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(-)-Epigallocatechin-3-gallate enhances anti-tumor effect of cytosine arabinoside on HL-60 cells¹

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KEY WORDS (-)-epigallocatechin-3-gallate; cytosine arabinoside; cell division; cell cycle; calcium; phytoantic agents; combined antineoplastic agents; Western blotting; flow cytometry

ABSTRACT

AIM: To study the potentiation of anti-tumor effect induced by cytosine arabinoside (AraC) with (-)-epigallocatechin-3-gallate (EGCG). **METHODS:** Growth curve method and MTT assay were used to measure the cytotoxic effect of AraC alone or in combination with EGCG on HL-60 cells. Flow cytometry was used to study the cell cycle distribution of HL-60 cells. Nullification assay was used to examine whether EGCG would nullify the rescue effect of deoxycytidine (dCdR) to AraC. Western blot analysis was employed to investigate bcl-2 expression. Intracellular Ca²⁺ assay was evaluated. **RESULTS:** Inhibition of HL-60 cell proliferation induced by AraC was enhanced by EGCG, with multiplication time prolonging from 48 h to 70 h and growth saturation density decreasing from 5.78 to 5.54. The MTT results indicated that IC₅₀ was decreased from (0.34±0.29) μmol/L (AraC alone) to (0.11±0.09) μmol/L (*P*<0.05) (in combination with EGCG). Cell cycle analysis indicated that AraC blocked HL-60 cells in G₁ phase, inhibited cells in S phase. EGCG had no effect on cell cycle at the current concentration, but enhanced the cell arrest by AraC. Nullification assay indicated that IC₅₀ was 0.03 μmol/L (AraC alone), increased to 0.02 mmol/L when rescued with dCdR, and finally decreased to 4.8 μmol/L when addition with EGCG. The expression of bcl-2 protein was down-regulated after treatment with AraC in combination with EGCG. The intracellular Ca²⁺ was increased after treatment by AraC in combination with EGCG. **CONCLUSION:** The combination with EGCG could enhance the anti-tumor effect of AraC on HL-60 cells.

INTRODUCTION

Intense interest in biochemical modulators has increased in tumor chemotherapy in recent years. Bio-

chemical modulators could enhance the anti-tumor activity and reduce the side effects of anti-tumor agents by acting on specific link or molecular target in biochemical metabolism^[1]. Based on our systematic research for more than 10 years, we advanced nucleoside transport as a new tactic of anticancer drug research, and have selected many biochemical modulators from the traditional Chinese medicines and other natural products. Green tea polyphenols, salvianolic acid A, antibiotic C3368-a, antibiotic C3368-b, and cinnamamide represent our research achievements^[2-6].

¹ Project supported by the National Key Basic Research Development Program (973), No G1998051212.

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Received 2002-09-16

Accepted 2002-10-22

In our previous studies we had found that green tea polyphenols inhibited nucleoside transport in tumor cells, enhanced cytotoxic activity of AraC and methotrexate on tumor cells, and potentiated the efficacy of AraC on leukemia L1210 and P388 in mouse^[2-3]. Green tea polyphenol is a multi-components extract containing catechins, 80 % of catechins is (-)-epigallocatechin-3-gallate (EGCG)^[7]. To explore the potential application of green tea polyphenols in the tumor chemotherapy, we investigated the modulative effect of EGCG, a unitary component, on anti-tumor activity of AraC on HL-60 cells.

MATERIALS AND METHODS

Drugs and chemicals EGCG was a kind gift from Prof CHENG Shu-Jun (Cancer Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). AraC injection, 50 mg/vial, was purchased from Shanghai Hua-Lian Pharmaceutical Cooperation (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), DNase were purchased from Sigma Chemical Co. Antibody of bcl-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). RPMI-1640 was purchased from GIBCO BRL (Grand Island, NY). EGCG was dissolved in serum-free RPMI-1640 just before use. AraC was first dissolved in sterile isotonic saline and diluted in serum-free RPMI-1640 just before use. The other chemicals used in this study were of highest purity available.

Cell culture Human acute promyelocytic cell line HL-60 was cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS), 2 mmol/L glutamine, penicillin (100 kU/L) and streptomycin (100 mg/L) at 37 °C in an incubator in a humidified atmosphere, with 5 % CO₂ in air. The cells were routinely passaged every 2 or 3 d.

Cell proliferation Briefly, 1×10⁸/L cells in the logarithmic growth phase were harvested and seeded in 25-cm² NUNC flasks (Denmark) overnight. The drugs were added and the cells were further incubated for various periods of time. At the end of incubation, cell viability was determined by the Trypan blue dye exclusion method. Growth curve was drawn according to the logarithmic number of cells/L with the incubation time. The multiplication time and growth saturation density were calculated based on the curve.

