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Differential effects of several phytochemicals and their derivatives on murine keratinocytes *in vitro* and *in vivo*: implications for skin cancer prevention

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The purpose of our study was to investigate *in vitro* the potential cancer preventive properties of several phytochemicals, i.e. grape seed extract (GSE), resveratrol (RES), ursolic acid (URA), ellagic acid (ELA), lycopene and *N*-acetyl-L-cysteine (NAC) to define the mechanisms by which these compounds may inhibit murine skin carcinogenesis. We measured quenching of peroxyl, superoxide and hydroxyl radicals by these phytochemicals. We also used adenosine triphosphate (ATP) bioluminescence, Caspase-Glo 3/7 and P450-Glo (CYP1A1 and CYP1B1) assays to study antiproliferative, proapoptotic and CYP-inhibiting effects of the phytochemicals. We next determined their effects on a 4 week inflammatory hyperplasia assay using 7,12-dimethylbenz[*a*]anthracene-induced murine skin carcinogenesis model to further understand their mechanism of action. Three murine keratinocyte cell lines, i.e. non-tumorigenic (3PC), papilloma-derived (MT1/2) and squamous cell carcinoma-derived (Ca3/7) cell lines, were used in *in vitro* assays. We have found that GSE, ELA and RES are potent scavengers of peroxyl and superoxide radicals. Statistically significant effects on activities of caspase-3 and -7 were observed only after GSE and URA treatments. All tested compounds protected cells from hydrogen peroxide-induced DNA damage. Using a short-term complete carcinogenesis assay, we have found that all selected compounds caused marked decreases of epidermal thickness and (except RES) reduced percentages of mice with mutation in codon 61 of Ha-*ras* oncogene. In conclusion, differential effects of tested phytochemicals on events and processes critical for the growth inhibition of keratinocytes *in vitro* and *in vivo* indicate that combinations of tested compounds may, in the future, better counteract both tumor initiation and tumor promotion/progression.

Introduction

The induction of cancer is a multistage process and its stages have been defined experimentally as initiation, promotion and progression. Carcinogenesis depends on inherited and acquired susceptibility factors, such as oncogenes and tumor suppressor genes (1,2), on exposure to initiation factors, i.e. exogenous and endogenous carcinogens, and on promotion and progression factors. The greatest understanding of the important cellular and molecular events involved in tumor initiation, promotion and progression has been provided by studies in the mouse skin carcinogenesis model (3,4). It is well known that a variety of chemical and physical agents can cause skin cancer in rodents and man. Repetitive treatment with known skin carcinogens will lead to skin damage followed by inflammation and regenerative hyperplasia, dysplasia, papillomas, basal and/or squamous cell carcinomas (3–5).

Abbreviations: ATP, adenosine triphosphate; CYP, cytochrome P450; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMBADE, 7,12-dimethylbenz[*a*]anthracene-3,4-diol-1,2-epoxide; ELA, ellagic acid; GSE, grape seed extract; LYC, lycopene; NAC, *N*-acetyl-L-cysteine; RES, resveratrol; URA, ursolic acid.

The mouse skin cancer model has provided an important system not only for studying the mechanisms involved in the various stages of carcinogenesis and for bioassay of tumor-promoting and carcinogenetic agents but also for the study of inhibitors of tumor formation and malignant conversion (5). The studies on the mechanisms of antitumor-initiating and antitumor-promoting properties of a variety of naturally occurring phytochemicals suggest that they are very important for the prevention of skin cancer as well as for the prevention of other epithelial cancers in humans (6). The mouse skin cancer model relates very well to other models where squamous cell carcinomas are induced. It is, however, important to choose for such studies compounds that act through one or more different mechanisms. The inhibitors may modify the carcinogen activation, enhance phase II enzymes detoxification, modify antioxidant enzymes, prevent oxidative damage to DNA bases and mutations, decrease inflammation, cell proliferation and hyperplasia, modulate the immune response and induce apoptosis.

In the present study, we initially used several standardized short-term assays to determine the mechanisms of action of selected natural compounds on murine skin carcinogenesis. Specifically, the assays used were the following very predictive short-term *in vitro* assays: adenosine triphosphate (ATP) bioluminescence assay (indicative of the overall cytostatic potential), sodium dodecyl sulfate–linoleic acid autoxidation assay (indicative of peroxyl radical scavenging abilities), cytochrome *c* reduction assay (indicative of superoxide anion scavenging abilities), 2-deoxyribose reduction assay (indicative of hydroxyl radical scavenging abilities), Caspase-Glo 3/7 assay (indicative of proapoptotic properties) and P450-Glo assay (indicative of CYP-inhibiting properties).

