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Antitumor Activity of Novel Fluoro-Substituted (–)-epigallocatechin-3-gallate Analogs

Huanjie Yang¹, Dong Kui Sun², Di Chen¹, Qiuzhi Cindy Cui¹, Yan Yan Gu², Tao Jiang², Wei Chen³, Sheng Biao Wan^{2,*}, and Q. Ping Dou^{1,*}

¹ The Prevention Program, Barbara Ann Karmanos Cancer Institute, and Department of Pathology, School of Medicine, Wayne State University, Detroit, Michigan, USA

² Key Laboratory of Marine Drug, Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, Qingdao, China

³ Biostatistics Core, Karmanos Cancer Institute, and Department of Internal Medicine, Wayne State University, Detroit, Michigan, USA

Abstract

Epidemiological studies support the cancer-preventive effects of green tea and its main constituent (–)-epigallocatechin gallate [(–)-EGCG], however, (–)-EGCG is unstable under physiological conditions. Here we report that two novel fluoro-substituted (–)-EGCG analogs inhibited tumor growth with similar potency to that of Pro-EGCG (1) which has improved potency over parental compound (–)-EGCG in human breast cancer MDA-MB-231 xenografts. MDA-MB-231 tumors treated with each fluoro-substituted (–)-EGCG analog showed proteasome inhibition and apoptotic cell death, suggesting that the proteasome might be one of the cellular targets of fluoro-(–)-EGCGs and that proteasome inhibition is partially responsible for the observed antitumor activity.

Keywords

Breast cancer; proteasome inhibitor; drug discovery; cancer prevention; tea polyphenol

1. Introduction

Green tea is widely consumed throughout the world, and is abundant in polyphenols, including (–)-epigallocatechin gallate (EGCG), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epicatechin (EC) [1]. The molecular mechanisms responsible for the cancer preventive and anticancer activities of (–)-EGCG, the major tea polyphenol with the most bioactive property, have been extensively studied and the involved multiple molecular

*Corresponding authors: Tel: (313)-576-8301 (Dou QP); 86-532-8203-2712 (Wan SB)., Fax: (313)-576-8307 (Dou QP); 86-532-82033054 (Wan SB)., doup@karmanos.org (Dou QP); biao@ouc.edu.cn (Wan SB).

Conflict of Interest

We confirmed that there is no potential conflict of interest regarding submission and publication of our manuscript entitled “The Antitumor Activity of Novel Fluoro-Substituted (–)-epigallocatechin-3-gallate Analogs”. All the authors have read and approved for submission to *Cancer Letters*.

Conflict of Interest

All the authors confirm that there is no potential conflict of interest regarding this publication.

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pathways have been identified, including inhibition of mitogen activated protein kinases (MAPKs), inhibition of growth factor signaling, suppression of nuclear factor- κ B (NF- κ B) pathway, and inhibition of the proteasome [2,3].

Clinical trials with prostate cancer patients demonstrated that green tea consumption produced promising effects against cancer development without inducing major toxicities [4,5]. However, data obtained from chemotherapeutic application of green tea extracts in clinic as treatment of established solid tumors are not impressive, with limited efficacy observed [6–8], suggesting that (–)-EGCG could not reach high concentration and potency at tumor sites. Under physiological conditions (–)-EGCG could be rapidly metabolized after absorbance by human body [9]. Furthermore, the hydroxyl groups of (–)-EGCG could be modified through biotransformation reactions, including methylation, glucuronidation, and sulfate formation, resulting in reduced biological activities of (–)-EGCG [10,11].

