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Epigallocatechin gallate, ellagic acid, and rosmarinic acid perturb dNTP pools and inhibit *de novo* DNA synthesis and proliferation of human HL-60 promyelocytic leukemia cells: Synergism with arabinofuranosylcytosine

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ABSTRACT

Epigallocatechin gallate (EGCG), ellagic acid (EA) and rosmarinic acid (RA) are natural polyphenols exerting cancer chemopreventive effects. Ribonucleotide reductase (RR; EC 1.17.4.1) converts ribonucleoside diphosphates into deoxyribonucleoside diphosphates being essential for DNA replication, which is why the enzyme is considered an excellent target for anticancer therapy.

EGCG, EA, and RA dose-dependently inhibited the growth of human HL-60 promyelocytic leukemia cells, exerted strong free radical scavenging potential, and significantly imbalanced nuclear deoxyribonucleoside triphosphate (dNTP) concentrations without distinctly affecting the protein levels of RR subunits (R1, R2, p53R2). Incorporation of ^{14}C-cytidine into nascent DNA of tumor cells was also significantly lowered, being equivalent to an inhibition of DNA synthesis. Consequently, treatment with EGCG and RA attenuated cells in the G0/G1 phase of the cell cycle, finally resulting in a pronounced induction of apoptosis. Sequential combination of EA and RA with the first-line antileukemic agent arabinofuranosylcytosine (AraC) synergistically potentiated the antiproliferative effect of AraC, whereas EGCG plus AraC yielded additive effects.

Taken together, we show for the first time that EGCG, EA, and RA perturbed dNTP levels and inhibited cell proliferation in human HL-60 promyelocytic leukemia cells, with EGCG and RA causing a pronounced induction of apoptosis. Due to these effects and synergism with AraC, these food ingredients deserve further preclinical and *in vivo* testing as inhibitors of leukemic cell proliferation.

Introduction

Epigallocatechin gallate (EGCG) is the major catechin found in green tea (*Camellia sinensis* (L.) Kuntze) (Lambert and Elias 2010). Accompanied by (−)-epicatechin-3-gallate, (−)-epigallocatechin, and (−)-epicatechin, EGCG accounts for 50–80% of the polyphenols in a brewed cup of green tea (Singh et al. 2011). These catechins show a pronounced radical scavenging activity and inhibit cell proliferation (Brown 1999). The potential health benefits ascribed to EGCG include cancer chemoprevention, amelioration of cardiovascular health and neurodegenerative maladies, preventive effects against diabetes, and protection of the skin from damage caused by ionizing radiation (Singh et al. 2011). Regarding its cancer chemopreventive abilities, numerous mechanisms have been proposed including antioxidant effects, inhibition of growth factor signaling, and induction of tumor suppressor genes (Lambert and Elias 2010).

Ellagic acid (EA) is present in fruits, nuts, and berries and belongs to a family of bioactive ellagitannins (ETs). All ETs share the ability to release EA upon hydrolysis, resulting in a prolonged release of EA into the bloodstream (Heber 2008). Pomegranate (*Punica granatum* (Horan.) S.A. Graham, Thorne & Reveal), an ancient fruit-bearing deciduous shrub represents the richest source of ETs and, subsequently, EA among fruits (Heber 2008; Viladomiu et al. 2013). Pomegranate juice has been reported to exert antioxidant, anticancer and

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chemopreventive effects by inhibition of cancer cell proliferation and modulation of inflammatory signaling pathways (Hagiwara et al. 2010). ETs and their hydrolysis product, EA, attenuate prostate cancer cell growth through cell cycle arrest and subsequent induction of apoptosis, including the inhibition of angiogenesis, the latter having been demonstrated *in vitro* and *in vivo* (Heber 2008; Lansky et al. 2005).

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid belonging to the main active compounds of rosemary (*Rosmarinus officinalis* (L.)), together with caffeic acid, ursolic acid, carnosic acid, and carnosol. The phenolic constituents of rosemary have also been found to exert preventive effects on several types of cancer through induction of cancer protective factors like apoptosis, or inhibition of tumor promoting events such as free radical generation (Ngo et al. 2011; Petersen and Simmonds 2003). In addition, RA possesses antioxidant as well as anti-inflammatory activities through which it has been shown to reduce the risk of myocardial infarction and to inhibit lipid peroxidation (Wu and Wang 2012). Due to its radical scavenging properties, RA was also able to exert protective effects against memory impairments induced by neurotoxins (Wu and Wang 2012).

Ribonucleotide reductase (RR; EC 1.17.4.1) is responsible for the reduction of ribonucleoside diphosphates into deoxyribonucleoside diphosphates, which are the building blocks for DNA synthesis and repair in all living organisms (Guarino et al. 2014; Shao et al. 2013).

RR is highly active in malignant tissues due to an increased need of deoxynucleoside triphosphates (dNTPs; the products of RR metabolism) of rapidly growing cells while showing low enzyme activity in slowly growing normal cells (Takeda and Weber 1981). RRs are divided into three classes depending on the mode of free radical generation. Class I RRs are aerobic enzymes being further subdivided depending on the metallocofactor employed (Guarino et al. 2014). Eukaryotes mainly use class Ia RRs, which comprise an $\alpha 2\beta 2$ complex made up of two subunits α (termed R1) and β (termed R2) that conglomerate to build the holoenzyme with the active form supposed to adopt $\alpha 2\beta 2$ quaternary state (Aye et al. 2014). Effector-binding R1 harbors substrate and allosteric effective sites for controlling enzyme activity and substrate specificity. Nonheme R2 contains two oxo-bridged dinuclear iron centers each stabilizing a protein tyrosyl radical. An inhibition of the nonheme iron subunit can thus be caused by chelation of the iron centers or by scavenging of the tyrosyl radical (Shao et al. 2013). Class II enzymes operate independently of oxygen and require 5′-deoxyadenosylcobalamin for radical generation, whereas class III RRs only work under strictly anaerobic conditions, thereby using a stable glycine radical for catalysis (Guarino et al. 2014). Hydroxyurea has been the first RR inhibitor being introduced in clinical practice and was used in chemotherapy regimens against chronic myeloid leukemia (CML) and a number of other malignant diseases (Saban and Bujak 2009; Tennant 2001). Another RR inhibitor, gemcitabine (difluordeoxycytidine; dFdC), is commonly used in the treatment of pancreatic cancer and non-small cell lung cancer (Noble and Goa 1997; Toschi et al. 2005).