Thus, we initially prescreened the phytochemicals chosen for this study for their cell growth inhibitory effects using 3PC, MT1/2 and Ca3/7 cell lines. The 3PC cell line derived from mouse keratinocytes treated *in vitro* with 7,12-dimethylbenz[*a*]anthracene (DMBA) (7) is non-tumorigenic. The MT1/2 cell line derived from mouse keratinocytes initiated *in vitro* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and promoted with 12-*O*-tetradecanoylphorbol-13-acetate produces papillomas (8). The results obtained with these cell lines may shed some light on the relative efficiency of the inhibitors in slowing down the growth of the initiated cells early during promotion of skin tumorigenesis. On the other hand, the information obtained with the Ca3/7 cell line, derived from a squamous cell carcinoma induced by DMBA/12-*O*-tetradecanoylphorbol-13-acetate, is useful in predicting the effectiveness of the compounds during late stages of skin carcinogenesis. We also evaluated oxygen free radical scavenging, proapoptotic and CYP inhibitory properties as well as oxidative DNA damage-reducing properties of the selected phytochemicals.

We have studied the effect of these phytochemicals on cytochrome P450 (CYP) enzymes because they are a superfamily of hemoprotein monooxygenases that catalyze the oxidation of a wide variety of both endogenous and xenobiotic compounds (9). They are involved in the metabolic activation of procarcinogens of the polycyclic aromatic hydrocarbons commonly found in our environment (10). The inhibition of CYP1 enzymes appears to be beneficial in the prevention of DMBA–DNA adducts formation *in vivo* and *in vitro* (11).

All the phytochemicals selected for this study occur in many medicinal herbs and plants. Grape seed extract (GSE) is a rich source of one of the most beneficial groups of plant flavonoids, proanthocyanidin oligomers. They exert many health-promoting effects (12), including antioxidant activity (13) or the inhibition of the growth of cancer cells in culture (14). Ellagic acid (ELA) is a phenolic compound presenting a variety of biological activities including potent antioxidant (15), anticancer (16) and antimutagen (17) properties. Resveratrol (RES) is a naturally occurring phytoalexin associated with many health benefits, most notably the mitigation of age-related

diseases, including neurodegeneration, carcinogenesis and atherosclerosis (18,19). Ursolic acid (URA) is a pentacyclic triterpenoid that has been shown to suppress tumorigenesis and angiogenesis (20,21). URA has also been found to induce apoptosis in a wide variety of cancer cells (22,23). *N*-acetyl-L-cysteine (NAC), a sulfur containing amino acid, is an *N*-acetyl derivative of L-cysteine. NAC acts as a source of cysteine and stimulates the production of glutathione, which protects the body against oxidative stress (24). Increased lycopene (LYC) levels have been associated with a decreased risk of various types of cancer and cardiovascular disease (25,26).

The overall goal of the present study was to determine, using both *in vitro* and *in vivo* approaches, the mechanisms by which the selected phytochemicals may affect murine skin tumorigenesis.

Materials and methods

Natural source antioxidants and chemicals

Powdered GSE was purchased from the Kalyx (Camden, NY). It was standardized to yield 95 mg (95%) proanthocyanidins. The proanthocyanidins extract as described by Llopiz *et al.* (27) obtained from *Vitis vinifera* grape seeds consisted of 21.3% monomers, 17.4% dimers, 16.3% trimers, 13.3% tetramers and 31.7% higher polymers. The concentration of the proanthocyanidin extract was calculated by taking a mean molecular weight = 1399. The calculations published in the above paper were used in our studies to provide micromolar concentration of GSE. RES, URA, ELA, LYC and NAC as well as other chemicals used in this study were obtained from Sigma Chemical Co. (St Louis, MO) and were of analytical grade or the highest grade available. Recombinant CYP1A1 and CYP1B1 expressed in baculovirus-infected insect microsomes (Supersomes®) were purchased from Gentest Corp. (Woburn, MA).

Cell lines and cell culture conditions

Three murine keratinocyte cell lines, non-tumorigenic (3PC), papilloma (MT1/2) and squamous cell carcinoma (Ca3/7) cell lines, representing various stages of malignant transformation were described previously in details (7,8). All cell lines were cultured at 37°C in the 5% CO_2 in Minimum Essential Medium Eagle Joklik Modification medium (Sigma Chemical Co.) supplemented with insulin (5 μg/ml), epidermal growth factor (5 ng/ml), transferrin (10 μg/ml), *O*-phosphoethanolamine (10 μM), ethanolamine (10 μM), penicillin (50 U/ml), streptomycin (50 ng/ml), gentamicin sulfate (50 μg/ml) and 4% fetal bovine serum (28). The cells were >90% viable as estimated by trypan blue exclusion.

Assessment of hydroxyl, peroxyl and superoxide radicals scavenging

Hydroxyl radical scavenging was measured by studying the competition between deoxyribose and the test compounds. The method is based on spectrophotometric measurement of thiobarbituric acid-reactive carbonyl compounds that are formed in the degradation reaction of deoxyribose by hydroxyl radicals (OH^*) generated from the Fe^{3+}–EDTA–H_2O_2 system (29).

