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Proteasome inhibition in human breast cancer cells with high catechol-*O*-methyltransferase activity by green tea polyphenol EGCG analogs

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ABSTRACT

A pro-drug **8** of a synthetic analog **7** is more active in its antiproliferative activity against human breast cancer MDA-MB-231 cells possessing high catechol-*O*-methyltransferase (COMT) activity than the pro-drugs of EGCG and the analog **5**. The higher activity of **8** is attributed to it not being a substrate of COMT.

1. Introduction

Regular drinking of green tea has been associated with reduced incidence of a variety of cancers. (−)-Epigallocatechin-3-gallate [**1**, (−)-EGCG] is the most abundant constituent in green tea extract and the most biologically active among the green tea polyphenols (GTPs). A number of epidemiological and biological studies have shown that (−)-EGCG can reduce or inhibit tumor growth in breast,[1–3] lung,[4] GI[5] and urinary[6,7] tracts.

We previously reported that the tumor cellular proteasome is an important target of (−)-EGCG.[8] The eukaryotic proteasome is a large multi-catalytic, multi-subunit protease complex. Inhibition of the chymotrypsin-like activity of the proteasome has been associated with induction of tumor cell apoptosis.[9–11] We had previously found that (−)-EGCG inhibits the chymotrypsin-like activity of the proteasome in vitro (IC_{50} = 86–194 nM) and in intact tumor cells (1–10 μM) (8). In silico docking studies have indicated that (−)-EGCG binds to the N-terminal threonine (Thr1) of the proteasomal chymotrypsin active site thus inhibiting the proteasomal chymotrypsin-like activity.[12] Acylation of the threonine hydroxy group by the gallate ester function in EGCG accounts for the inhibition. This explains why green tea polyphenols without the ester function, such as epigallocatechin (EGC, **2**), is essentially inactive (IC_{50} = 12 mM).[8] Synthetic (+)-EGCG (**3**)[13], the enantiomer of the natural (−)-EGCG, and other synthetic analogs of green tea catechins with an ester bond have also been found to inhibit the proteasomal chymotrypsin-like activity.[14,15] The fact that the synthetic (+)-EGCG (**3**) is equally potent a proteasome inhibitor (IC_{50} = 170 nM) as the natural (−)-EGCG and that they both bind to the same active site in proteasome suggested that the active site may be nearly symmetrical and can accommodate both enantiomers equally well.[12] On that basis, we designed a simple synthetic analog **5** which is symmetrical and contains the gallate ester function. Interestingly, compound **5** was found to be nearly as potent a proteasome inhibitor as either **1** or **3**, with an IC_{50} = 340 nM.[14]

The challenge in developing (−)-EGCG for cancer prevention and therapy is its low bioavailability, partly due to its poor absorption and its instability under neutral or alkaline conditions (i.e., physiologic pH) and partly due to biologically inactivating processes such as methylation.[16,17] We have previously tried to improve the bioavailability of (−)-EGCG through a pro-drug approach.[18] Acetylation of (−)-EGCG gave the peracetate of (−)-EGCG (**4**, pro-EGCG). Even though **4** itself is not active as a proteasome inhibitor, it exhibited enhanced growth inhibitory activity relative to EGCG in a number of cancer cell lines.[19] Improved bioavailability was observed in vivo: intragastric administration of **4** to CF-1 mice led to higher concentration of EGCG in plasma, small intestinal and colonic tissues compared with administration of equimolar doses of EGCG.[19] More importantly, the enhanced bioavailability also manifested in enhanced bioactivity in vivo. In animal xenograft models, Pro-EGCG (**4**) was found to be more effective than EGCG (**1**) at equivalent dosages in inhibiting tumor growth for breast tumors[20] and for androgen-independent prostate cancer.[21]

Methylation of (−)-EGCG occurs by catechol-*O*-methyltransferase (COMT), an enzyme widely distributed throughout the body.[22] In humans, a single gene for COMT encodes both a soluble COMT (S-COMT) and a membrane-bound COMT (MB-COMT). A single nucleotide polymorphism (G to A) in codon 108 (S-COMT) or 158 (MB-COMT) results in a valine to methionine (Val to Met) substitution, leading to a high- (Val/Val [H/H]), intermediate- (Val/Met [H/L]), or low-activity (Met/Met [L/L]) form of COMT.[23] There is a three-to-four-fold difference in enzyme activity between the high- and low-activity expressed genes.[24] A recent case-control study of

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breast cancer in Asian–American women revealed that women who consumed green tea and who also carried the low activity COMT polymorphism had a reduced risk of breast cancer.[25] In contrast, among those who were homozygous for the high activity COMT allele, breast cancer risk did not differ between tea drinkers and non-tea drinkers. These data suggest that EGCG and other tea polyphenols may be less cancer-protective upon methylation. In support of this possibility, catechins were known to be substrates of human COMT.[26,27] Recently, we found that, using synthetic methylated catechins which are metabolites or potential metabolites of tea EGCG in biomethylation,[28] the proteasome inhibition potency decreased as the number of methyl groups in methylated EGCG increased.[29] Metabolic *O*-methylation of EGCG may therefore reduce the effectiveness of EGCG in its anti-cancer activity[29] in support of the human study.[25]

In this study, we wish to build on the observation that the simple analog **5** is almost as effective a proteasome inhibitor as the natural EGCG. We want to know if acetylation of **5** would enhance the cytotoxic activity of **5** just as pro-EGCG exhibited enhanced growth inhibitory activity relative to EGCG. We also want to see if the problem of metabolic O-methylation can be circumvented by using analogs of EGCG. We hypothesize that an analog such as **7**, being devoid of the *o*-catechol structure, should not be a substrate of COMT. We report here the proteasome inhibition and cytotoxic activity of **7** as well as its peracetate **8** against breast cancer MDA-MB-231 cells possessing high COMT activity. Their activities are compared with the natural EGCG (**1**) and the analogs **5** as well as their peracetates **4** and **6**, respectively.

