramatic round-up, detachment, and cellular fragmentation (FIG. 11B). 1 induced mild morphological changes, while (−)-EGCG treatment led to enlarged, flattened cells, indicating growth arrest.

Soft agar assay is used to determine the transforming activity of tumor cells. Abrogation of colony formation is linked to GI arrest and/or apoptosis. 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 (FIG. 11C). 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 (FIGS. 11C and 11D). Protected polyphenols also inhibited tumor cell transforming activity, with 2a and 4a being the most potent inhibitors of colony formation (FIG. 11D).

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. To determine whether the protected compounds also affect normal cells, both VA-13 and WI-38 cells were treated with 25 μM of (−)-EGCG, 2, or 2a for 24 h and examined proteasome activity, nuclear morphological changes, and detachment. A differential decrease was found in the chyrnotrypsin-like activity of the proteasome in VA-13 cells over normal WI-38 cells (FIG. 12A). A 42-48% decrease in proteasome activity was observed in VA-13 cells treated with (−)-EGCG and 2, while 2a inhibited 92% of the proteasomal activity. Conversely, the proteasome activity in WI-38 cells was decreased by only −5% with all three polyphenol treatments.

Next, apoptotic nuclear morphology is examined after treatment with natural (−)-EGCG, 2, and 2a (FIG. 12B). While (−)-EGCG and 2 exhibited little or no apoptosis, 2a markedly induced apoptosis in SV-40-transformed VA-13 cells. In contrast, normal WI-38 fibroblasts treated with all the compounds did not undergo apoptosis and very little detachment was visible. A comparison among all the protected analogs was then performed using 25 μM for 24 h. After 36 h, (−)-EGCG did initiate apoptosis in VA-13 cells (FIG. 12C). All of the protected analogs induced apoptosis in transformed (VA-13), but not normal (WI-38) cells (FIGS. 12B and 12C). 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 PAW cleavage (FIG. 12D).

Discussion

Natural (−)-EGCG from green tea has been converted to its peracetate compound 1. In addition, several synthetic analogs to (−)-EGCG that possess deletions of the hydroxyl groups on the gallate ring are synthesized. Additionally, the hydroxyl groups were converted to acetate groups to create a prodrug, which could be cleaved via esterases inside the cell and converted to the parent drug. Surprisingly, the protected analogs were much more potent proteasome inhibitors in intact tumor cells than their unprotected counterparts. Consistently, the protected analogs were also more potent apoptosis inducers than unprotected partners, when tested in leukemic (Jurkat), solid tumor and transformed cell lines. SAR analysis of the protected analogs revealed that the order of their potency is as follows: 2a=4a>3a>1>)-EGCG.

Protected analogs of (−)-EGCG were designed and synthesized. Unexpectedly, these compounds appear to have proteasome inhibitory activity in vivo.

This invention provides a variety of derivatives of (−)-EGCG that is at least as potent as (−)-EGCG. The carboxylate protected forms of (−)-EGCG and its derivatives are found to be more stable the unprotected forms, which can be used as proteasome inhibitors to reduce tumor cell growth. Further, from the structures of 1, 2, 3, and 4, it can be seen that some of the hydroxyl groups of the gallate ring of (−)EGCG may not be important to the potency.

While the preferred embodiment of the present invention has been described in detail by the examples, it is apparent that modifications and adaptations of the present invention will occur to those skilled in the art. Furthermore, the embodiments of the present invention shall not be interpreted to be restricted by the examples or figures only. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims. For instance, features illustrated or described as part of one embodiment can be used on another embodiment to yield a still further embodiment. Thus, it is intended that the present invention cover such modifications and variations as come within the scope of the claims and their equivalents.

The invention claimed is:

1. A compound for inhibiting proteasome having the formula:

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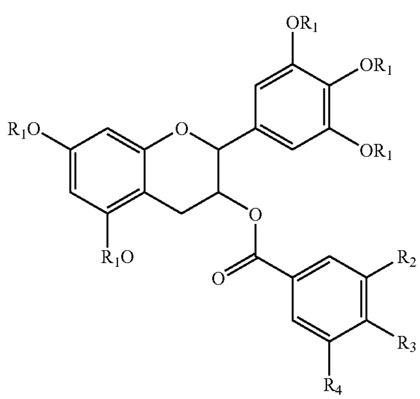


wherein

R_1 is selected from the group consisting of —H and C_1 to C_6 acyl group;

R_2 is selected from the group consisting of —OH and C_1 to C_6 acyloxyl group; and

R_3 and R_4 are —H.

2. The compound of claim **1**, wherein R_1 is —(CO)—CH_3.

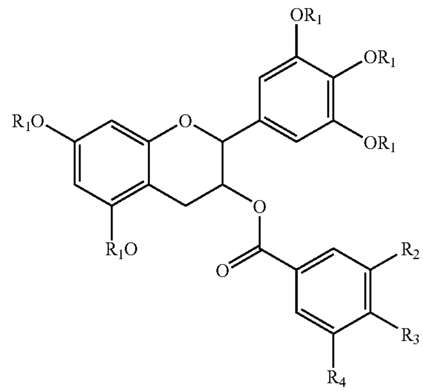
3. The compound of claim **2**, wherein R_2 is —O—(CO)—CH_3.

4. The compound of claim **1**, wherein R_1 is —H.

5. The compound of claim **4**, wherein R_2 is —OH.

6. A method of reducing tumor cell growth comprising a step of administering an effective amount of a compound having the formula:

10



wherein

R_1 is selected from the group consisting of —H and C_1 to C_6 acyl group;

R_2, R_3, and R_4 are each independently selected from the group consisting of —H, —OH, and C_1 to C_6 acyloxyl group;

if R_1=H and R_2=R_3=R_4, then R_2 is not —OH; and

if R_1=H, then R_3 is —H or C_1 to C_6 acyloxyl group.