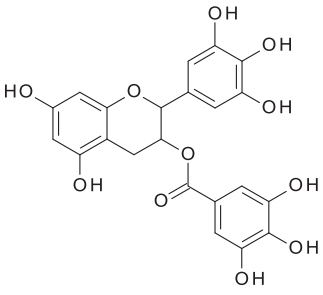
EGCG, EA, and RA have never been tested for their RR inhibiting capabilities but all of them have a number of free hydroxyl groups being located at their aromatic rings which might be able to scavenge the tyrosyl radical harbored in the R2 subunit of the enzyme thus attenuating its activity. Following this strategy, we now investigated the antiproliferative and biochemical effects of these natural compounds in the human HL-60 promyelocytic leukemia cell line, which already served as a cellular model for testing extracts of ethnopharmacological healing plants (Gridling et al. 2009). The free radical scavenging potential of EGCG, EA, and RA was measured by DPPH• assay, the DNA synthesis activity was determined by incorporation of radio-labeled ^{14}C-cytidine into nascent DNA of tumor cells, and the steady state of dNTPs was analyzed by HPLC. The protein levels of RR subunits R1, R2, and p53R2 were examined by western blotting. Cell cycle perturbations were evaluated by FACS, and the induction of apoptosis was quantified employing a specific Hoechst 33258/propidium iodide double staining method developed by our group.

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) mainly characterized by specific morphology, leucopenia, and a life-threatening coagulopathy being the most prominent manifestation of the disease (Tallman and Altman 2009). Anthracycline plus arabinofuranosylcytosine (AraC) combination chemotherapy has been the only treatment option for APL until the late 1980s (Ades et al. 2006), with AraC still being in charge of reducing relapses in APL (Kelaidi et al. 2009). Hence, we combined all drugs with AraC in order to elucidate potential additive or synergistic behavior.

Materials and methods

Chemicals and supplies

EGCG, EA, RA, solvent DMSO, and all other chemicals and reagents were obtained from Sigma–Aldrich GmbH (Vienna, Austria) and of highest purity. Structural formulas of EGCG, EA, and RA including nomenclature, chemical formula, and molecular weight (MW) are given in Fig. 1.

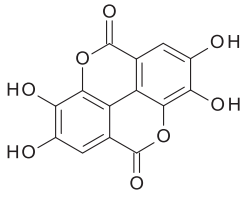


Epigallocatechin gallate (EGCG)

(2R,3R)-2-(3,4,5-Trihydroxyphenyl)-
-3,4-dihydro-1[2H]-benzopyran-
-3,5,7-triol-3-(3,4,5-trihydroxybenzoate)

$C_{22}H_{18}O_{11}$

MW = 458.36

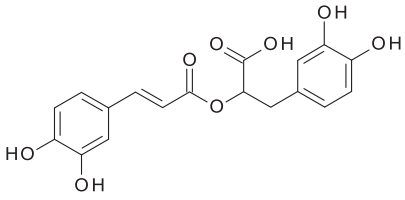


Ellagic acid (EA)

2,3,7,8-Tetrahydroxy-chromeno-
-[5,4,3-cde]chromene-5,10-dione

$C_{14}H_6O_8$

MW = 302.20



Rosmarinic acid (RA)

(2"R")-2-[[(2"E")-3-(3,4-Dihydroxyphenyl)-
-1-oxo-2-propenyl]]oxy]-3-(3,4-dihydroxy-
-phenyl)propanoic acid

$C_{18}H_{16}O_8$

MW = 360.33

Fig. 1. Structural formulas of EGCG, EA, and RA including nomenclature, chemical formula, and molecular weight (MW).

DPPH• radical scavenging activity assay

The radical scavenging activity of drugs was determined using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•). DPPH• absorbs at 515 nm but when reduced by an antioxidant or radical species, its absorption decreases. The reaction was initiated by adding EGCG, ES, and RS (10 μl; 1–100 μM final concentration) to 3.0 ml of 0.1 mM DPPH• in methanol. The bleaching of DPPH• was followed using an HP 8453 diode array spectrometer equipped with a magnetically stirred quartz cell. Absorbance was recorded for up to 15 min, although steady states of reaction were reached within 5 min in most cases. The reference cuvette contained up to 0.1 mM DPPH• in 3.0 ml of methanol. The DPPH• radical scavenging activity obtained for each compound was compared with that of α-tocopherol or ascorbic acid, both of which served as reference compounds. Data are means ± standard errors of three determinations.

Cell culture

The human HL-60 promyelocytic leukemia cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2 using a Heraeus cytoperm 2 incubator (Heraeus, Vienna, Austria). All media and supplements were purchased from Gibco Life Technologies (Paisley, Scotland, UK). Cell counts were determined using a microcellcounter CC-110 (SYSMEX, Kobe, Japan). Cells growing in the logarithmic phase of growth were used for all experiments described below.

Growth inhibition assay

HL-60 cells (0.1×10^6 cells/ml) were seeded in 25 cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of EGCG, EA, or RA at 37 °C under cell culture conditions. Cell counts and IC_{50} values (IC_{50} = 50% growth inhibition of tumor cells) were determined after 24, 48, and 72 h using a microcellcounter CC-110. Viability of cells was determined by staining with trypan blue. Results were calculated as number of viable cells. Data are means ± standard errors of three determinations.

Determination of deoxyribonucleoside triphosphates (dNTPs)

HL-60 cells were seeded in 175 cm^2 tissue culture flasks (1×10^8 per flask) and then incubated with increasing concentrations of EGCG, EA, or RA for 24 h. The cells were then centrifuged at 1800 *g* for 5 min, resuspended in 100 μl of PBS, and extracted with 10 μl of trichloracetic acid (90%). The lysate was allowed to rest on ice for 30 min and neutralized by the addition of 1.5 volumes of freon containing 0.5 M tri-*n*-octylamine. Concentrations of dNTPs were then determined using the method initially described by Garrett and Santi (1979), which has been slightly modified by our group (Saiko et al. 2007a). In brief, aliquots (100 μl) of the samples were analyzed using a Merck "La Chrom" high-performance liquid chromatography (HPLC) system (Merck, Darmstadt, Germany). Detection time was set at 80 min, with the detector operating on 280 nm for 40 min and then switched to 260 nm for another 40 min. Samples were eluted with a 3.2 M ammonium phosphate buffer (pH 3.6, adjusted by the addition of 0.32 M H_3PO_4) containing 2% acetonitrile using a 4.6 × 250 mm PARTISIL 10 SAX column (Whatman Ltd., Kent, UK). Separation was performed at a flow rate of 2 ml/min. The concentration of each dNTP was calculated as percentage of the total area under the curve for each sample. Data are means ± standard errors of three determinations.