2. Results

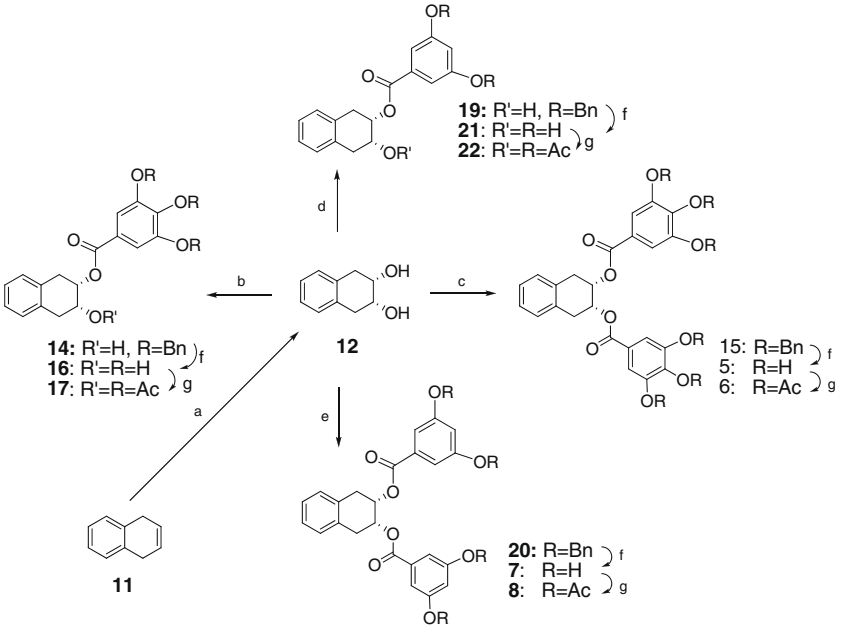
2.1. Chemical synthesis

The synthesis of compounds **5** and **7** as well as their peracetates **6** and **8** is outlined in Scheme 1. 1,4-Dihydronaphthalene (**11**) was dihydroxylated with osmium tetraoxide to give the *cis*-diol **12**. Acylation of **12** with DCC/DMAP and an equivalent of benzyl-protected gallic acid **13** gave the corresponding monobenzoate **14**. When more than 2 equiv of **13** were used, the dibenzoate **15** was obtained. Removal of the benzyl protection of **14** and **15** by palladium catalyzed hydrogenolysis gave compounds **16** and **5**, respectively. Acetylation of **16** and **5** gave the corresponding acetates **17** and **6**. Similar sequence of reactions of **12** with 3,5-dibenzyloxybenzoic acid (**18**) gave the series **7** and **21** as well as their acetates **8** and **22**.

2.2. Bioassays

We first examined the ability of the analogs to inhibit the chymotrypsin-like activity of purified 20S proteasome (Fig. 2). EGCG potently inhibited the proteasomal chymotryptic activity consistent with our previous observation.[8] As shown in Fig. 2, compound **5** which can be considered as an analog of EGCG, inhibited the proteasomal chymotrypsin-like activity with an IC_{50} value of 19 μM (Fig. 2A). Compound **16**, which can be considered as the analog of (−)-EGC, is not active even at a concentration of 50 μM. On the other hand, compound **7**, with an IC_{50} of 29 μM, is less active than EGCG or **5** (Fig. 2A). This is consistent with our previous observation that for EGCG, when the number of hydroxyl groups in the B ring or D ring is reduced, their proteasomal inhibition activity is also reduced.[15,30] Not surprisingly, compound **21** is not active in proteasome inhibition even at 50 μM. As expected, the peracetates **4**, **6**, **8**, **17** and **22** are all not active in proteasome inhibition under in vitro conditions (Fig. 2B).