Peroxyl radical quenching was measured using the linoleic acid autoxidation assay. It is based on the rate of autoxidation of linoleic acid to its conjugated diene hydroperoxide (30).

Superoxide radical scavenging activity of compounds was measured as the capacity to inhibit the reduction of cytochrome *c* by a superoxide radical (31,32).

Determination of cell viability

For the quantitative estimation of cell proliferation and cytotoxicity of tested compounds, we have used the ATPLite assay (PerkinElmer, Boston, MA). Cells were plated in 96-well microplates at 5000 cells per well, and various concentrations of tested compounds dissolved in ethanol or dimethyl sulfoxide were administered for 24 h. At the end of the treatment, cellular ATP was released by direct lysis of the cells with a detergent supplied with ATPLite assay. We have used camptothecin as positive control.

Determination of caspase-3 and -7 activities

For estimation of caspase-3/-7 activity (marker for apoptosis) in cell cultures, we have used the Caspase-Glo 3/7 assay (Promega Corp., Madison, WI). The assay provides a proluminescent caspase-3/-7 substrate that is cleaved to aminoluciferin. The released aminoluciferin is a substrate that is consumed by the luciferase, generating a luminescent signal. The signal is proportional to caspase-3/-7 activity. We have used camptothecin as apoptotic positive control.

Single-cell gel electrophoresis (comet assay)

DNA damage was assessed using the comet assay (33,34). After seeding cells (2×10^5) in six-well plate for overnight, the medium was removed, and the cells were treated with ELA (10 or 50 μM), RES (1 or 5 μM), GSEs (1, 4 or 20 μM) or URA (0.2, 0.5 or 1 μM) for 24 h. Cells were treated with hydrogen peroxide (2 mM) for 10 min. Fifty randomly selected cells for each slide were electronically captured on a fluorescence microscope Olympus BX41 (Olympus Corp., Center Valley, PA) with an excitation filter of BP545-580 and a barrier filter of BA610IF using an image analysis system Image-Pro Discovery (Olympus Corp.). The tail length that means the migration of the DNA from the right edge of the comet head was used as a measure of DNA damage.

Measuring of CYP activities

To check the effects of compounds on CYP activities, we have used P450-Glo assays (Promega Corp) for CYP1A1 and CYP1B1. The kit P450-Glo contained the substrates and the experiments were conducted according to the manufacturer's instructions. The substrates in the P450-Glo™ assays are derivatives of beetle luciferin [(4S)-4,5-dihydro-2-(6′-hydroxy-2′-benzothiazolyl)-4-thiazolecarboxylic acid] and are CYP enzyme substrates but not substrates for luciferase. For CYP1A1 and 1B1, the substrate is luciferin 6′ chloroethyl ether. We used 0.5 pmol of CYP1A1 and 1 pmol of CYP1B1.

In this system, the CYP reaction is performed by incubating the CYP with a luminogenic CYP substrate and an nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt regeneration system. The substrate is converted by CYP enzyme to a luciferin product that is detected in a second reaction with the luciferin detection reagent. The amount of produced light is directly proportional to the activity of the CYP. Compounds were tested at different concentrations to determine the concentration at which 50% inhibition (IC_{50}) occurred for each compound. Assays were run using 96-well microplates (5000 cells per well) and bioluminescence was measured with microplate reader (BioTek Instruments, Winooski, VT).

Animals

Female SENCAR mice, 5 weeks old, were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD), and placed into quarantine for 1 week prior to beginning of carcinogen treatment. Mice were housed in groups of five under conditions of constant temperature and humidity and maintained on a 12 h light–dark cycle with *ad libitum* access to food and water. All animal procedures were performed in accordance with the National Institutes of Health Guidelines and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Carcinogen administration

At 6–7 weeks of age, the backs of mice were shaved. The dose of 100 nmol DMBA dissolved in acetone (200 μl) was applied twice weekly and the inhibitors GSE (1, 2.5 and 5 μmol), RES (1, 2.5, 5 and 10 μmol), ELA (0.5, 1, 2.5 and 5 μmol), URA (1, 2.5 and 5 μmol), NAC (1, 2.5 and 5 μmol) and LYC (0.5, 1 and 2.5 μmol) were applied in a total volume of 200 μl of acetone topically 20 min prior to DMBA. The inhibitors and DMBA were given twice a week for 4 weeks. Two days after the last treatment, mice were killed and samples of skin were collected for immunostaining and DNA isolations.

Tissue preparation

Mice were killed by spinal dislocation and skin samples were collected from the dosed area of each animal. Sections specified for histological evaluation were fixed in 10% neutral-buffered formalin overnight and transferred to 70% ethanol the next day for paraffin embedment using standard procedures. Skin sections (5 μm) were cut on a microtome and mounted on poly-lysine-treated glass slides until processing for immunohistochemistry. Two-thirds of the dorsal skin tissues were frozen using liquid nitrogen for DNA extraction.