Incorporation of ^{14}C-labeled cytidine into DNA

To determine the effect of drug treatment on DNA synthesis, we performed an assay as described previously (Saiko et al. 2007b). Radio-labeled ^{14}C-cytidine has to be reduced by RR in order to be incorporated into the DNA of tumor cells following incubation with a given compound. Cells (0.4×10^6 cells/ml) were incubated with effective concentrations of EGCG, EA, or RA for 24 h. Subsequently, cells were counted and pulsed with ^{14}C-cytidine (0.3125 μCi, 5 nM) for 30 min at 37 °C. Cells were then collected by centrifugation and washed with PBS. Total DNA from 5×10^6 cells was purified by phenol–chloroform–isoamyl alcohol extraction and specific radioactivity of the samples was measured using a Wallac 1414 liquid scintillation counter (PerkinElmer, Boston, MA), whose read out was normalized by a Hitachi U-2000 Double Beam Spectrophotometer to ensure equal amounts and purity of DNA. Data are means ± standard errors of three determinations.

Cell cycle distribution analysis

Cells (0.4×10^6 cells/ml) were seeded in 25 cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of EGCG, EA, or RA at 37 °C under cell culture conditions. After 48 h, cells were harvested and suspended in 5 ml cold PBS, centrifuged, resuspended and fixed in 3 ml cold ethanol (70%) for 30 min at 4 °C. After two washing steps in cold PBS RNase A and propidium iodide were added to a final concentration of 50 μg/ml each and incubated at 4 °C for 60 min before measurement. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA). Data are means ± standard errors of three determinations.

Hoechst dye 33258 and propidium iodide double staining

The Hoechst staining was performed according to the method described by our group (Grusch et al. 2002). Cells (0.2×10^6 cells/ml) were seeded in 25 cm^2 Nunc tissue culture flasks and exposed to increasing concentrations of EGCG, EA, or RA for 24 or 48 h. Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma, St. Louis, MO, USA) were added directly to the cells to final concentrations of 5 μg/ml and 2 μg/ml, respectively, followed by 90 min of incubation at 37 °C. Cells were examined on a Nikon Eclipse TE-300 Inverted Epi-Fluorescence Microscope (Nikon, Tokyo, Japan) equipped with a Nikon DS-5M-L1 Digital Sight Camera System including appropriate filters for Hoechst 33258 and PI. This method allows distinguishing between early apoptosis, late apoptosis, and necrosis and is therefore superior to TUNEL assay that fails to discriminate among apoptosis and necrosis (Grasl-Kraupp et al. 1995) and does not provide any morphological information. In addition, the HO/PI staining is more sensitive than a customary FACS based Annexin V binding assay (Grusch et al. 2001). Cells were judged according to their morphology and the integrity of their cell membranes, counted under the microscope and the number of apoptotic cells was given as percentage value. Experiments were repeated twice.

Western blotting

After incubation with 30 μM EGCG, HL-60 cells (2×10^6 cells/ml) were harvested, washed twice with ice-cold PBS (pH 7.2) and lysed in a buffer containing 150 mM NaCl, 50 mM Tris–buffered saline (Tris pH 8.0), 1% Triton X-100, 2.5% 100 mM phenylmethylsulfonylfluoride (PMSF) and 2.5% protease inhibitor cocktail (PIC; from a 100× stock). The lysate was centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was stored at −20 °C until further analysis as reported previously (Saiko et al. 2011). Equal numbers of cells were lysed

for each sample and PVDF membranes were stained with Ponceau S to ensure equal sample loading. The latter was also controlled by β-actin expression, which appeared to be stable when inspected in short term exposures to X-ray films. Antibodies directed against R1 (T-16), R2 (I-15), p53R2 (N-16), and donkey anti-goat IgG were from Santa Cruz (Santa Cruz, CA, USA), and the antibody directed against β-actin was from Sigma (St. Louis, MO, USA). The primary and secondary antibodies were used at dilutions of 1:500 and 1:2000, respectively.

Sequential growth inhibition assay

HL-60 cells (0.1×10^6 cells/ml) were seeded in 25 cm^2 Nunc tissue culture flasks and first incubated with effective concentrations of EGCG, EA, or RA for 24 h. Concentrations were considered effective when significantly depleting at least one dNTP pool. Then the respective compound was washed out and cells were further exposed to various concentrations of AraC for another 48 h. Since AraC is still being in charge of reducing relapses in APL (Kelaidi et al. 2009), we decided to combine all natural compounds with this clinically established antileukemic drug. Concentrations were chosen such that they were strictly in the IC_{10}–IC_{30} range to avoid exaggerated growth inhibition after 72 h when being applied in combination. Cells were counted every 24 h using a microcellcounter CC-110. Experiments were repeated twice.

Calculations of combination effects

The calculations of combination effects were performed using the Calcusyn 2.0 software designed by Chou and Talalay (Biosoft, Ferguson, MO) (Chou and Talalay 1984). The analytical method of Chou and Talalay (1981, 1984) yields two parameters that describe the interactions among drugs in a given combination: the combination index (CI) and the dose reduction index (DRI). DRI measures by what factor the dose of each drug in a combination may be reduced at a given effect level compared with the dose when each drug is used alone. DRI may be influenced by the combination ratio and the number of drugs. Toxicity towards the host may be avoided or reduced when the dose is reduced. The advantage of this method is that it takes into account not only the potency (median effect dose values [D_m] or drug concentration at 50% neutralization [EC_{50}]), but also the shape (sigmoidicity) of the dose–effect curve, based on the median effect equation of Chou. The latter correlates drug dose and cytostatic effect using the following form:

$$f_a/f_u = (D/D_m)^m \text{ or } D = D_m[f_a/(1-f_a)]^{1/m}$$

D represents the dose of the drug; D_m is the median effect dose meaning the potency, determined from the x-intercept of the median effect plot; f_a is the fraction affected by the dose; f_u is the fraction unaffected ($f_u = 1 - f_a$); and m is an exponent that signifies the shape (sigmoidicity) of the dose–effect curve, which is given by the slope of the median effect plot. The median effect equation is utilized to calculate D_x, which is the dose of a drug that inhibits x% of cells. For drugs with mutually nonexclusive mechanisms of action (i.e. drugs that have a different mode of action, thus being not competitive inhibitors of each other), the CI is then calculated by the following equation:

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + [(D)_1(D)_2]/[(D)_1(D)_2]$$

The CI equation determines the additive effect of drug combinations, such that a CI of <0.9 indicates synergism, a CI of 0.9–1.1 indicates additive effects, and a CI of >1.1 indicates antagonism. Experiments were repeated twice.