To test whether COMT could affect the proteasome inhibitory effects of the two EGCG analogs **5** and **7**, we tested proteasome inhibition by compounds **5** and **7** in cells containing COMT. Human breast cancer MDA-MB-231 cell lysates that contains high COMT activity were treated with different concentrations of compound **5** or **7**. Compound **7** inhibited 18–51% proteasomal activity at 1–10 μM while compound **5** only inhibited 10–16% proteasomal activity at the same conditions (Fig. 3A). Since compound **5** was more potent in inhibiting purified 20S proteasome than compound **7** (Fig. 2) and compound **5** but not **7** contains COMT-recognizable structure (Fig. 1), these results suggested that compound **5** may be more susceptible to COMT modification than compound **7**. As a control, EGCG at 10 μM only inhibited ~22% of the chymotrypsin-like activity in these cells, also not as active as compound **7**



Scheme 1. Reagents and conditions: (a) OsO_4, NMO, acetone/H_2O; (b) 3,4,5-tris(benzyloxy)benzoic acid (1.1 equiv), DCC, DMAP, CH_2Cl_2; (c) 3,4,5-tris(benzyloxy)benzoic acid (2.1 equiv), DCC, DMAP, CH_2Cl_2; (d) 3,5-bis(benzyloxy)benzoic acid (1.1 equiv), DCC, DMAP, CH_2Cl_2; (e) 3,5-bis(benzyloxy)benzoic acid (2.1 equiv), DCC, DMAP, CH_2Cl_2; (f) Pd/C, H_2, THF/MeOH; (g) Ac_2O, Py.

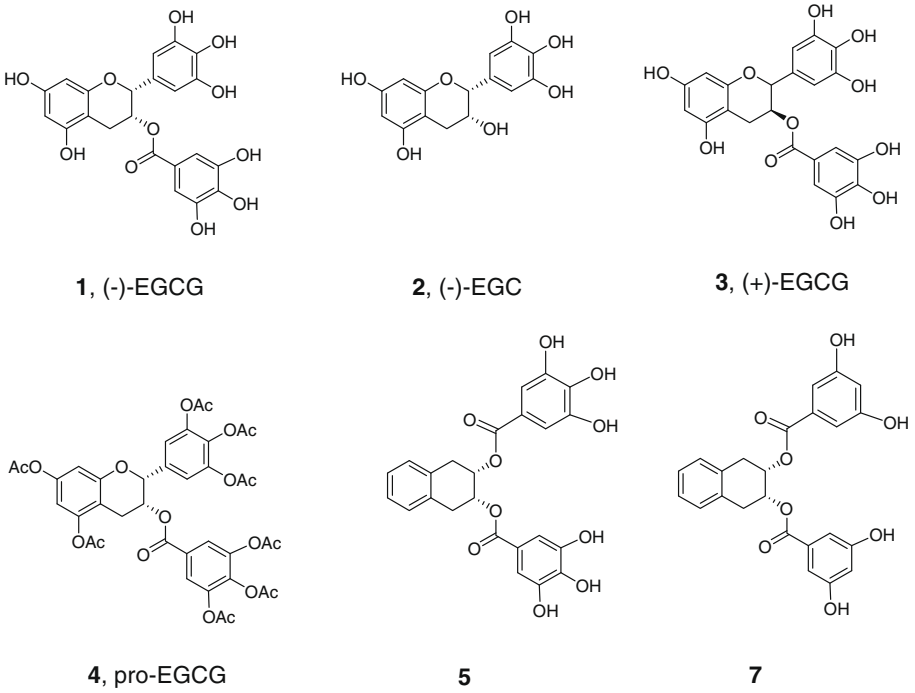


Figure 1. Structures of EGCG and analogs.

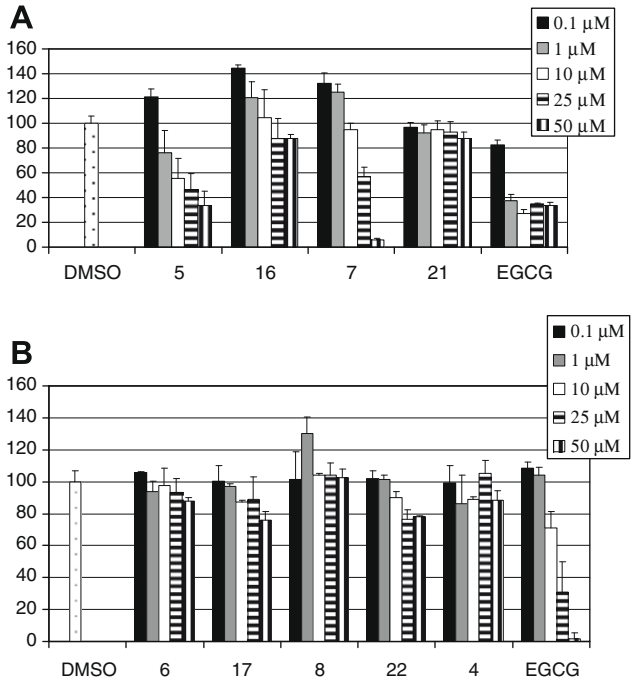


Figure 2. Proteasome inhibition by EGCG analogs. Purified 20S proteasome (35 ng) was incubated with compound **5**, **7**, **16**, or **21** (A) or MDA-MB-231 cell extract (5.7 μg) was incubated with compound **6**, **8**, **17**, or **22** (B) at indicated concentrations for 2 h, followed by measuring the proteasomal chymotrypsin-like activity. EGCG was used as a comparison.