MTT assay Briefly, cells in the logarithmic growth

phase were harvested and seeded in 96-well plates (Costar, Cambridge, MA) overnight. The cell number was diluted to 5000/well. The drugs were added and cells were further incubated for 72 h. The cell population was determined using MTT according to the method described by Carmichael *et al*^[8]. Combination drug index (CDI) was used to evaluate whether the combination of drug A and drug B was synergistic or not, which was calculated according to the formula^[9]:

$$CDI = \frac{\text{Survival \% (drug A+drug B)}}{\text{Survival \% (drug A)} \times \text{Survival \% (drug B)}}$$

The value of CDI ≤1 meant synergistic drug interaction.

Flow cytometry Cell harvest and seeding were the same as described above. After the appropriate treatment, cells were harvested by centrifugation and washed with phosphate buffered saline (PBS). The cells were fixed with ice-cold 75 % ethanol at 4 °C for 18 h. The fixed cell suspensions were washed with PBS, and then treated with 800 μL 50 mg/L PI dye and 50 mg/L RNase for 30 min in the dark. Samples were run through a FACScan (ELITE). Results were presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence.

Nullification assay Briefly, cells in the logarithmic growth phase were harvested and seeded in 24-well plates (Costar, Cambridge, MA) overnight. The cell number was diluted to 1×10⁵/well. AraC alone, or in combination with dCdR, or further plus EGCG were added and cells were further incubated for 72 h. The cell viability was determined by the Trypan blue dye exclusion method. Cell viability IC₅₀ was calculated.

Western blot analysis The cells were lysed in lysis buffer at 4 °C with sonication. The lysates were centrifuged at 15000×g for 15 min and the concentration of the protein in each lysate was determined with Coomassie brilliant blue G-250. Loading buffer was added to each lysate, which was subsequently boiled for 3 min and then electrophoresed on a SDS-PAGE. Proteins were transferred to nitrocellulose and incubated with anti-bcl-2 antibody and then with peroxidase-conjugated secondary antibody in the second reaction. Detection was performed with enhanced chemiluminescence agent. The results on Western blot analysis represented the average of the three individual experiments.

Intracellular Ca²⁺ assay Briefly, the cells were

collected by centrifugation, washed with Hanks' solution and re-suspended with DMEM. The cell suspension was added with Fura-2 AM and loaded for 45 min in 37 °C water bath. The cells were washed with Hanks' solution twice and entered alternative time-scan assay to determine the intracellular Ca²⁺ concentration at the condition of excitation wavelength A340 nm, B380 nm and emission wavelength 500 nm. The intracellular Ca²⁺ concentration was calculated according to the following formula^[10]:

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}}$$

Statistical analysis The data were mean values of experiments for at least 3 times and expressed as mean±SD. The student's *t*-test was used to compare data. *P*<0.05 was considered to be statistically significant.

RESULTS

Effect of EGCG on AraC-induced inhibition of cell proliferation The effect of EGCG on AraC-induced inhibition of cell proliferation on HL-60 cells was shown in Fig 1.

The HL-60 cell growth curve appeared linear from d 1 to d 5. The mortalities of four groups were 0 % (control), 9.9 % (EGCG 20 μmol/L alone), 32.8 % (AraC 0.20 μmol/L alone), and 43.6 % (AraC 0.20 μmol/L in combination with EGCG 20 μmol/L), respectively. The

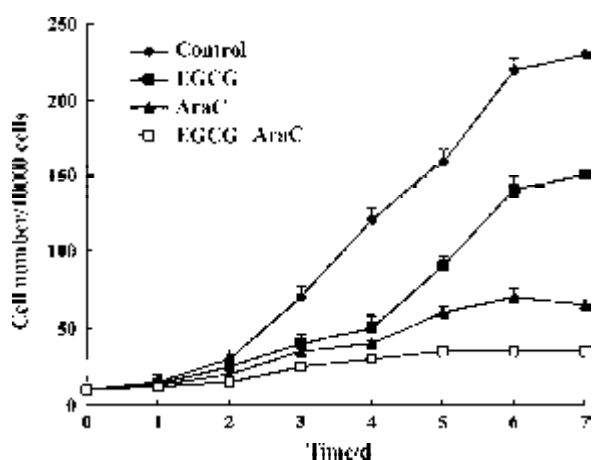


Fig 1. HL-60 cell growth curves by different treatments. Control: RPMI-1640; EGCG: 20 mmol/L; AraC: 0.20 mmol/L; EGCG+AraC: EGCG 20 mmol/L+AraC 0.20 mmol/L. *n*=3. Mean±SD.