Histological evaluation

The tissues were prepared for histological evaluation by using conventional paraffin sections and hematoxylin–eosin staining. Approximately, 1 cm^2 of each skin was preserved in formalin for immunohistochemistry. Epithelial thickness was determined, using an Olympus microscope B45 (Olympus Corp.; Leeds Instruments, Irving, TX), from at least 20 randomly selected sites in formalin-fixed skin samples.

Analysis of Ha-ras mutations in codon 61

Reverse transcription–polymerase chain reactions were carried out by using genomic DNA isolated from freshly frozen skin tissue by using DNAzol (MRC, Cincinnati, OH) according to the instructions provided. Detection of CAA → CTA transversion in codon 61 of Ha-*ras* gene was performed by DNA amplification using two different sets of primers: the first was able to amplify only wild-type of Ha-*ras* and the second, with mutated primer, was capable to amplify only mutated DNA (35). Substitution at the 3′ end, for example in a mutated primer, enables it to anneal only to a sequence with mutation, but in the case of wild-type DNA, it is impossible for polymerase to start DNA synthesis. For reverse transcription–polymerase chain reaction, the following primers were used: forward primer for both wild-type and mutant Ha-*ras*,

5′-CTAAGCCTGTTGTTTTGCAGGAC-3′; reverse primer for wild-type Ha-*ras*, 5′-CATGGCACTATACTCTTCTT-3′ and reverse primer for mutant Ha-*ras*, 5′-CATGGCACTATACTCTTCTA-3′. Polymerase chain reaction was performed at 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 58°C for 20 s and 78°C for 20 s.

Statistical analysis

The results were expressed as means ± SDs. For comparison of the differences between the groups, a two-tailed, unpaired, Student's *t*-test was used. A *P* value <0.05 was considered statistically significant.

Results

Quenching of free radicals

Many forms of cancer are the result of reactions between free radicals and DNA, resulting in mutations that can lead to malignancy. Free radicals and their metabolites are increasingly recognized for their contribution to tissue injury leading to both initiation and promotion of multistage carcinogenesis (36,37). Therefore, the antioxidants—which prevent free radicals from oxidizing sensitive biological molecules or reduce their formation—slow down the aging process and prevent disease. Antioxidants/free radical scavengers function as inhibitors of both tumor initiation and promotion (36).

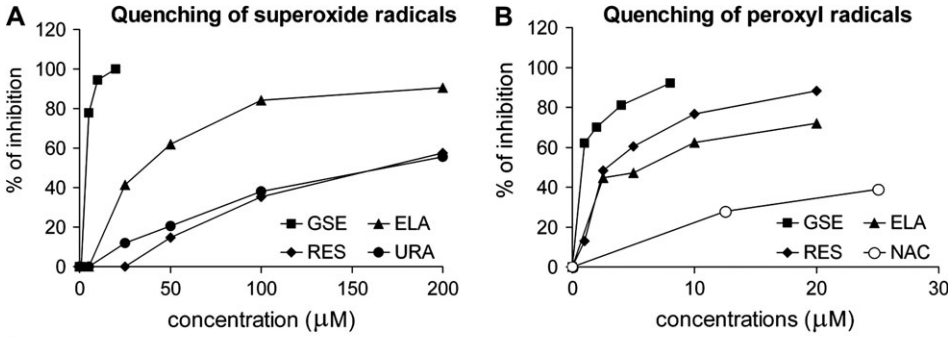
In the assay used to determine quenching of superoxide radicals, only four tested compounds showed the ability to quench superoxide radicals. The GSE showed the strongest effect with IC_{50} value of 0.8 μM (Figure 1A). ELA was next with IC_{50} of 31 μM. RES and URA were slightly less effective in this assay. They had IC_{50} of 162 and 167 μM, respectively. LYC and NAC were inactive in this assay.

To determine what compounds have the ability to scavenge the hydroxyl radical, we used a Fenton-like reaction. In this particular system, the majority of solvents demonstrate strong activity by themselves. Our data were achieved using only water-soluble compounds (data not shown). The ELA had the best effect with IC_{50} value of 60 μM. GSE also showed some activity at a higher concentration (>1 mM). NAC was a good scavenger in this assay, but its IC_{50} was ∼6 mM. RES was inactive at all tested concentrations. URA and LYC were not evaluated in this particular assay because of their insolubility in water.

The results shown in Figure 1B compare the peroxyl scavenging activity of GSE, ELA, RES and NAC using an assay based on autoxidation of linoleic acid at 40°C. The concentration of GSE required to cause 50% inhibition was 0.5 μM. RES and ELA also had strong scavenging abilities with IC_{50} of 2.8 and 4.4 μM, respectively. The IC_{50} for NAC was 41 μM, but for URA it was much higher (28.2 mM).