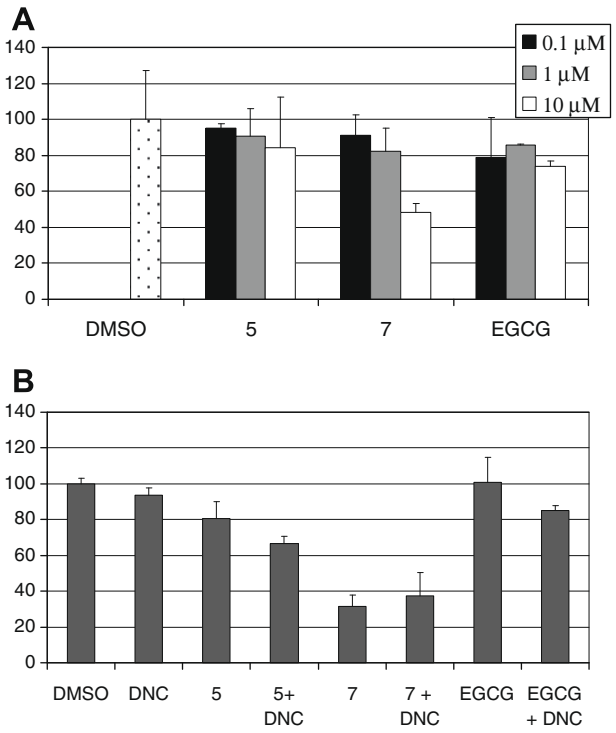


Figure 3. Inhibition on cellular proteasome by EGCG and EGCG analogs. (A) MDA-MB-231 cell extracts (5.7 μg) were incubated with different concentrations of compound **5** or **7** or EGCG for 2 h, followed by performance of proteasomal chymotrypsin-like activity assay. EGCG was used as a control. (B) MDA-MB-231 cell extracts (5.7 μg) were pre-treated with 10 μM DNC for 20 min, followed by co-incubation with **5**, or **7** or EGCG for 2 h. The proteasomal chymotrypsin-like activity was measured.

(Fig. 3A). Presumably, EGCG is also susceptible to COMT from the lysate. Indeed, compound **5** or EGCG activity was improved when the lysate was pre-treated with a COMT inhibitor, 3,5-dinitrocatechol (DNC). In contrast, compound **7**'s activity was only slightly increased at the same condition (Fig. 3B), further confirming that compound **7** is resistant to COMT modification.

It was previously reported that pro-EGCG (**4**) exhibited enhanced growth inhibitory activity relative to EGCG (**1**) in a number of cancer cell lines.[18,20,21,30] We now found that the peracetates **6**

and **8** were also more potent in inhibiting cell growth compared to their non-acetylated precursors **5** and **7** (Fig. 4). It was reasonable to assume that the peracetates **6** and **8** function similarly as pro-drugs to release **5** and **7** intracellularly after absorption. We next compared the growth inhibitory activity of **4** with the peracetates **6** and **8** on human breast cancer MDA-MB-231 cells. The results are summarized in Fig. 4. Among these pro-drugs, compound **8**, the pro-drug of compound **7**, was the most potent analog, showing 70–79% inhibition in MDA-MB-231 cells (Fig. 4) at 25–50 μM. Next to compound **8**, compound **6**, the pro-drug of compound **5** induced about 50% inhibition in MDA-MB-231 cells (Fig. 4). Both are more potent than pro-EGCG (**4**) itself which showed 0–32% inhibition at these concentrations. It is tempting therefore to suggest that the higher potency of compound **8** in inhibition of MDA-MB-231 proliferation is due to the resistance of compound **7** to COMT methylation. By contrast, EGCG, though more potent an inhibitor for purified 20S proteasome, is less potent in the inhibition of MDA-MB-231 proliferation because it is readily subject to biomethylation by the COMT in MDA-MB-231 cells.[29]

To test the validity of the above argument, we examined whether the inhibitory activity of compounds **8** (as pro-drug of **7**) or **6** (as pro-drug of **5**) as well as that of pro-EGCG (**4**) is affected by the addition of 3,5-dinitrocatechol (DNC), a tight-binding inhibitor of COMT.[37] We hypothesize that if DNC inhibits COMT-mediated methylation of compound **5** or EGCG, we would observe increased growth inhibitory activity of compound **6** or pro-EGCG (**4**) on the addition of DNC. On the other hand, the growth inhibitory activity of compound **8** would not be significantly affected by the addition of DNC as compound **7** presumably should not be a substrate of COMT. To test this idea, MDA-MB-231 cells were treated with compounds **8** or **6**, the pro-drugs of compounds **7** or **5**, in the presence or absence of DNC. Compound **6** alone at 50 μM inhibited 48% cell proliferation. With the co-addition of 10 μM DNC, compound **6** efficacy was increased, showing 88% inhibition on cell proliferation (Fig. 5). Similarly, Pro-EGCG (**4**) efficacy was greatly improved from 42% inhibition to 89% inhibition when combined with DNC. However, compound **8** efficacy was not greatly improved by co-treatment with DNC, showing 69% and 84% inhibition on cell proliferation without or with the presence of DNC. These results are consistent with the hypothesis and suggest that for the compounds which could be methylated by COMT in cells, inhibition of COMT could increase their activity by reducing biomethylation. Compared with compound **7**, compound **5** and Pro-E are more susceptible towards COMT modification.