With the purpose of improving the stability and potency of (–)-EGCG, we synthesized the prodrug of (–)-EGCG, Pro-EGCG (1), with all the reactive hydroxyl groups protected by peracetate groups. We showed that Pro-EGCG (1) had improved potency to induce apoptosis in human breast, prostate and leukemic cancer compared to (–)-EGCG [12,13]. In order to discover new potent tea polyphenol analogs as proteasome inhibitors, we have designed and synthesized several novel fluoro-substituted (–)-EGCG analogues with eliminating –OH groups from D-ring and replaced with one or two fluorine(s), named F-EGCGs [14]. Our *in vitro* screening indicates that prodrug of fluoro-substituted (–)-EGCG at meta-position on the phenyl ring (Pro-F-EGCG2, Fig. 1A) or difluoro-substituted (–)-EGCG at both meta- and para-positions on the phenyl ring (Pro-F-EGCG4, Fig. 1A) had similar or even more potency as Pro-EGCG (1) (Fig. 1A) to induce apoptosis in cultured human breast cancer cells [14]. In the current study, we investigated the *in vivo* effects of these fluoro-substituted (–)-EGCG analogs in terms of their antitumor and proteasome-inhibitory activities using breast cancer xenograft model.

2. Materials and Methods

2.1. Materials

Cremophor and DMSO were purchased from Sigma-Aldrich. RPMI 1640, fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen. The fluorogenic peptide substrate Suc-LLVY-AMC (for the proteasomal chymotrypsin-like) was from Calbiochem. Mouse monoclonal antibody against human poly(ADP-ribose) polymerase (PARP) was purchased from BIOMOL International LP. Mouse monoclonal antibodies against Bax (B-9), p27 (F-8) and ubiquitin (P4D1), goat polyclonal antibodies against actin (C-11), and secondary antibodies were from Santa Cruz Biotechnology, Inc.

2.2. Synthesis of Pro-F-EGCG2 and Pro-F-EGCG4

Pro-F-EGCG2 and Pro-F-EGCG4 were semi-synthesized from (–)-EGCG as described [14].

2.3. Establishment of human breast tumor xenografts

Female athymic nude mice aged 5-week were purchased from Taconic Research Animal Services and housed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of Wayne State University. Human breast cancer MDA-MB-231 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified incubator with 5% CO₂. MDA-MB-231 cells (5×10^6) suspended in 0.1 mL of serum-free RPMI 1640 were inoculated *s.c.* in the right flank of each mouse. When tumors reached a size of ~ 120 mm³, the mice were randomly grouped and treated by daily *s.c.* injection with 50 mg/kg of Pro-F-EGCG2

or Pro-F-EGCG4 (nine mice each group). Vehicle [20% DMSO and 80% cremophor/ethanol (3:1)] (nine mice) and same dose of Pro-EGCG (1) (six mice) treatment were used as control [13]. Tumor size was measured every other day using calipers and their volumes were calculated according to a standard formula: $\text{width}^2 \times \text{length} / 2$. Mice were sacrificed after 31 days of treatment when control tumors reached $\sim 1,500 \text{ mm}^3$. The tumors were weighed and tumor tissues were collected for different assays.

2.4. Terminal nucleotidyl transferase-mediated nick end labeling (TUNEL), immunostaining of p27 and Hematoxylin and Eosin (H & E) assays using tumor tissues

Terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay using *in situ* apoptosis detection kit and immunostaining of p27 were done as previously described [15]. H & E staining in tumor tissues was performed following the manufacturer's protocols.

2.5. Proteasome activity assay

Whole tissue extracts (10 μg) from MDA-MB-231 tumors were incubated with Suc-Leu-Leu-Val-Tyr-AMC (20 $\mu\text{mol/L}$) fluorogenic substrate at 37°C in 100 μL of assay buffer (50 mmol/L Tris-HCl, pH 8.0) for 2 h. After incubation, production of hydrolyzed 7-amino-4-methylcoumarin (AMC) groups was measured using a Victor³ Multilabel Counter with an excitation filter of 380 nm and an emission filter of 460 nm (Perkin-Elmer).

2.6. Western blot analysis

Tumor tissue extracts (40 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, followed by visualization using the enhanced chemiluminescence kit (Amersham Biosciences) as previously described [16].