Growth inhibitory effects of individual test compounds

ATP is a marker for cell viability because it is present in all metabolically active cells and the concentration declines rapidly when the



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ATP (IC_{50})	GSE	RES	ELA	URA
3PC	21.6 μM	4.1 μM	75.1 μM	9 μM
MT1/2	19.9 μM	10.3 μM	79.7 μM	10.4 μM
Ca3/7	34.7 μM	5.2 μM	51.1 μM	11.5 μM

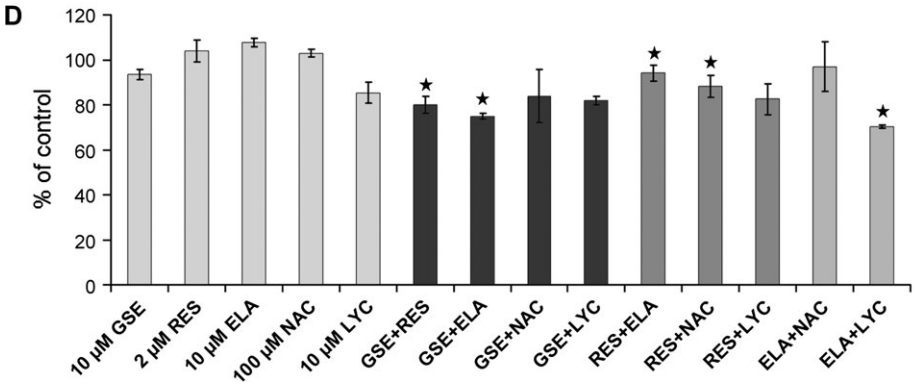

Fig. 1. (**A**) Superoxide scavenging (percentage inhibition of the cytochrome *c* reduction) by GSE (filled square), ELA (filled triangle), RES (filled diamond) and URA (filled circle) at 21°C. (**B**) Inhibition of auto-oxidation of linoleic acid to its conjugated diene hydroperoxide in the presence of GSE (filled square), ELA (filled triangle), RES (filled diamond) and NAC (open circle). (**C**) The viability of keratinocytes exposed to the compounds tested for 24 h in the ATP assay. Cells were treated with different concentrations of the compounds and the dose–response curves provided an IC_{50} values. (**D**) The growth inhibitory effect of the combined test compounds on MT1/2 papilloma cells. The concentrations 10 μM for GSE, 2 μM for RES, 10 μM for ELA, 100 μM for NAC and 10 μM for LYC were chosen. Interaction was examined after 24 h of treatment using the ATP assay. The cell viability is presented as a percent of control. *P* value (★) less than 0.05 was considered statistically significant.

cells undergo necrosis or apoptosis. The selected compounds were screened for their growth inhibitory effects using three keratinocyte cell lines. The ATP bioluminescence assay allowed us to determine the concentrations of these compounds inhibiting cell proliferation as well as the concentrations that are cytotoxic to a non-tumorigenic immortalized mouse skin cell line, a tumorigenic but non-malignant mouse papilloma cell line or a mouse skin carcinoma cell line.

Cells were treated with different concentrations of the selected compounds and the dose–response curves (data not shown) provided the IC_{50} values (Figure 1C). For GSE, the IC_{50} values ranged from 20 to 35 μM for all the three cell lines. The lowest effective concentration for RES was between 4 μM for 3PC and 10 μM for MT1/2 cells. Higher concentrations were needed in case of ELA. This compound appears to have the strongest inhibitory effect for Ca3/7 squamous carcinoma cells. The IC_{50} values for URA oscillated ∼10 μM. LYC and NAC showed only slight effects.

The growth inhibitory effect of combinations of the phytochemicals

To check the effect of combinations of two compounds on cell proliferation, we have chosen the following concentrations: 2 μM for RES, 10 μM for GSE, 10 μM for ELA, 10 μM for LYC and 100 μM for NAC. Cotreatment effect was examined after 24 h of treatment. Our results as shown in Figure 1D revealed that almost all combinations of RES and ELA caused a decrease in proliferation of MT1/2 cells when compared with individual treatments. A statistically significant effect was observed only for the MT1/2 papilloma cell line. The combined compounds appeared to have some effect on Ca3/7 cells, but not all combinations showed a statistically significant effect (data not shown).

Protective effect of tested phytochemicals against oxidative DNA damage induced by H_2O_2

There is a growing evidence that DNA is one of the most important targets of oxidative attack. If repair mechanisms fail to eliminate oxidative DNA damage, deleterious consequences for the cells may occur, including age-related dysfunctions and later development of malignancies (38). A useful method of assessing DNA damage is the single-cell gel electrophoresis, or 'comet' assay, which detects DNA strand breaks in individual cells (39).