Next, we would like to see if these pro-drugs indeed function by proteasome inhibition and if compound **8** is more potent to hit the target, the proteasome. Indeed, higher levels of ubiquitinated proteins were accumulated by compound **8** compared with compound **6** and Pro-EGCG (**4**) in MDA-MB-231 cells (Fig. 6), indicating that more proteasome activity was inhibited by compound **8**.

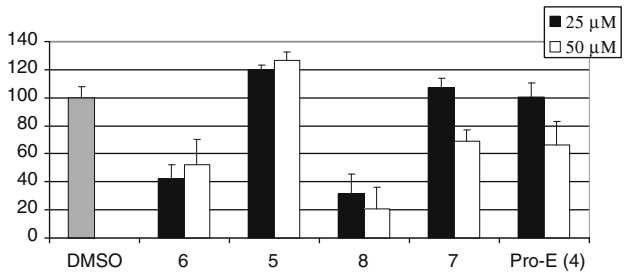


Figure 4. Inhibition on cell proliferation by EGCG analogs. Human breast cancer MDA-MB-231 cells were treated with 25 or 50 μM compound **5** or its pro-drug **6**, or compound **7** or its pro-drug **8** for 24 h, followed by MTT assay. Pro-EGCG (**4**) was used as a comparison.

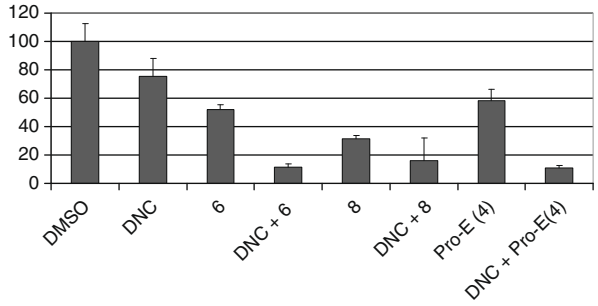


Figure 5. Effects of DNC on EGCG analogs efficacy against cell proliferation. Human breast cancer MDA-MB-231 cells with high COMT activity were treated with 50 μM EGCG analogs for 24 h in the absence or presence of 10 μM DNC, followed by MTT assay. Pro-EGCG was used as a comparison.

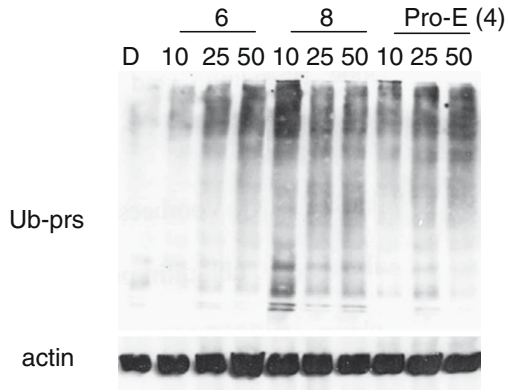


Figure 6. Accumulation of proteasome substrates. MDA-MB-231 cells were treated with 50 μM EGCG analogs for 22 h. Extracted proteins were subject to Western blotting analysis using antibodies against ubiquitinated proteins and actin.

3. Discussion

The health benefits of green tea and its main constituent (−)-EGCG have been widely supported by results from epidemiological, cell culture, and animal studies.[31] Since there are no toxic effects associated with tea drinking, the attraction of using green tea extract or EGCG as therapeutic agents is considerable. Yet, the U. S. Food and Drug Administration (FDA), after reviewing the human data, concluded in 2005 that 'it is highly unlikely that green tea reduces the risk of breast cancer or prostate cancer'.[32] A major challenge in extrapolating the biological activities of green tea polyphenols in vitro to possible benefits in vivo is its bioavailability,[33,35,36] The poor bioavailability of EGCG can be attributed to the following factors: (a) the ease of degradation of EGCG in alkaline or neutral conditions,[34] (b) poor cellular uptake because of the high aqueous solubility of EGCG and poor hydrophobicity to be absorbed by cells and (c) metabolic transformations such as methylation, glucuronidation and sulfation after absorption.[16]

In the present study, we demonstrated that a pro-drug of a simple synthetic analog **5**, the acetate **6**, enhanced the cytotoxic activity of **5** just as pro-EGCG (**4**) enhanced the growth inhibitory activity relative to EGCG (Fig. 4). An even simpler analog **7**, though not as potent an inhibitor of purified proteasome as EGCG or **5** (Fig. 2), was found to be a better proteasome inhibitor when incubated with MDA-MB-231 cell extracts (Fig. 3). Furthermore, the pro-drug of **7**, the acetate **8**, was found to be more potent in growth inhibition than either **6** or pro-EGCG (Fig. 4). In order to explain the enhanced cytotoxicity of **7** (from **8**) towards MDA-MB-231cells, we postulated that this is due to the biomethylation of **5** or EGCG by COMT in MDA-MB-231cells. We have

recently assayed the COMT genotype of various breast cancer cell lines via DNA sequencing and MDA-MB-231 was found to express the COMT-*H* allele.[37] Biomethylation of **5** or EGCG by COMT would lead to methylated metabolites. We have previously showed that the proteasome inhibition potency decreased as the number of methyl groups in methylated EGCG increased.[30] Compound **7**, in contrast, does not have the *o*-catechol structure and should not be a substrate of COMT and biomethylated. Supporting evidence for the role of COMT in reducing the proteasome inhibition activity of EGCG and **5** is provided by the addition of DNC, a known COMT inhibitor[37] (Fig. 3B). The activity of either EGCG or **5** was enhanced in the presence of DNC. By contrast, the proteasome inhibition activity of **7** was not affected by the addition of DNC. Similarly, the antiproliferative activities of pro-EGCG and **6** but not **8** are affected by co-treatment with DNC. Figure 5 showed that addition of DNC potently enhanced the antiproliferative activities of both pro-EGCG and **6** but not **8**. Inhibition of COMT by DNC restored the potencies of EGCG and **5** because they are better proteasome inhibitors than **7**.