Statistical calculations

Dose–response curves were calculated using the Prism 5.01 software (GraphPad, San Diego, CA, USA) and significant differences between controls and each drug concentration applied were determined by unpaired t-test. All p-values below 0.05 were considered significant and marked with an asterisk (*).

Results

Reduction of HL-60 cell numbers

HL-60 cells (0.1×10^6 cells/ml) were seeded in 25 cm^2 Falcon tissue culture flasks and treated with increasing concentrations of drugs. After the incubation period, the cell number of viable leukemia cells was determined using a microcellcounter. Exposure to EGCG for 24, 48, and 72 h resulted in IC_{50} values of 30, 18, and 16 μM, respectively (Fig. 2a). Treatment with EA for 72 h led to an IC_{50} value of 35 μM (Fig. 2b). Incubation with RA for 24, 48, and 72 h yielded IC_{50} values of 147, 74, and 69 μM, respectively (Fig. 2c). IC_{50} values after 72 h are also listed in Table 1.

Antioxidant activity of EGCG, EA, and RA

The *in vitro* free radical-scavenging activity of EGCG, EA, and RA was determined by trapping the DPPH• radical. After incubation for 15 min EGCG, EA, and RA reduced DPPH• absorption with IC_{50} values of 5.8, 6.5, and 22.2 μM, respectively. Therefore, the potential of EGCG, EA, and RA to inhibit HL-60 cell expansion correlated directly with their potential to scavenge free radicals. Tocopherol and ascorbic acid were used as reference compounds resulting in IC_{50} values of 21.3 and 16.2 μM, respectively (Table 1).

Alterations of intracellular dNTP pools

Under physiological conditions, RR maintains stable deoxyribonucleotide production, which is essential for DNA synthesis; accordingly, inhibition of RR activity eliminates this balance. EGCG, EA, or RA treatment for 24 h each caused a significant imbalance of dNTPs in HL-60 cells, which was reminiscent of the imbalances caused by the RR inhibitors hydroxyurea and gemcitabine (Hakansson et al. 2006; Heinemann et al. 1995; Ostruszka and Shewach 2003; Smid et al. 2001). Incubation with 20 and 40 μM EGCG resulted in a depletion of intracellular dATP pools to 51 and 37% of controls, respectively, whereas dTTP pools were increased to 141% of untreated cells upon 40 μM EGCG (Fig. 3a). Treatment with 20, 30, and 60 μM EA reduced dCTP pools to 77, 57, and 49% of untreated controls, respectively, and 20 and 30 μM EA depleted dTTP pools to 72 and 65%, respectively (Fig. 3b). Exposure to 50 μM RA led to a depletion of dTTP pools to 71%, and 50, 100, and 150 μM RA reduced dATP pools to 68, 48, and 45% of controls, respectively (Fig. 3c). All dGTP pools remained beyond the detectability limit of the method.

Inhibition of incorporation of ^{14}C-cytidine into DNA of HL-60 cells

To quantify DNA synthesis, the incorporation of ^{14}C-cytidine into nascent DNA of tumor cells was measured after incubation with effective concentrations of EGCG, EA, or RA (i.e. doses that have been shown to significantly reduce dNTP pools). Exposure of cells to 20 μM EGCG, 60 μM EA, and 100 μM RA significantly lowered the incorporation of radiolabeled cytidine to 45%, 62%, and 46% of control values, respectively (Fig. 4).

Expression levels of RR subunits R1, R2, and p53R2 after treatment with EGCG

To monitor the effect of EGCG on the expression of RR subunits R1, R2, and p53R2, HL-60 cells were incubated with 30 μM EGCG for 0.5, 2, 4, 8, and 24 h and subjected to western blot analysis. R1 and R2 protein levels remained almost unchanged throughout the time course being in line with the fact that enzyme activity can be attenuated without