2.7. Statistical analysis

ANOVA was applied to analyze the differences between multiple groups, followed by pairwise Student's *t* test to compare the difference between each two groups. $p < 0.05$ was set up as difference and $p < 0.01$ was set up as significant difference.

3. Results

3.1. Synthetic fluoro-substituted EGCG analogs inhibit the growth of human breast cancer xenografts

To investigate the antitumor effect of the novel EGCG analogs, Pro-F-EGCG2 and Pro-F-EGCG4 (Fig. 1A) [14], human breast cancer MDA-MB-231 xenografts were established in 5-week-old female athymic nude mice. When the tumors reached a palpable size ($\sim 120 \text{ mm}^3$), the mice were *s.c.* daily treated with either the vehicle control or 50 mg/kg of Pro-F-EGCG2, Pro-F-EGCG4 or Pro-EGCG (1) as a comparison. At the end of 31 days, control tumors grew to an average size of $1,620.6 \pm 562.6 \text{ mg}$ (Fig. 1B). Pro-EGCG (1)-treated tumors grew to $597.9 \pm 603.1 \text{ mg}$, showing 63% tumor growth inhibition ($p = 0.015$) which is similar to our previous report (54% tumor growth inhibition) [13]. Similarly, Pro-F-EGCG4 treated tumors grew to $537.4 \pm 706.7 \text{ mg}$ (Fig. 1B), showing $\sim 67\%$ tumor growth inhibition which is statistically different from control treatment ($p = 0.03$). Pro-F-EGCG2 treated tumors grew to $480.5 \pm 517.6 \text{ mg}$, showing $\sim 70\%$ tumor growth inhibition which is significantly different from vehicle treatment ($p < 0.001$). No statistic differences were obtained when compare Pro-EGCG (1) with either of these two fluoro-substituted EGCG analogs or compare Pro-F-EGCG2 with Pro-F-EGCG4 ($p > 0.3$). These results indicate that the prodrug of fluoro-substituted (–)-EGCG analog, Pro-F-EGCG2 and Pro-F-EGCG4 are similar to Pro-EGCG (1) on tumor growth inhibition in MDA-MB-231 breast tumor model. The mice showed endurable to 50 mg/kg/day

Pro-F-EGCG treatment during the 31-day period. No weight loss and decreased activity were observed in both Pro-F-EGCG2 and Pro-F-EGCG4 treated animals.

3.2. Fluoro- substituted EGCG analogs induce apoptosis in breast tumor xenograft

These two novel prodrugs of fluoro-substituted EGCG induced apoptosis *in vitro* [14]. To determine whether they can reach the tumor cells and induce apoptosis *in vivo*, multiple assays were performed using tumor tissue samples. PARP cleavage, an indicator of apoptosis [17] was detected to the greater extent in the extracts of tumors treated with Pro-F-EGCG2 or Pro-F-EGCG4 compared to that treated with vehicle (Fig. 2A). Furthermore, apoptotic cells, as indicated by TUNEL positivity were observed in tumors from animals treated with Pro-F-EGCG2 or Pro-F-EGCG4 (Fig. 2B). Apoptotic nuclei, as shown by nuclear condensation using H&E staining were observed in tumors from animals treated with Pro-F-EGCG2 or Pro-F-EGCG4 (Fig. 2B). In comparison, control tumors were observed in good status of cell proliferation as shown by multiple nuclei, mitotic chromosomes (Fig. 2B). Similar results were observed in multiple tumor samples of each group.