These results suggest that COMT does play an important role in affecting the antiproliferative activities of EGCG and its analogs. This is especially critical in cells having high COMT activities, such as the MDA-MB-231 breast cancer cells. The results are also in agreement with the epidemiological study which showed that for Asian–American women who were homozygous for the high activity COMT allele, breast cancer risk did not differ between tea drinkers and non-tea drinkers.[25] Such information will have to be taken into consideration for any effort to develop green tea polyphenols or analogs into therapeutics for cancer treatment.

4. Experimental

4.1. General experimentals for chemical synthesis

The starting materials and reagents, purchased from commercial suppliers, were used without further purification. Anhydrous methylene chloride was distilled under nitrogen from CaH_2. Anhydrous DMF was distilled under vacuum from CaH_2. Reaction flasks were flame-dried under a stream of N_2. All moisture-sensitive reactions were conducted under a nitrogen atmosphere. Flash chromatography was carried out using Silica Gel 60 (70–230 mesh). The melting points were uncorrected. ^{1}H NMR and ^{13}C NMR (300 MHz) spectra were measured with TMS as an internal standard when $CDCl_3$, CD_3OD and acetone-d_6 were used as solvent. High-resolution (ESI) MS spectra were recorded using a QTOF-2 Micromass spectrometer.

4.1.1. Preparation of *cis*-1,2,3,4-tetrahydro-naphthalene-2,3-diol (12)

To a solution of 1,4-dihydronaphthalene (500 mg, 3.84 mmol) in acetone/H_2O (3.0/1.0 mL) was added a solution of NMO in H_2O (1.43 mL, 50 wt %, 6.90 mmol) and a solution of OsO_4 in 2-methyl-2-propanol (313 μL, 2.5 wt %, 25 μmol). The mixture was stirred at room temperature for 16 h. Saturated Na_2SO_3 aqueous solution (10 mL) was added and stirred for an additional 15 min. H_2O (10 mL) and EtOAc (30 mL) was added and stirred for 5 min. The aqueous phase was extracted with EtOAc (4 × 30 mL). The combined organic phase was washed with brine (20 mL) and dried over anhydrous Na_2SO_4. The solution was concentrated by rotary evaporator and vacuum drying to give the crude product which was purified by silica gel chromatography (hexane/EtOAc/CH_2Cl_2 = 5/1/1) to afford 521.7 mg (83%) of the title compound as a white solid. ^{1}H NMR (acetone-d_6, 300 MHz) δ 7.13 (m, 2H), 7.09 (m, 2H), 4.11 (t, J = 5.4, 2H), 3.01 (m, 4H), 2.36 (s, 2H); ^{13}C NMR (acetone-d_6, 75 MHz) δ 132.87, 129.11, 126.25, 69.24, 34.32.

4.1.2. Preparation of monobenzoates 14 and 19

To a solution of the corresponding benzoic acid (0.22 mmol) in dry CH_2Cl_2 (20 mL), dicyclohexylcarbodiimide (DCC, 45 mg, 0.22 mmol) was added. The resulting mixture was stirred at room temperature for 4 h, 4-dimethylaminopyridine (DMAP, 3 mg, 0.025 mmol.) was added, then a solution of diol **12** (33 mg, 0.2 mol) in CH_2Cl_2 (5 mL) was added dropwise. The mixture was stirred at room temperature overnight. Then the mixture was concentrated, EA (1 mL) was added and cooled in fridge, the urea byproduct was filtered and the filtrate was evaporated. The resulting residue was purified by column chromatography (EA/*n*-hex = 1:3) to afford the desired compound as a pale yellow amorphous solid.

4.1.2.1. Compound 14. White solid (61% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.43–7.13 (m, 22H), 5.48 (br s, 1H), 5.15 (s, 2H), 5.11 (s, 4H), 4.33 (s, 1H), 3.28–3.03 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.4, 152.7, 142.7, 137.6, 136.9, 133.3, 132.7, 129.5, 129.3, 128.9, 128.8, 128.5, 128.3, 127.8, 126.7, 126.6, 125.3, 109.4, 75.4, 73.4, 71.4, 69.8, 68.1, 35.0, 32.2.

4.1.2.2. Compound 19. White solid (65% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.44–7.13 (m, 16H), 6.82 (s, 1H), 5.50 (br s, 1H), 5.14 (s, 1H), 5.05 (s, 4H), 4.35 (s, 1H), 3.31–3.08 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.6, 160.0, 136.8, 133.4, 132.8, 132.3, 129.6, 129.3, 129.0, 128.5, 128.0, 126.7, 126.7, 108.9, 107.4, 73.7, 70.6, 68.0, 35.0, 32.1.