Figure 2A shows that pretreatment of carcinoma cells Ca3/7 cells with the various compounds for 24 h resulted in a substantially reduced level of H_2O_2-induced DNA strand breaks as determined by the comet assay. The average tail length of the comets was 27 ± 9 μm in untreated cell culture, whereas the positive control (treated with hydrogen peroxide at 2 mM) showed a significant increase in the mean tail length (72 ± 14 μm). All the tested compounds resulted in comets with decreased lengths compared with those observed in the positive control. GSE and RES in a dose-dependent manner showed the most pronounced results. The tail lengths for the highest concentrations of the tested compounds were even shorter than in negative control. ELA and

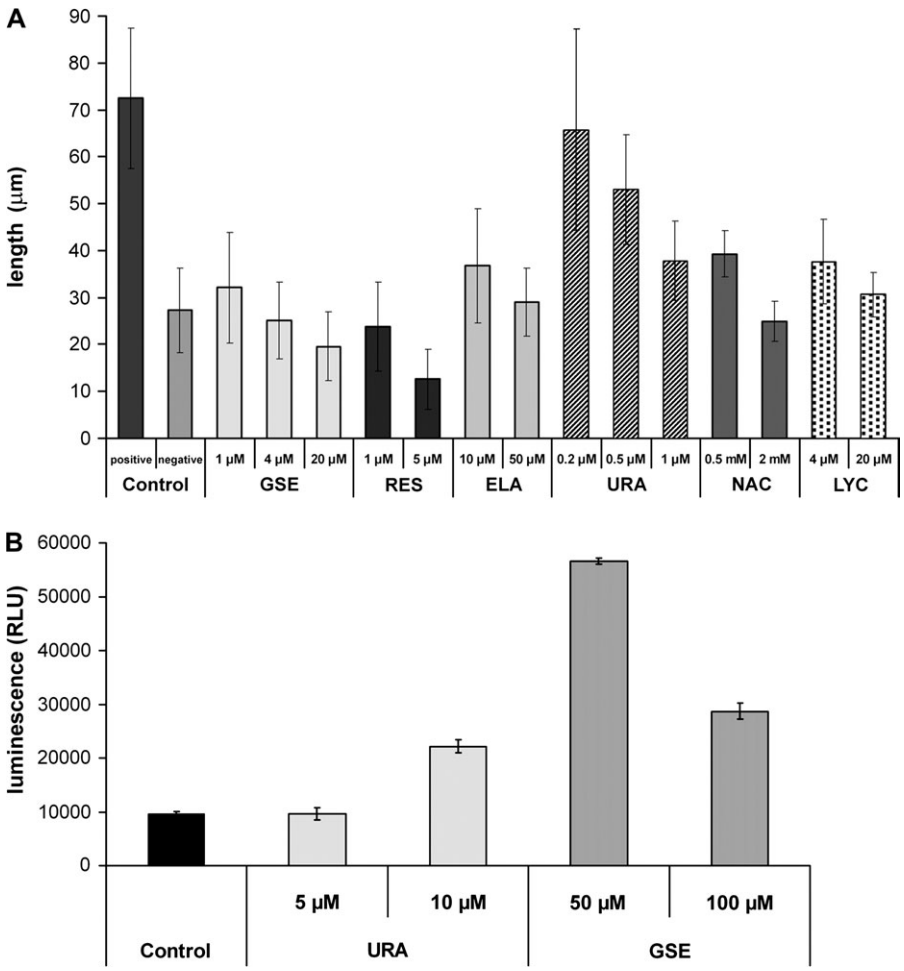

Fig. 2. (**A**) Effects of preincubation of the compounds tested on the rate of H_2O_2-mediated DNA damage in Ca3/7 cells, as determined by the comet assay. All compounds lowered the rate of H_2O_2-mediated DNA damage compared with the positive control. In each experiment, 50 cells were analyzed per sample and values are means ± SEM of the comet tail length. (**B**) To show the effect of GSE and URA on cellular caspase-3 and -7 activity, Ca3/7 cells were exposed to different concentrations of these compounds for 24 h. Activated caspase-3/-7 level was measured by conversion of aminoluciferin into a luminescent signal.

LYC also showed a good protective activity and at 50 and 20 μM, respectively, reached the tail length close to the value of negative control. URA showed the strongest effect at 1 μM concentration but higher doses were not more effective. NAC showed some effect at 2 mM. These results suggest that the tested compounds have a powerful potential to protect the genomic DNA of skin cells against oxidative stress.

Analysis of active caspase-3 and -7 level in Ca3/7 cells treated with the selected compounds

Because caspase-3 and -7 have a pivotal role in the intrinsic apoptosis pathway (40), the effect of the tested phytochemicals on cleavage of caspase-3/-7 by Caspase-Glo 3/7 assay was investigated. The Ca3/7 cell line was treated with different concentrations of the selected compounds for 24 h. RES and LYC had no effect on the activation of caspases in Ca3/7 carcinoma cells, whereas ELA showed a very small effect; NAC showed a 10% increase at 20 μM level. On the other hand, URA that showed a powerful effect on the viability of the cells in this assay increased caspases level 2-fold at 10 μM (Figure 2B). The best result was observed for GSE, which increased the level of caspases 6-fold at 50 μM.