7. The method of claim **6**, wherein R_1 is —(CO)—CH_3.

8. The method of claim **7**, wherein R_2 is —O—(CO)—CH_3, and R_3=R_4=—H.

9. The method of claim **7**, wherein R_3 is —O—(CO)—CH_3, and R_2=R_4=—H.

10. The method of claim **7**, wherein R_3 is —H, and R_2=R_4=—O—(CO)—CH_3.

11. The method of claim **6**, wherein R_1 is —H.

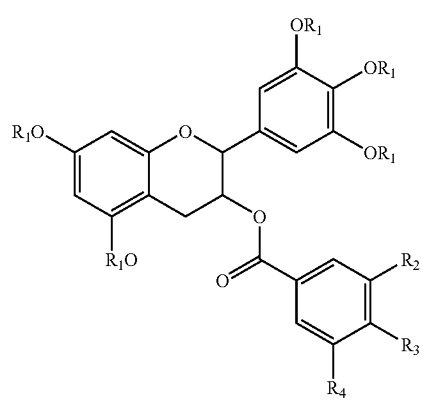
12. The method of claim **11**, wherein R_2 is —OH, and R_3=R_4=—H.

13. The method of claim **11**, wherein R_3 is —OH, and R_2=R_4=—H.

14. The method of claim **11**, wherein R_3 is —H, and R_2=R_4=—OH.

15. A compound for inhibiting proteasome having the formula:

10



wherein

R_1 is selected from the group consisting of —H and C_1 to C_6 acyl group;

R_2 and R_4 are each independently selected from the group consisting of —OH and C_1 to C_6 acyloxyl group; and

R_3 is —H.

16. The compound of claim **15**, wherein R_1 is —(CO)—CH_3.

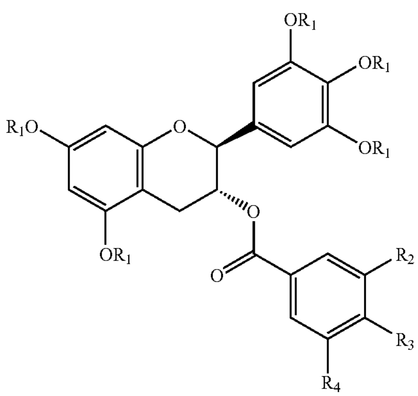
17. The compound of claim **16**, wherein R_2═R_4═—O—(CO)—CH_3.

18. The compound of claim **15**, wherein R_1 is —H.

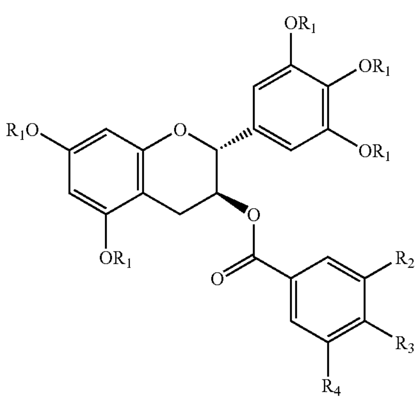
19. The compound of claim **18**, wherein R_2═R_4═—OH.

20. A compound for inhibiting proteasome having one of the following formulae 10A or 10B:

10A



10B



wherein

R_1 is selected from the group consisting of —H and C_1 to C_6 acyl group;

R_3 is selected from the group consisting of —OH and C_1 to C_6 acyloxy group; and

R_2 and R_4 are —H.

21. The compound of claim **20**, wherein R_1 is —(CO)—CH_3.

22. The compound of claim **21**, wherein R_3 is —O—(CO)—CH_3.

23. The compound of claim **20**, wherein R_1 is —H.

24. The compound of claim **23**, wherein R_3 is —OH.

25. A method of inhibiting proteasome comprising a step of administering an effective amount of a compound having the formula:

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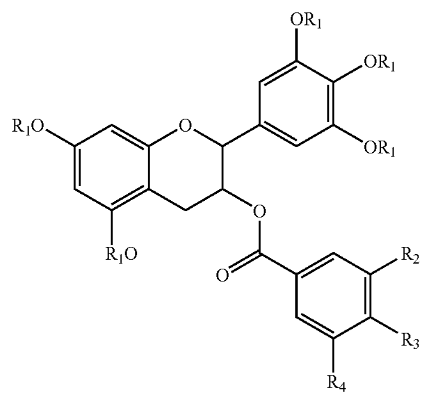

wherein

R_1 is selected from the group consisting of —H and C_1 to C_6 acyl group;

R_2, R_3 and R_4 are each independently selected from the group consisting of —H, —OH and C_1 to C_6 acyloxy group;

if R_1═H and R_2═R_3═R_4, then R_2 is not —OH; and

if R_1═H, then R_3 is —H or C_1 to C_6 acyloxy group.

26. The method of claim **25**, wherein R_1 is —(CO)—CH_3.

27. The method of claim **26**, wherein R_2 is —O—(CO)—CH_3, and R_3═R_4═—H.

28. The method of claim **26**, wherein R_3 is —O—(CO)—CH_3, and R_2═R_4═—H.

29. The method of claim **26**, wherein R_3 is —H, and R_2═R_4═—O—(CO)—CH_3.

30. The method of claim **25**, where R_1 is —H.

31. The method of claim **30**, wherein R_2 is —OH, and R_3═R_4═—H.

32. The method of claim **30**, wherein R_3 is —OH, and R_2═R_4═—H.

33. The method of claim **30**, wherein R_3 is —H, and R_2═R_4═—OH.

34. A method of inhibiting proteasome comprising a step of administering an effective amount of the compound according to claim **20**.

* * * * *