4.1.3. Preparation of dibenzoates 15 and 20

To a solution of **12** (33 mg, 0.2 mmol) in CH_2Cl_2 (3.0 mL) were added the corresponding benzoic acid (0.42 mmol), 4-dimethylaminopyridine (DMAP, 6 mg, 0.05 mmol) and dicyclohexylcarbodiimide (DCC, 87 mg, 0.42 mmol). The mixture was stirred at room temperature overnight. Then the mixture was concentrated, EA (1 mL) was added and cooled in fridge, the urea byproduct was filtered and the filtrate was evaporated. The resulting residue was purified by column chromatography (EA/*n*-hex = 1:6) to afford the desired compound as white amorphous solid.

4.1.3.1. Compound 15. White solid (59% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.43–7.22 (m, 38H), 5.72 (br s, 1H), 5.01–4.96 (m, 12H), 3.38 (dd, J = 17.4 Hz, J = 4.8 Hz, 1H), 3.25 (dd, J = 17.4 Hz, J = 6.9 Hz, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.7, 152.7, 142.7, 137.7, 136.7, 132.6, 129.4, 128.8, 128.7, 128.5, 128.3, 128.2, 127.8, 126.9, 125.2, 109.2, 75.3, 71.2, 70.5, 32.5.

4.1.3.2. Compound 20. White solid (71% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.42–7.18 (m, 28H), 6.74 (s, 1H), 5.72 (br s, 1H), 4.91 (m, 9H), 3.34 (m, 4H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.9, 160.0, 136.6, 132.5, 132.2, 129.4, 128.8, 128.4, 127.9, 126.8, 108.6, 107.8, 70.6, 70.4, 32.3.

4.1.4. General procedures for palladium catalyzed hydrogenolysis: preparation of 5, 7, 16 and 21

To a solution of benzylated substrate (0.1 mmol) in THF/MeOH (3 mL, 1:2) was added palladium hydroxide (20 mg, 20% on carbon). The resulting mixture was stirred at room temperature until TLC showed that the reaction was completed (within 6 h). Then the reaction mixture was filtered to remove the catalyst. The filtrate was evaporated to afford product as a white solid.

4.1.4.1. Compound 16. White solid (95% yield). ^{1}H NMR (CD_3OD, 300 MHz) δ 7.13–7.01 (m, 6H), 5.39 (m, 1H), 4.25 (m, 1H), 3.14–3.06 (m, 4H); ^{13}C NMR (CD_3OD, 75 MHz) δ 167.1, 145.2, 138.6, 133.5, 132.8, 129.0, 128.8, 126.1, 126.0, 120.6, 109.0, 72.5, 67.4,

34.4, 32.1; HRMS *m/z* calcd for $C_{17}H_{16}O_6Na$ 339.0840, found 339.0839.

4.1.4.2. Compound 5. White solid (95% yield). ^1H NMR (CD_3OD, 300 MHz) δ 7.21–7.16 (m, 4H), 7.09 (s, 4H), 5.61 (m, 2H), 3.37–3.25 (m, 4H); ^{13}C NMR (CD_3OD, 75 MHz) δ 165.3, 145.1, 138.0, 132.8, 129.0, 126.3, 120.9, 109.0, 69.7, 31.9; HRMS *m/z* calcd for $C_{24}H_{20}O_{10}Na$ 491.0947, found 491.0949.

4.1.4.3. Compound 21. White solid (95% yield). ^1H NMR (CD_3OD, 300 MHz) δ 7.14–7.07 (m, 4H), 6. 92 (s, 2H), 6.44 (s, 1H), 5.43 (m, 1H), 4.27 (m, 1H), 3.17–3.10 (m, 4H); ^{13}C NMR (CD_3OD, 75 MHz) δ 170.6, 166.0, 134.2, 133.2, 132.9, 129.3, 129.1, 126.3, 126.2, 108.2, 107.4, 73.2, 67.2, 35.0, 32.1; HRMS *m/z* calcd for $C_{17}H_{16}O_5Na$ 323.0891, found 323.0890.

4.1.4.4. Compound 7. White solid (95% yield). ^1H NMR (CD_3OD, 300 MHz) δ 7.16 (s, 4H), 6.88 (s, 4H), 6.46 (s, 2H), 5.66 (m, 2H), 3.31 (m, 4H); ^{13}C NMR (CD_3OD, 75 MHz) δ 166.3, 158.6, 132.5, 131.9, 128.9, 126.4, 107.7, 107.3, 70.4, 31.8; HRMS *m/z* calcd for $C_{24}H_{20}O_8$ Na 459.1047, found 459.1050.

4.1.5. Preparation of the acetates 6, 8, 17 and 22

To a solution of the corresponding substrate (0.1 mmol) and Ac_2O (0.5 mL) in pyridine (0.5 mL) at room temperature. The resulting mixture was stirred overnight. Then EA (50 mL) was added and 1 N HCl (1 mL) and washed with CuSO4 solution (3 × 10 mL), water (2 × 10 mL) and brine (10 mL), dried over sodium sulfate and evaporated. The residue was purified by column chromatography over silica gel (EA/*n*-hex = 3:2) to afford the title product as white solid.