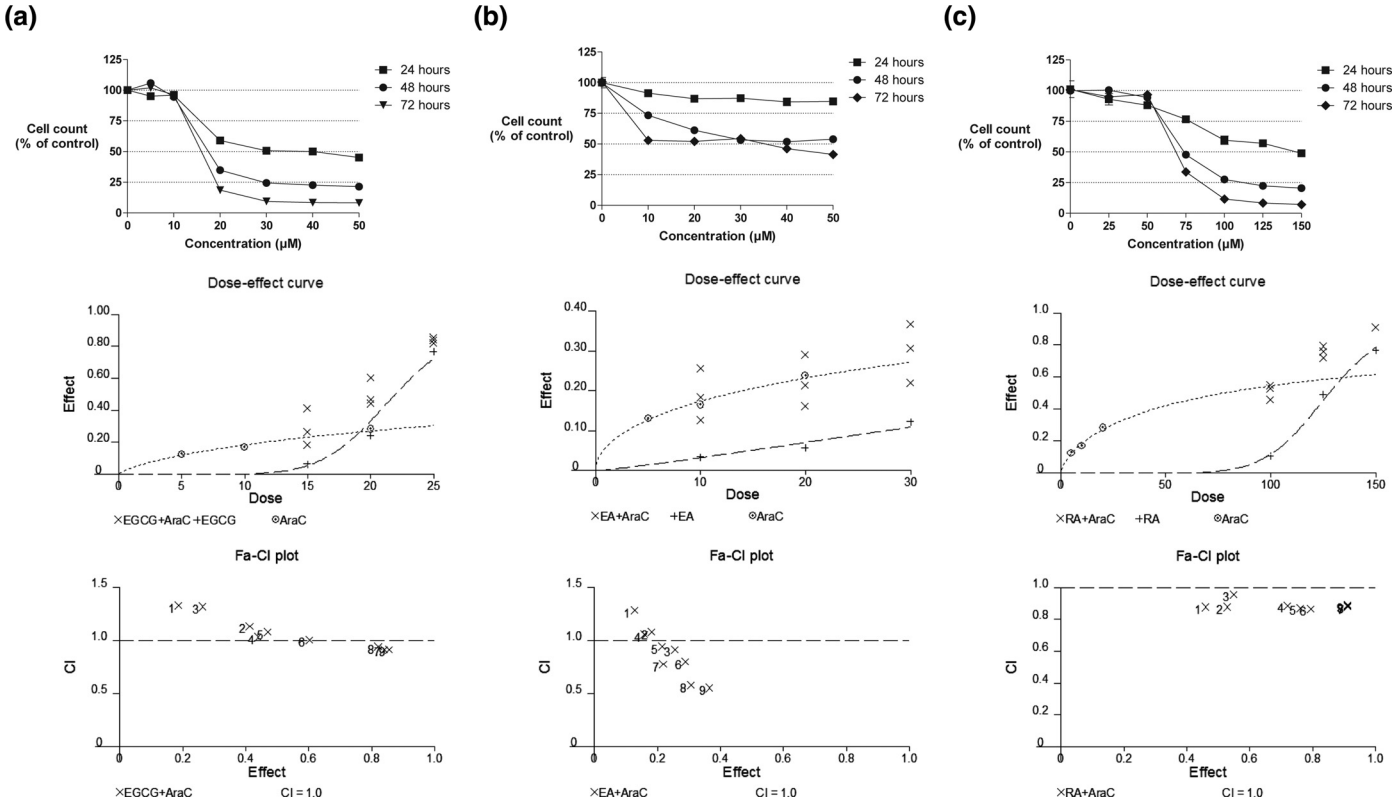


Fig. 2. Reduction of cell numbers, dose–effect curves, and Fa–CI plots after incubation of HL-60 cells with (a) EGCG, (b) EA, and (c) RA. HL-60 cells (0.1×10^6 cells/ml) were seeded in 25 cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of EGCG, EA, or RA at 37 °C under cell culture conditions. Cell counts and IC_{50} values (IC_{50} = 50% growth inhibition of tumor cells) were determined after 24, 48, and 72 h using a microcellcounter CC-110. Viability of cells was determined by staining with trypan blue. Results were calculated as number of viable cells. In another set of experiments, HL-60 cells were first incubated with effective concentrations of EGCG, EA, or RA for 24 h. Concentrations were considered effective when significantly depleting at least one dNTP pool. Then the respective compound was washed out and cells were further exposed to various concentrations of AraC for another 48 h. Concentrations were chosen such that they were strictly in the IC_{10}–IC_{30} range to avoid exaggerated growth inhibition after 72 h when being applied in combination. The calculations of dose–effect curves and Fa–CI plots were performed using the Calcusyn 2.0 software designed by Chou and Talalay (Biosoft, Ferguson, MO) (Chou and Talalay, 1984).

influencing the protein levels of its subunits (Saiko et al. 2011, 2013), whereas p53R2 expression was slightly decreased (Fig. 5).

Cell cycle distribution in HL-60 cells after treatment with EGCG, EA, and RA

Treatment with EGCG, EA, or RA led to significant alterations of the cell cycle distribution. Incubation with 50 μM EGCG attenuated cells in the G0-G1 phase of the cell cycle, increasing this cell population from 34.6% to 48.2%, whereas S phase cells decreased from 48.5% to 40.1%. In contrast, exposure to 60 μM EA resulted in an accumulation of cells in the S phase, thereby augmenting these cells from 21.8% to 38.8% while reducing cells in the G0–G1 phase from 62.8% to 43.9%. RA acted similarly to EGCG; 150 μM RA attenuated cells in the G0-G1 phase, increasing this population from 33.1% to 47.4% while decreasing cells mainly in the G2-M phase from 17.3% to 9.0% (Fig. 6).

Table 1
DPPH• radical scavenging activity after treatment for 15 min and growth inhibition of HL-60 cells after incubation for 72 h.

Compound	DPPH• activity IC_{50} (μM)	HL-60 cells IC_{50} (μM)
EGCG	5.8 ± 0.18	16.0 ± 0.23
EA	6.5 ± 0.31	35.0 ± 1.53
RA	22.2 ± 0.65	69.0 ± 2.28
Ascorbic acid	21.3 ± 1.51	–
Tocopherol	16.2 ± 0.90	–

Induction of apoptosis in HL-60 cells after exposure to EGCG, EA, and RA

Tumor cells were incubated with increasing concentrations of drugs for 24 or 48 h and then double stained with Hoechst 33258 and propidium iodide to quantify apoptotic cells death. Treatment with 30, 40, and 50 μM EGCG led to 16.7, 56.3, and 84.3% apoptotic cells, respectively, but after incubation with EA, we could not observe a prominent induction of apoptosis at any concentration employed. Exposure to 50 and 100 μM RA did not result in apoptosis as well, whereas 150 μM RA led to 60.7% apoptotic cells (Table 2).

Combination effects with AraC on the growth of HL-60 cells

To examine the impact of EGCG, EA, or RA in combination with AraC, HL-60 cells were seeded at a density of 0.1×10^6 cells/ml and incubated with increasing concentrations of the respective drug for 24 h, followed by AraC treatment for 48 h as described in the "Materials and methods" section. Dose–effect curves and Fa–CI plots for EGCG, EA, and RA are given in Fig. 2a, b, and c, respectively. Regarding EGCG, six out of nine combinations applied yielded additive effects according to the equation of Chou and Talalay (1984) (Table 3). In contrast, four out of nine EA combinations and eight out of nine RA combinations applied showed synergism according to the equation of Chou and Talalay (1984) (Tables 4 and 5).

Discussion

EGCG, EA, and RA are polyphenolic plant ingredients that have been shown to possess antioxidant capabilities (Lambert and Elias