Inhibitory effects of the selected phytochemicals on CYP1A1 and CYP1B1 activity

One of the most important factors determining the sensitivity of a tissue to carcinogenesis is the activation of the carcinogenic agent by the cytochrome p450 system. DMBA is a procarcinogen and requires metabolic conversion to its ultimate carcinogenic metabolite by oxidation by CYP1A1 and 1B1. CYP enzymes are known to be expressed at high level in various human cancers, including breast, colon, lung, skin and brain. On the other hand, CYP1B1 is expressed at a high frequency in various cancers, but not in normal tissues (41). CYP1B1 is not required for mouse development because CYP1B1-null mice show no obvious change in phenotype (42). However, CYP1B1 is critical for carcinogenesis because CYP1B1 knockout mice are more resistant to DMBA-induced tumor formation than wild ones. Blocking CYP1A1 in CYP1B1(−/−) mice eliminated the metabolic activation of DMBA to DNA-binding products (43).

CYP1A1 and CYP1B1 activities were measured *in vitro*. All the tested compounds (except NAC) suppressed their activity in a concentration-dependent manner (Figure 3). The best results were obtained for RES. We found that IC_{50} for RES is 10-fold higher for CYP1B1 than for 1A1. Interestingly, the effect of 7,8-benzoflavone also displays a 10-fold difference in potency against 1B1 versus 1A1 as published by Kleiner *et al.* (11). We are now planning to study if the RES is able to inhibit metabolic activation of DMBA in murine skin similar to 7,8-benzoflavone, in order to be able to explain 10-fold difference in the activity of 1B1 > 1A1. As to the other phytochemicals, GSE showed good inhibitory activities with IC_{50} of ∼2.5 μM for both cytochromes. ELA inhibited both CYP1A1 and CYP1B1 but showed the strongest effect for CYP1B1 (IC_{50} 2.1 μM). LYC showed similar strength, but its activity was directed more to CYP1A1 (IC_{50} 9.9 μM). The suppressive effect of URA was much less pronounced.

Effects of the selected compounds on sustained epidermal hyperplasia in SENCAR mice

The induction of epidermal hyperplasia after multiple treatments with each compound tested is showed in Figure 4A. The results are the averages of 20 measurements at various locations along the epidermis of the skin specimen from each treatment group. We have found out that DMBA alone increased epidermal thickness 4.6 times from on average 15.5 ± 2.7 μm in normal mouse skin to 69.7 ± 4.7 μm in DMBA-treated skin. All phytochemicals inhibited hyperplasia in a dose-dependent manner. The most potent inhibition was observed in the group of mice treated with ELA that reduced the DMBA-induced epidermal thickness in a clear dose-dependent manner from 0.5 to 5.0 μmol of ELA. The observed decrease of epidermal thickness in this group reached almost the normal skin epidermal thickness level. URA was also able to reduce the DMBA-induced epidermal thickness by up to 50%; however, we did not observe significant differences between different concentrations used in this study. RES had the weakest effect on decreasing DMBA-induced epidermal thickness. However, almost all tested phytochemicals showed statistically significant ($P < 0.05$), dose-dependent inhibitory effects on DMBA-induced hyperplasia.

Effect of phytochemicals on mutations in codon 61 of Ha-ras oncogene in skin of SENCAR mice

After initiation with DMBA *in vivo*, the majority of the papillomas that appear have an A to T mutation in the second position of codon 61