4.1.5.1. Compound 17. White solid (92% yield). ^1H NMR ($CDCl_3$, 300 MHz) δ 7.74 (s, 2H), 7.21–7.11 (m, 4H), 5.64 (m, 1H), 5.42 (m, 1H), 3.26–3.16 (m, 4H), 2.30 (s, 9H), 2.07 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.9, 167.8, 166.6, 164.1, 143.7, 139.0, 132.6, 132.3, 129.3, 128.6, 126.8, 126.7, 122.5, 70.9, 69.7, 32.5, 31.7, 21.4, 20.8, 20.4; HRMS *m/z* calcd for $C_{25}H_{24}O_{10}Na$ 507.1266, found 507.1262.

4.1.5.2. Compound 6. White solid (94% yield). ^1H NMR ($CDCl_3$, 300 MHz) δ 7.73–7.70 (m, 4H), 7.22–7.14 (m, 4H), 5.68 (m, 2H), 3.31 (m, 4H), 2.28 (s, 18H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.9, 166.6, 164.1, 143.7, 139.1, 132.2, 129.4, 128.4, 126.9, 122.6, 71.1, 32.1, 20.8, 20.4; HRMS *m/z* calcd for $C_{36}H_{32}O_{16}Na$ 743.1576, found 743.1583.

4.1.5.3. Compound 22. White solid (90% yield). ^1H NMR ($CDCl_3$, 300 MHz) δ 7.60 (s, 2H), 7.21–7.11 (m, 5H), 5.64 (m, 1H), 5.44 (m, 1H), 3.26–3.17 (m, 4H), 2.31 (s, 6H), 2.08 (s, 3H),; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 170.9, 169.0, 164.6, 151.1, 132.6, 132.5, 132.3, 129.3, 126.8, 126.7, 120.7, 120.6, 70.8, 69.7, 32.4, 31.8, 21.4, 21.3; HRMS *m/z* calcd for $C_{23}H_{22}O_8Na$ 449.1201, found 449.1207.

4.1.5.4. Compound 8. White solid (91% yield). ^1H NMR ($CDCl_3$, 300 MHz) δ 7.58 (s, 4H), 7.23–7.14 (m, 6H), 5.70 (m, 2H), 3.32 (m, 4H), 2.28 (s, 12H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.0, 164.6, 151.1, 132.3, 132.2, 129.4, 126.9, 120.8, 120.6, 71.0, 32.1, 21.2; HRMS *m/z* calcd for $C_{32}H_{28}O_{12}Na$ 627.1468, found 627.1473.

4.1.6. Biological assays

4.1.6.1. Materials. Purified rabbit 20S proteasome and fluorogenic substrate Suc-LLVY-AMC for the proteasomal chymotrypsin-like (CT-like) activity were obtained from Calbiochem, Inc. (San Diego, CA). Fetal bovine serum (FBS) was from Tissue Culture Biologicals (Tulare, CA). Penicillin and streptomycin were purchased from Invitrogen Co. (Carlsbad, CA). RPMI 1640 medium was purchased from Invitrogen Co. (Carlsbad, CA). MTT (3-4, 5-dimethyltiazol-2-yl-2.5-diphenyl-tetrazolium bromide) was purchased from Sigma-Aldrich.

4.1.6.2. Cell culture. Human breast cancer MDA-MB-231 cells were purchased from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium supplemented with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cells were maintained at 37 °C and 5% CO_2.

4.1.6.3. Inhibition of purified 20S proteasome activity by EGCG and its analogs. A purified rabbit 20S proteasome (35 ng) was incubated with 20 μM of substrate Suc-LLVY-AMC in 100 μL assay buffer (20 mM Tris–HCl, pH 7.5), in the presence of EGCG or EGCG analogs at different concentrations or the solvent for 2 h at 37 °C, followed by measurement of hydrolysis of the fluorogenic substrates using a Wallac Victor3™ multi-label counter with 355-nm excitation and 460-nm emission wavelengths.

4.1.6.4. Inhibition of 26S proteasome activity by EGCG and its analogs in vitro. Human breast cancer MDA-MB-231 cell extract (5.7 μg) was incubated in 20 μM of substrate Suc-LLVY-AMC in 100 μL assay buffer (20 mM Tris–HCl, pH 7.5) in the presence of EGCG or EGCG analogs at different concentrations or the solvent for 2 h at 37 °C, followed by measurement of hydrolysis of the fluorogenic substrates as described above.

4.1.6.5. Inhibition of cellular 26S proteasome by EGCG and its analogs. Human breast cancer MDA-MB-231 cells were treated with compound **5** or **7** for 24 h. Cell lysates were subjected to chymotrypsin activity assay and Western blotting analysis as described before.[20]

4.1.6.6. MTT assay. Cells were grown in a 96-well plate. Triplicate wells of cells were treated with indicated concentrations of EGCG or EGCG analogs for 24 h. After aspiration of medium, MTT (1 mg/mL) was then added to the cell cultures, followed by incubation for 3 h at 37 °C. After cells were crystallized, MTT was removed and DMSO was added to dissolve the metabolized MTT product. The absorbance was then measured on a Wallac Victor3 1420 Multi-label counter at 540 nm.

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