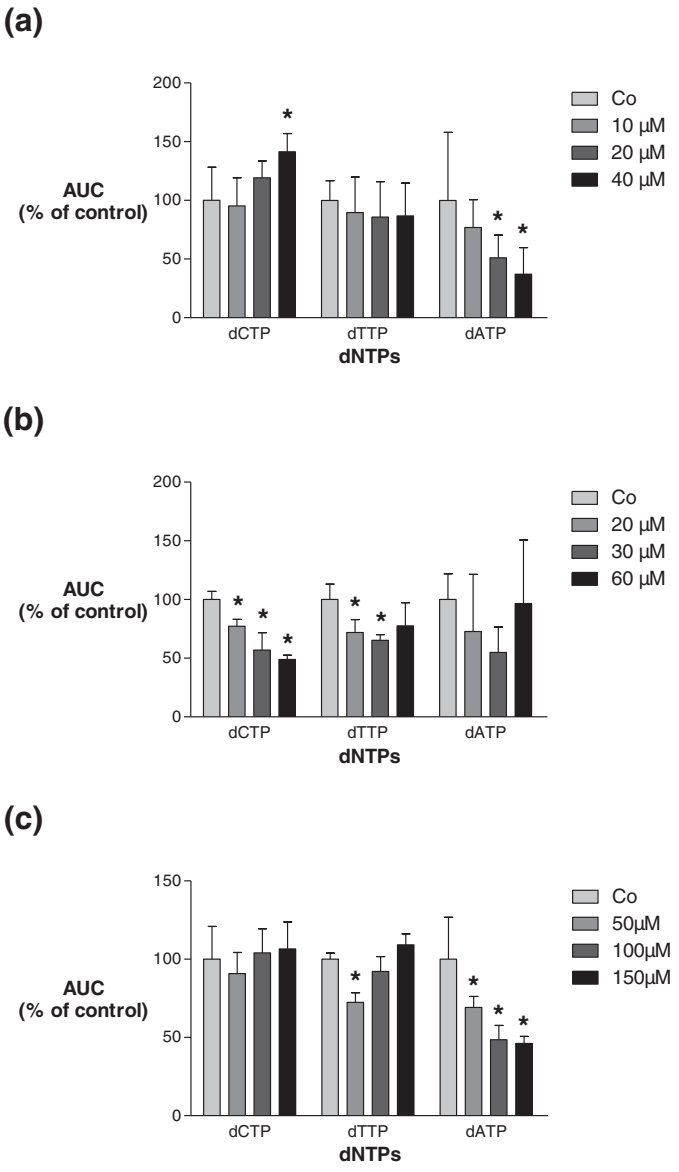


Fig. 3. Concentration of dNTP pools in HL-60 cells upon treatment with (a) EGCG, (b) EA, and (c) RA. Cells (0.8×10^6 cells/ml) were incubated with EGCG (10, 20, and 40 μM), EA (20, 30, and 60 μM) or RA (50, 100, and 150 mM) for 24 h. Afterwards, 1×10^8 cells were separated for the extraction of dNTPs. The concentration of dNTPs was calculated as percent of total area under the curve for each sample. Values significantly ($p < 0.05$) different from control are marked with an asterisk (∗).

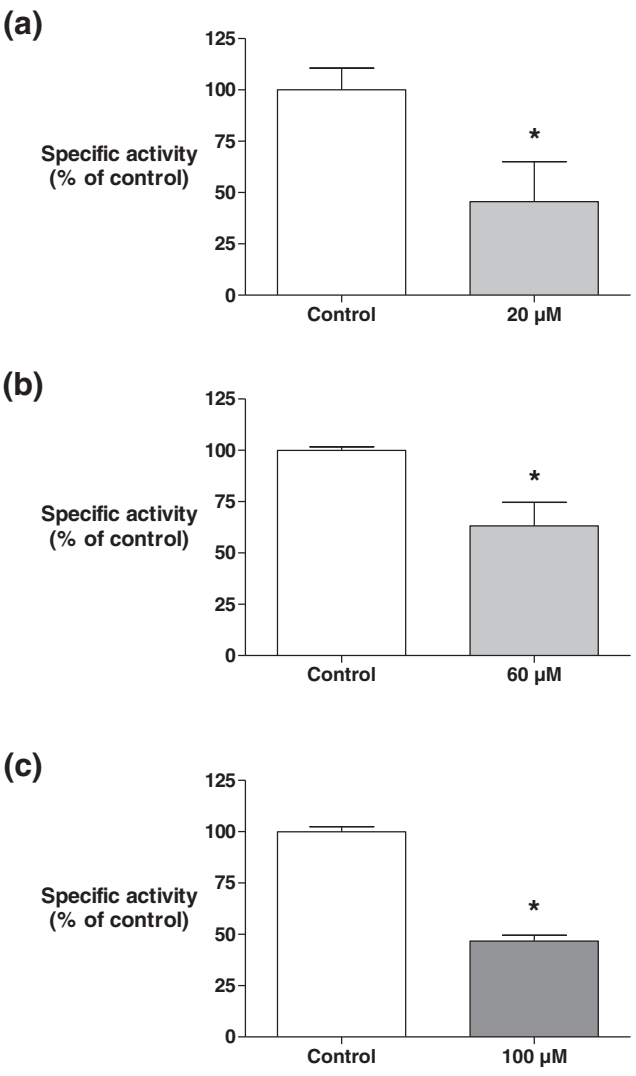


Fig. 4. Inhibition of ^{14}C-cytidine incorporation into DNA of HL-60 cells after exposure to (a) EGCG, (b) EA, and (c) RA. Cells (0.4×10^6 cells/ml) were incubated with EGCG (20 μM), EA (60 μM), or RA (100 μM) for 24 h. After the incubation period, cells were counted and pulsed with ^{14}C-cytidine (0.3125 μCi, 5 nM) for 30 min at 37 °C. Then cells were collected by centrifugation and washed with PBS. Total DNA was extracted from 5×10^6 cells and specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (PerkinElmer, Boston, MA). Values significantly ($p < 0.05$) different from control are marked with an asterisk (∗).

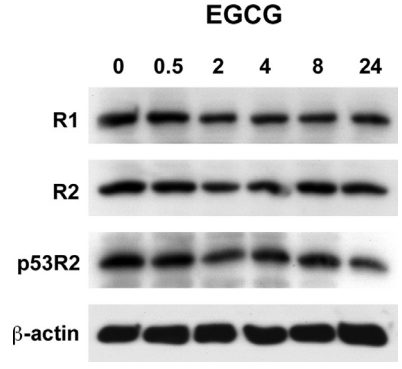


Fig. 5. Expression levels of RR subunits R1, R2, and p53R2 in HL-60 cells upon treatment with EGCG. After incubation with 30 μM EGCG for 0.5, 2, 4, 8, and 24 h, HL-60 cells (2×10^6 cells/ml) were harvested, washed twice with ice-cold PBS (pH 7.2) and lysed in a buffer containing 150 mM NaCl, 50 mM Tris–buffered saline (Tris pH 8.0), 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (PIC; from a 100× stock). The lysate was centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant was subjected to western blot analysis.

2010), to induce cell cycle arrest and apoptosis, and to act as cancer chemopreventive agents in a multitude of preclinical and animal studies (Hagiwara et al. 2010; Heber 2008; Ngo et al. 2011; Petersen and Simmonds 2003; Singh et al. 2011; Viladomiu et al. 2013; Wu and Wang 2012).

In this study, we report for the first time the effects of these natural compounds on ribonucleotide reductase (RR) metabolism in human HL-60 promyelocytic leukemia cells.

Acute promyelocytic leukemia is a distinct subtype of acute myeloid leukemia and represents a relatively rare hematological disease, accounting for approximately 5–8% of all AML cases (Mi 2011). The balanced reciprocal translocation t(15;17) generates a PML (promyelocytic leukemia)-RARα (retinoic acid receptor alpha) fusion (onco)gene (Mi et al. 2012). This PML–RARα transcript initiates APL by blocking myeloid differentiation and by increased self-renewal of leukemic progenitor cells (Ades et al. 2006; de The and Chen 2010).