3.3 Fluoro- substituted EGCG analogs inhibit the proteasome in tumor tissues

Whether Pro-F-EGCG2 or Pro-F-EGCG4 targets the proteasome *in vivo* as we observed *in vitro* [14] was determined by measurement of the proteasome activity in the extracts of tumor tissues. As shown in Fig. 3A, both fluoro-substituted (–)-EGCGs inhibited the chymotryptic activity of the proteasome (Fig. 3A). In this experiment, the proteasomal chymotrypsin-like activity was inhibited by 42% or 33% in the tumor treated with Pro-F-EGCG2 or Pro-F-EGCG4, respectively, compared with control vehicle treatment (Fig. 3A). Inhibition of the proteasome should result in accumulation of ubiquitinated proteins and the proteasome substrates [18,19]. Indeed, the level of ubiquitinated proteins was increased in both Pro-F-EGCG2- and Pro-F-EGCG4-treated tumors with comparison to control tumors (Fig. 3B). Consistently, an ubiquitinated form of p27 [20] was also increased in tumors treated with Pro-F-EGCG2 and Pro-F-EGCG4 (Fig. 3B, indicated by an arrow). The proteasome target proteins p27 and Bax were also increased in the tumors after both treatments compared to that with vehicle treatment (Fig. 3B). As shown in Fig. 3B, two forms of Bax protein were detected, Bax/p18 and Bax/p21, both of which were increased by Pro-F-EGCG2- or Pro-F-EGCG4 treatment (Fig. 3B). In addition, extensive expression of p27 protein was detected in the sections from Pro-F-EGCG2 or Pro-F-EGCG4 treated tumors by immunohistochemistry (Fig. 3C). In comparison, much less p27 immuno-positive cells were observed in vehicle treated tissues (Fig. 3C). Therefore, treatment by Pro-F-EGCG analogs potentially inhibited tumor proteasome activity *in vivo*.

In summary, our results indicate that the prodrugs of fluoro-substituted EGCG analogs induced proteasome inhibition and apoptosis induction *in vivo*, as observed *in vitro* [14], and similar to Pro-EGCG (1), the prodrugs of fluoro-substituted (–)-EGCG analogs possessed potent anti-breast tumor activity *in vivo*.

4. Discussion

The proteasome inhibitor Bortezomib has been used as a treatment for hematological malignancies in clinical trials [21], supporting that targeting the proteasome is an effective strategy. However frequent toxic side effects associated with bortezomib treatment were observed [21]. Furthermore Bortezomib showed limited effects against solid tumors [21], which encourages the researchers to develop novel proteasome inhibitors for the treatment of solid tumors including breast cancer.

(-)-EGCG, the main component of green tea is a potent proteasome inhibitor [22]. It inhibited the purified 20S proteasome with an IC_{50} value of 0.2–0.6 $\mu\text{mol/L}$. However it always needs high concentration to inhibit the cellular proteasome, suggesting that the cellular potency of (-)-EGCG was reduced. Indeed, (-)-EGCG is subject to rapid degradation and several biological modifications, resulting in decreased activity of (-)-EGCG [23]. Compared to (-)-EGCG, its prodrug, Pro-EGCG (1) with peracetate protection at all reactive hydroxyl groups of phenol rings showed increased antitumor activity associated with increased activity on inhibition of the proteasome and induction of apoptosis [13].

In this study, we tested the antitumor activity of two novel synthesized prodrugs of fluoro-substituted (-)-EGCG. We notice that although Pro-F-EGCG2 and Pro-F-EGCG4 showed potent antitumor activity in most of treated mice, one mouse in the group treated with either Pro-F-EGCG2 or Pro-F-EGCG4 grew a tumor comparable to control, suggesting that there was individual variation in response to fluoro-substituted (-)-EGCGs treatment. Despite of this, statistical analysis suggest that fluoro-substituted (-)-EGCG analogs significantly inhibited breast tumor growth, similar to Pro-EGCG (1) (Fig. 1).

At the tested dose (50 mg/kg/d), both mono and difluoro-substituted EGCG analogs reached an effective concentration at which they induced apoptosis in tumors evidenced by PARP cleavage, TUNEL positivity and condensed nuclei (Fig. 2) and inhibit the cellular proteasome as shown by decreased level of proteasomal chymotryptic activity and increased levels of proteasomal substrates such as p27, Bax as well as ubiquitinated proteins (Fig. 3).