multiplication time of 4 groups was 30 h (control), 37 h (EGCG alone), 48 h (AraC alone), and 70 h (AraC+EGCG) respectively, based on the cell number of d 5. The growth saturation density of 4 groups was 6.35 (control), 6.17 (EGCG alone), 5.78 (AraC alone), and 5.54 (AraC+EGCG) respectively. The results indicated that AraC 0.2 μmol/L had stronger cytotoxic activity in combination with EGCG, prolonged the multiplication time, and weakened the cell proliferation ability.

Effect of EGCG on AraC-induced cytotoxic activity The effect of EGCG on AraC-induced cytotoxic activity on HL-60 cells was shown in Tab 1.

Tab 1. Inhibitory percentage (%) of HL-60 cell proliferation induced by AraC alone or in combination with EGCG (20 mmol/L). *n*=3. Mean±SD.

AraC concentration/μmol·L ⁻¹	AraC alone	AraC+EGCG	Combination Drug Index
0.80	74±17	80±7	1.15
0.40	39±45	73±11	0.67
0.20	22±42	55±27	0.87
0.10	11±20	40±20	1.01
0	0	33±5	

The cytotoxic activity of AraC at different concentrations was enhanced in combination with EGCG, compared with AraC alone. Synergism was shown between AraC 0.40 μmol/L and 0.20 μmol/L in combination with EGCG 20 μmol/L respectively, because CDI was less than 1. IC₅₀ of AraC in combination with EGCG was (0.11±0.09) μmol/L, decreasing from (0.34±0.29) μmol/L (AraC alone) (*P*<0.05).

Cell cycle distribution HL-60 cells treated with EGCG 20 μmol/L demonstrated a normal distribution pattern. The cells treated with AraC 0.20 μmol/L produced G₁ arrest and blocked the cells in S phase. Furthermore 0.20 μmol/L AraC-induced G₁ arrest, S inhibition, and apoptosis were enhanced in the presence of EGCG 20 μmol/L. (Tab 2)

Nullification assay Nullification assay results were shown in Fig 2. The results showed that AraC more than 0.20 μmol/L almost inhibited cell proliferation of HL-60 cells. dCdR 10 mg/L could reverse the inhibition by AraC markedly. However, EGCG 20 μmol/L nullified the reversal of dCdR to a certain degree, which indicated that EGCG enhanced the cytotoxic

Tab 2. Comparison of cell cycle distribution of HL-60 cells treated with AraC 0.20 mmol/L alone or in combination with EGCG 20 mmol/L at different time points.

Group	Time (h)	G ₁ (%)	G ₂ /M (%)	S (%)	Sub-G ₁ (%)
Control		30.4	10.7	59.0	
AraC	3	46.8	6.9	46.5	3.4
	6	50.6	6.5	43.0	5.1
	12	52.0	4.7	43.4	7.9
	24	47.9	12.8	39.4	30.5
EGCG	3	30.5	16.7	52.8	
	6	32.2	15.2	52.6	
	12	37.7	20.7	41.7	8.1
	24	26.1	15.8	58.1	6.8
AraC+EGCG	3	42.0	12.5	45.5	13.5
	6	45.8	11.9	42.3	16.1
	12	46.6	8.7	44.8	22.4
	24	53.5	18.9	27.7	40.5

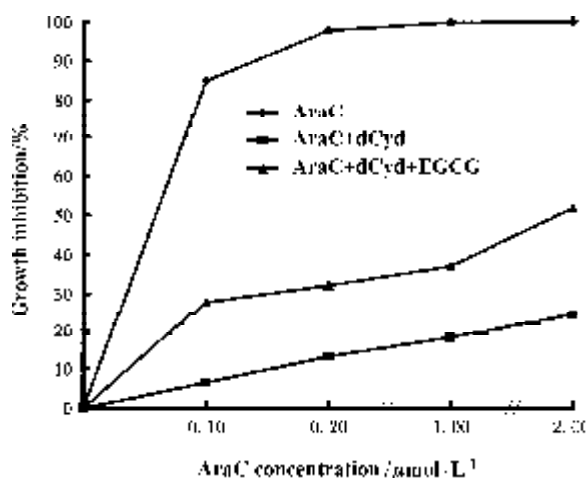


Fig 2. EGCG nullified the reversal of dCyd on AraC anti-tumor effect. The concentrations of dCyd and EGCG were 20 mmol/L.

activity of AraC by nullifying the rescue effect of dCdR. IC₅₀ of AraC (alone) in nullification assay was (0.03±0.01) μmol/L, increasing to (0.02±0.01) mmol/L when dCdR was added, and finally reducing to (4.8±4.0) μmol/L when EGCG was added.