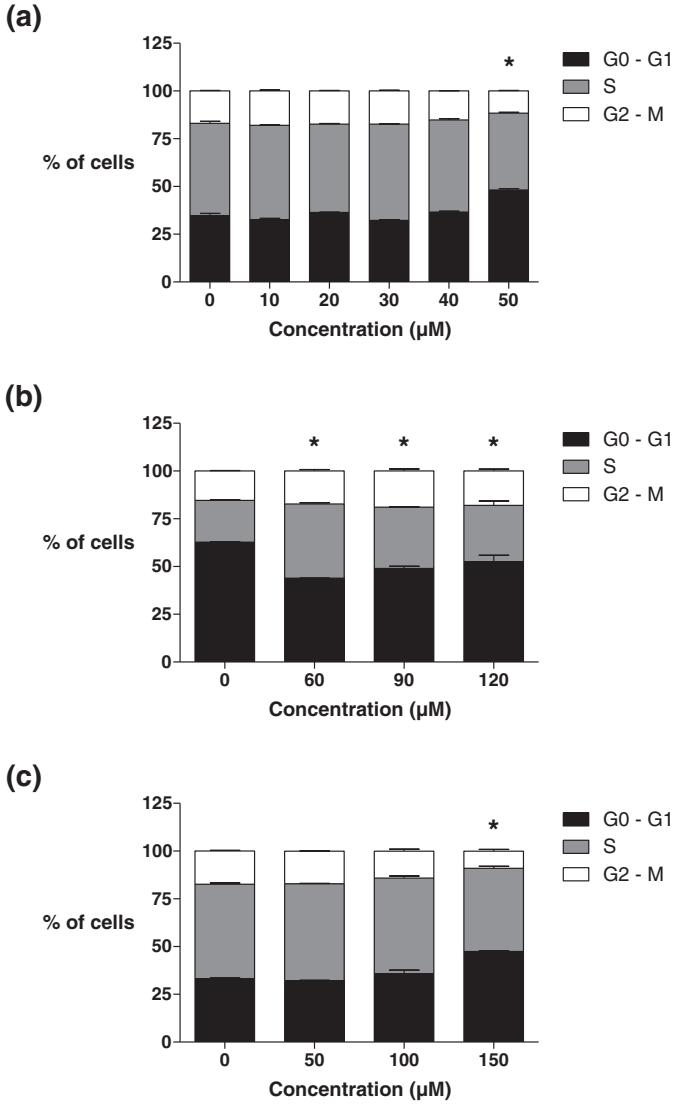


Fig. 6. Cell cycle distribution in HL-60 cells after incubation with (a) EGCG, (b) EA, and (c) RA. Cells (0.4×10^6 cells/ml) were seeded in 25 cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of EGCG, EA, or RA for 48 h under cell culture conditions. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA). Values significantly ($p < 0.05$) different from control are marked with an asterisk (*).

Table 2
Induction of apoptosis in HL-60 cells after exposure to EGCG, EA, and RA.

EGCG	Control	10 μM	20 μM	30 μM	40 μM	50 μM
24 h	2%	3%	3%	17%	41%	84%
48 h	3%	6%	3%	17%	56%	84%
EA	Control	30 μM	45 μM	60 μM	90 μM	120 μM
24 h	3%	–	–	5%	7%	7%
48 h	1%	2%	2%	2%	–	–
RA	Control	50 μM	100 μM	150 μM		
24 h	2%	2%	7%	5%		
48 h	1%	1%	3%	61%		

Table 3
Combination effects of EGCG and AraC in HL-60 cells employing a sequential growth inhibition assay.

Compound	Concentration (μM/nM)	Cell number (% of control)	Combination index[a]
EGCG (μM)	15	93.2	
	20	75.6	
	25	23.1	
AraC (nM)	5	87.3	
	10	83.0	
	20	71.6	
EGCG + AraC	15		
	5	81.3	**1.328**
EGCG + AraC	15		
	10	73.6	**1.319**
EGCG + AraC	15		
	20	58.7	**1.136**
EGCG + AraC	20		
	5	55.9	**1.030**[b]
EGCG + AraC	20		
	10	53.0	**1.078**[b]
EGCG + AraC	20		
	20	39.7	**1.004**[b]
EGCG + AraC	25		
	5	15.8	**0.914**[b]
EGCG + AraC	25		
	10	17.8	**0.941**[b]
EGCG + AraC	25		
	20	14.4	**0.916**[b]

Cells were sequentially incubated with (1) EGCG for 24 h and (2) AraC for 48 h, and then the cell number was determined. Data are means of two determinations.
[a] Combination indices according to the equation of Chou and Talalay (1984).
[b] Additive combination effect.

Table 4
Combination effects of EA and AraC in HL-60 cells employing a sequential growth inhibition assay.

Compound	Concentration (μM/nM)	Cell number (% of control)	Combination index[a]
EA (μM)	10	96.5	
	20	94.2	
	30	87.7	
AraC (nM)	5	86.9	
	10	83.6	
	20	76.3	
EA + AraC	10		
	5	87.3	**1.285**
EA + AraC	10		
	10	81.7	**1.079**
EA + AraC	10		
	20	74.4	**0.916**
EA + AraC	20		
	5	84.0	**1.055**
EA + AraC	20		
	10	78.5	**0.939**
EA + AraC	20		
	20	71.0	**0.797**[b]
EA + AraC	30		
	5	78.0	**0.777**[b]
EA + AraC	30		
	10	69.5	**0.580**[b]
EA + AraC	30		
	20	63.4	**0.555**[b]

Cells were sequentially incubated with (1) EA for 24 h and (2) AraC for 48 h, and then the cell number was determined. Data are means of two determinations.
[a] Combination indices according to the equation of Chou and Talalay (1984).
[b] Synergistic combination effect.

Table 5
Combination effects of RA and AraC in HL-60 cells employing a sequential growth inhibition assay.

Compound	Concentration (μM/nM)	Cell number (% of control)	Combination index[a]
RA (μM)	100	88.9	
	125	50.8	
	150	23.1	
AraC (nM)	5	87.3	
	10	83.0	
	20	71.6	
RA + AraC	100		
	5	54.0	**0.878**[b]
RA + AraC	100		
	10	47.1	**0.880**[b]
RA + AraC	100		
	20	45.1	**0.959**
RA + AraC	125		
	5	27.9	**0.886**[b]
RA + AraC	125		
	10	24.0	**0.873**[b]
RA + AraC	125		
	20	20.6	**0.867**[b]
RA + AraC	150		
	5	8.7	**0.880**[b]
RA + AraC	150		
	10	8.8	**0.884**[b]
RA + AraC	150		
	20	8.9	**0.890**[b]

Cells were sequentially incubated with (1) RA for 24 h and (2) AraC for 48 h, and then the cell number was determined. Data are means of two determinations.
[a] Combination indices according to the equation of Chou and Talalay (1984).
[b] Synergistic combination effect.