The prodrugs of fluoro-substituted (-)-EGCG induced ~67–70% inhibition on tumor growth (Fig. 1). However, they caused 33–42% inhibition on proteasomal chymotryptic activity (Fig. 3), suggesting that the proteasome might be only one of the cellular targets of fluoro-substituted (-)-EGCG prodrugs and that proteasome inhibition might be partially responsible for tumor growth inhibition. Other molecules involved in the observed tumor growth inhibition need to be discovered in the future.

In our nude mice model, no weight loss and decreased activity were observed in fluoro-substituted (-)-EGCG-treated mice during the 31-day period of treatment, suggesting that fluoro-substituted (-)-EGCG is tolerate in mice model.

Taken together, our *in vivo* observation in nude mice is consistent with our *in vitro* results, showing that the prodrugs of fluoro-substituted EGCG are potent to inhibit breast cancer growth at the dose tolerate to mice, which is partially associated with proteasome inhibition in tumor tissue. Whether fluoro-substituted (-)-EGCG and their prodrugs can be biotransformed will be determined in the future.

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A

B

Figure 1. Antitumor effects of fluoro-substituted EGCG analogs

A, Chemical structures of two novel prodrugs of fluoro-substituted EGCG. The chemical structures of Pro-F-EGCG2, Pro-F-EGCG4 and peracetate-protected EGCG [(Pro-EGCG (1))] were shown. B, Inhibition of MDA-MB-231 tumor growth. Nude mice were *s.c.* inoculated with human breast cancer MDA-MB-231 cells (5×10^6). When the tumors were detectable, nude mice were *s.c.* daily treated with either drug vehicle (n=9), or 50 mg/kg Pro-F-EGCG2 (Pro-F2) (n=9), Pro-F-EGCG4 (Pro-F4) (n=9) or control peracetate-protected EGCG (Pro-E) (n=6), as described in **Materials and Methods**. By the end of 31 days treatment, mice were euthanized and tumors were weighed. *Points*, mean of tumor weights; *bars*, SE. **, $p < 0.01$; *, $p < 0.05$.

Figure 2. Cell death induction by prodrugs of fluoro-substituted EGCGs in MDA-MB-231 tumors Tumor tissues were treated with vehicle (V) or Pro-F-EGCG2 (Pro-F2) or Pro-F-EGCG4 (Pro-F4). Proteins were extracted from tumor tissues, followed by Western blotting analysis using antibody against PARP (A). The full length of PARP is 116 kDa, while the cleaved form of PARP is 65 kDa. B, tumor tissues were sectioned, followed by TUNEL assay and H&E staining. Pro-F-EGCGs treated tumors showed TUNEL positive cells with brown color and condensed nuclei in H&E staining. Magnifications, $\times 400$.

Figure 3. Inhibition of the proteasome by prodrugs of fluoro-substituted EGCG in MDA-MB-231 tumors

Tumors were treated with vehicle (V) or Pro-F-EGCG2 (Pro-F2) or Pro-F-EGCG4 (Pro-F4) as described in Figure 1 and tissues were used. A, Proteasomal chymotrypsin-like activity assay. Proteins were extracted from tumor tissues and subject to proteasome activity assay using Suc-LLVY substrate for chymotrypsin-like activity, as described in **Materials and Methods**. Protein level of ubiquitinated protein and proteasome targets p27 and Bax were analyzed by Western blotting (B). An ubiquitinated form of p27 (70 kDa) was indicated by an arrow. Bax, both p21 and p18 forms were shown. Actin was used as loading control. C,

Immunohistochemistry with p27 antibody. Tumor tissues were sectioned for immunohistochemistry using p27 antibody. Magnifications, $\times 400$.