Effect of EGCG and AraC on the expression of bcl-2 protein The bands were scanned with light density. Bcl-2 expression after treatment with EGCG 20 μmol/L was almost the same as that of the control. After treatment with AraC 0.20 μmol/L bcl-2 expression was 71.3 % of the control. Furthermore the bcl-2

expression after treatment with AraC+EGCG was 14 % of the control. (Fig 3)

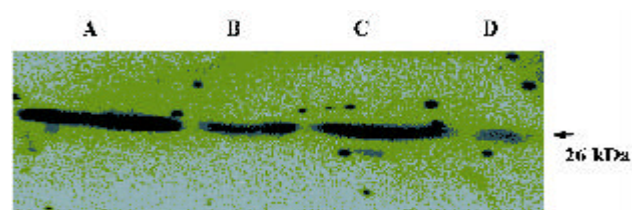


Fig 3. Bcl-2 expression of HL-60 cells treated by different drugs. A: control; B: AraC 0.20 mmol/L alone; C: EGCG 20 mmol/L alone; D: AraC 0.20 mmol/L+EGCG 20 mmol/L. The result presented is typical of 3 separate experiments.

Effect on intracellular Ca²⁺ Intracellular Ca²⁺ levels were not influenced after treatment with EGCG 20 μmol/L for 6 and 12 h. AraC 0.20 μmol/L treatment increased the intracellular Ca²⁺ a little. However, treatment with AraC in combination with EGCG for 6 and 12 h increased the intracellular Ca²⁺ to 1.8 and 2.1 fold of the control respectively (Tab 3).

Tab 3. Effect of EGCG 20 mmol/L and AraC 0.20 mmol/L on intracellular Ca²⁺ concentration (nmol/L) of HL-60 cells. n=3. Mean±SD. ^bP<0.05 vs AraC+EGCG group.

Group	6 h	12 h
Control	99±3	97±3
AraC	110.4±1.4 ^b	145±2.9 ^b
EGCG	71±6 ^b	81±6 ^b
AraC+EGCG	177±33	206±60

DISCUSSION

While chemotherapeutic agents are extremely useful in the tumor treatment, their doses are limited for their toxicity. Recently biochemical modulators that enhance the effects of known anti-tumor agents and reduce their toxicity bring extensive attention in tumor chemotherapy^[1-6]. Thus the search for novel anti-tumor biochemical modulators from natural origin becomes an important research problem and has a wide future application^[11,12].

It has been reported that EGCG showed some activities of biochemical modulator. The combination of EGCG and curcumin synergistically inhibited cell lines

derived from dysplastic leukoplakia and squamous cell carcinoma, for EGCG blocked cells in G₁ whereas curcumin blocked cells in S and G₂/M^[13]. Sulindac at concentrations up to 100 μmol/L did not induce apoptosis of human lung cancer cell line PC-9, whereas sulindac 10 μmol/L in combination with EGCG 75 μmol/L induced apoptosis, providing a new application method which by drinking green tea enhanced the preventive effects of sulindac while reducing its toxicity^[14]. No report on the potentiation of EGCG to AraC anti-tumor effect has yet been published. Our study indicated that EGCG 20 μmol/L alone had nearly no anti-tumor activity on HL-60 cells, however, it could enhance inhibitory effects on cell proliferation, G₁ phase arrest, S phase inhibition, and apoptosis induced by AraC on HL-60 cells. Hastak pointed out recently that EGCG exerted a little or no effect on normal cells, its anti-tumor effect was independent of the status of p53 protein^[15].

It is well known that bcl-2 protein inhibits the cell apoptosis. ATRA (all-*trans* retinoic acid), a natural compound, sensitized the response of acute myeloblastic leukemia to chemotherapy by down-regulation of bcl-2 expression^[16]. Similarly, our research showed that EGCG enhanced the sensitivity of HL-60 cells to chemotherapy through down-regulation of bcl-2 and increase of the intracellular Ca²⁺ level of cells treated with AraC, as well as nullification of the rescue effect of dCdR in HL-60 cells treated with AraC.

Further study on the mechanism of biochemical modulation of EGCG will focus on effect of EGCG on apoptosis signal transduction.

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