Amelioration of chemotherapy was gained by the application of high-dose AraC to high-risk patients (Mi et al. 2012) with the majority of studies suggesting a reduction of relapse risk through addition of AraC (Sanz and Lo-Coco 2011).

The quantification of deoxyribonucleoside triphosphate (dNTP) pools, which are the products of RR metabolism, revealed that EGCG, EA, and RA significantly depleted dATP pools with EA also causing a decrease of dCTP pools. A similar reduction of dATP concentrations could already be observed with gemcitabine and is characteristic for the cytostatic effects of this clinically established anticancer agent (Robinson et al. 2003). The analysis of ^{14}C-cytidine incorporation into nascent DNA of cells confirmed that all compounds are pronounced inhibitors of DNA synthesis in the lower micromolar range. Perturbed dNTP balance followed by incomplete DNA synthesis attenuates cell cycle progression as it was monitored for EGCG, EA, and RA. The main effect of EGCG and RA was an accumulation of cells in the G0-G1 phase of the cell cycle, whereas EA caused an increase of cells in the S phase, the latter being in line with the fact that RR is the rate-limiting enzyme for S-phase transit (Chimploy et al. 2009). However, cell cycle progression can already be suspended prior to S phase in G0-G1 phase, as it was recently shown for resveratrol (3,4′,5-trihydroxy-*trans*-stilbene; RV), a naturally occurring RR inhibitor derived from grapes (Quoc Trung et al. 2013). Since RV also accumulated cells in the S phase in a multitude of studies, it is likely that the type of cell cycle arrest very much depends on the experimental setting and/or on the cell line employed (Bernhard et al. 2000; Larrosa et al. 2003).

The expression of the constitutively expressed R1 subunit and the S phase specific R2 subunit remained almost unchanged during the whole time course being consistent with the fact that enzyme activity can be effectively halted without influencing the protein levels of its subunits (Saiko et al. 2013) or even elevated, thereby indicating a compensatory up-regulation through which the cells try to reestablish their dNTP levels (Saiko et al. 2011). After EGCG treatment, only the protein level of the p53 inducible p53R2 subunit was slightly decreased, which could argue for the superior potential of EGCG in terms of inhibiting the ^{14}C-cytidine incorporation into DNA and to arrest HL-60 cells in the G0-G1 phase.

Following disruption of the cell cycle, growth inhibition and subsequent apoptosis are the expected consequences. It has been proposed that tumor cells experiencing RR inhibition caused by hydroxyurea may enter the (early) S phase and accumulate there until they undergo programmed cell death (Yarbro 1992). Examination of the apoptosis inducing properties revealed that EGCG was the most potent compound in this regard, resulting in more than 80% of apoptotic cells at 50 μM. Treatment with 150 μM RA led to 60% of cells showing morphological signs of apoptosis, whereas EA could not raise any prominent apoptotic cell fractions, indicating that cell cycle attenuation rather than induction of programmed cell death seems to be the primary cytostatic effect of this natural compound.

EGCG, EA, and RA hold a number of free hydroxyl groups being located at their aromatic rings, which render them possible radical scavengers. We have demonstrated earlier that gallic acid (3,4,5-trihydroxybenzoic acid; GA) and digalloylresveratrol (3,5-O-digalloyl-resveratrol; DIG) inhibit RR by scavenging the tyrosyl radical being essential for its function (Madlener et al. 2007; Saiko et al. 2013), suggesting that EGCG, EA, and RA might act in a similar manner. Our experiments revealed that the *in vitro* radical scavenging activity of EGCG and EA was much more pronounced than that of ascorbic acid and tocopherol, both of which are commonly used antioxidants. This finding is no direct proof for any tyrosyl radical scavenging property by itself since the latter could be reduced by steric hindrance. However, given the fact that all three natural compounds significantly reduced ^{14}C-cytidine incorporation into nascent DNA of tumor cells, we hypothesize that EGCG, EA, and RA may, nevertheless, inhibit RR activity through scavenging of the tyrosyl radical. In line with this is our observation that the inhibition of *in situ* ^{14}C-incorporation by EGCG (IC_{50} ~ 20 μM) was in the same range as its effects on cell number reduction being attributed to inhibition of cell proliferation (IC_{50} = 30 μM). Regarding RA, there was a similar relation between inhibition of *in situ* ^{14}C-incorporation (IC_{50} ~ 100 μM) and inhibition of cell proliferation (IC_{50} = 147 μM), whereas for EA, this relationship was less evident. Although we provide no direct evidence, the present data indirectly support the notion that DNA synthesis and HL-60 cell proliferation were attenuated most likely by inhibiting RR.

The most apparent advantage of combining drugs is the achievement of additive and/or synergistic effects through alteration of specific molecular pathways resulting in a decrease of drug resistance, drug dosage and, accordingly, drug toxicity. AraC is well-known especially for affecting intracellular dCTP pools (Gandhi et al. 1997; Seymour et al. 1996; Wills et al. 2000) thus causing synergism with various inhibitors of RR (Fritzer-Szekeres et al. 2002, 2008; Horvath et al. 2005, 2006; Saiko et al. 2007a). The different findings described in the clinical studies led us to the assumption that combining AraC, an inhibitor of DNA polymerases (Park et al. 1991), with putative inhibitors of RR such as EGCG, EA, and RA could enhance its growth inhibitory potential, thereby reducing its toxicity thus preserving the beneficial effects seen with this treatment option. Indeed, sequential growth inhibition experiments with AraC revealed that EGCG yielded additive effects in the majority of combinations applied, whereas RA and EA synergistically potentiated the cytostatic activity seen with AraC.

Taken together, EGCG, EA, and RA exerted strong radical-scavenging effects and diminished cell cycle progression due to significant inhibition of DNA synthesis. All three agents were able to enhance the antitumor effects of AraC, with EGCG and RA also causing a pronounced induction of apoptosis. These natural compounds may be administered singularly or as a triple component phytotherapeutic approach not only to act as cancer preventive agents, but also to

support conventional chemotherapy regimens and therefore deserve further preclinical and *in vivo* testing.

Conflict of interest

The authors declare that they have no conflict to disclose.

Acknowledgments

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