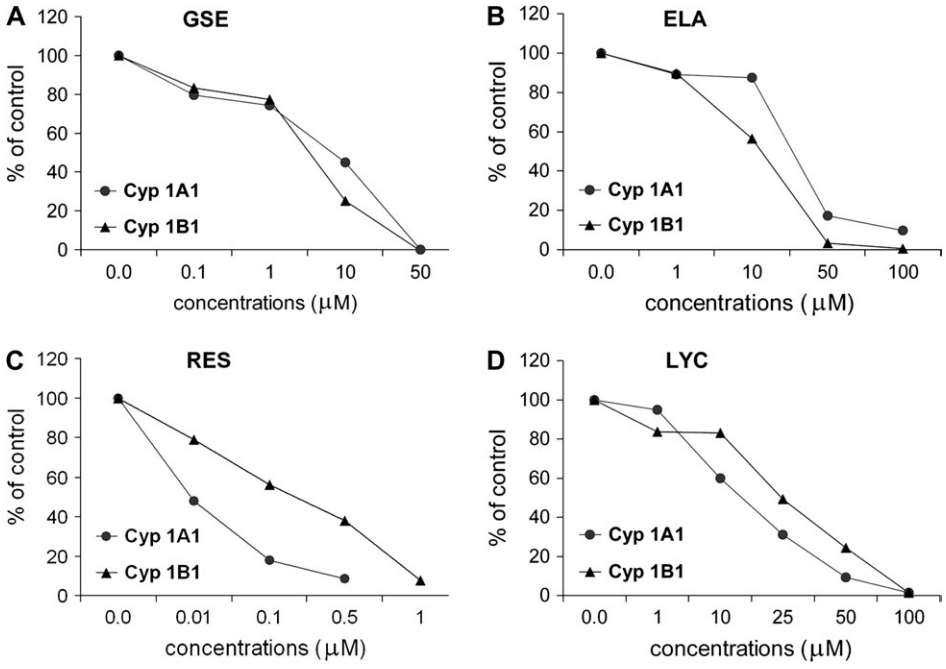

Fig. 3. Inhibition of CYP1A1 and CYP1B1 activities by GSE (**A**), ELA (**B**), RES (**C**) and LYC (**D**).

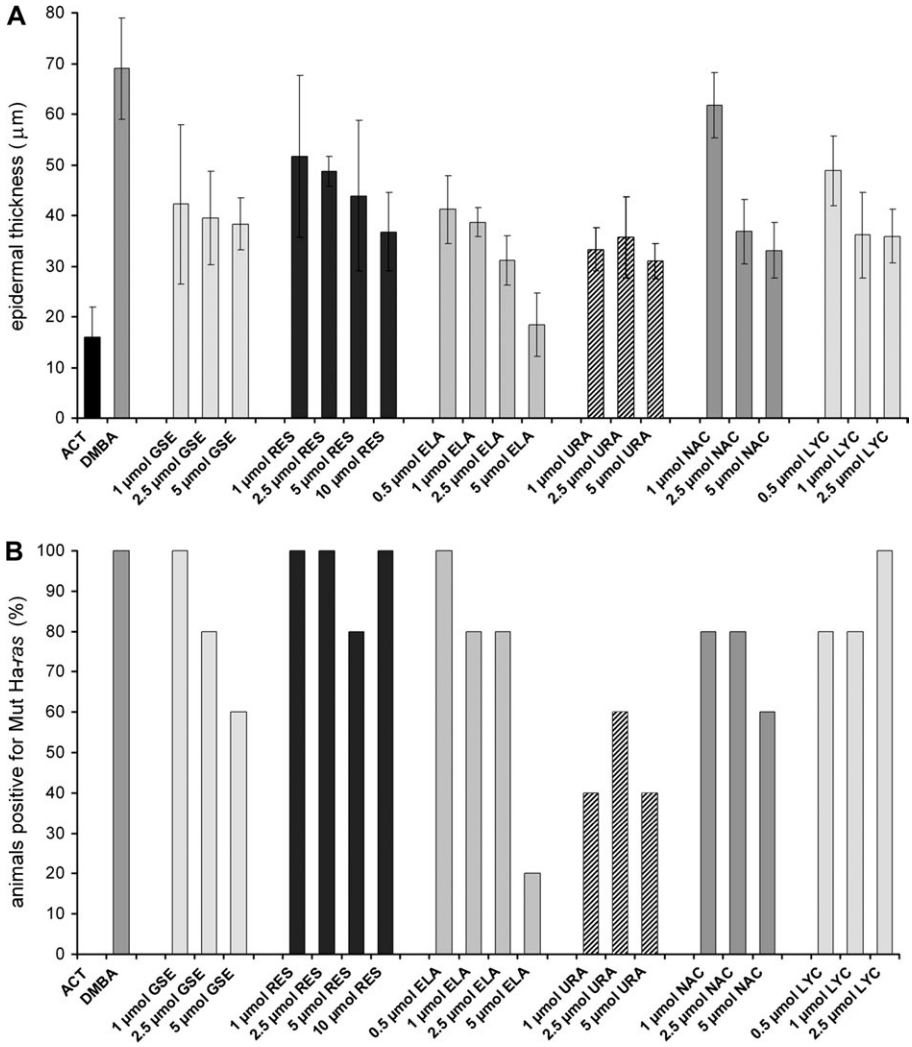

Fig. 4. (**A**) Effects of the test compounds on sustained epidermal hyperplasia after multiple treatments with each compound. The results are the averages of 20 measurements at various locations along the epidermis of the skin specimen from each treatment group. (**B**) Mutations in codon 61 of Ha-*ras* oncogene were analyzed in skin of SENCAR mice treated with the test compounds.

of the Ha-*ras* oncogene. This transversion is thought to be the initiating event (44). The mutation can be detected very early after the initiating treatment. Most of the tested compounds showed a dose-dependent inhibitory effect on the number of animals with Ha-*ras* mutations (Figure 4B). However, treatments with RES or LYC seem to be ineffective in inhibiting Ha-*ras* mutations. The best results were obtained for URA and ELA (60 and 80% inhibition, respectively), at 5 μmol doses. Both URA and ELA showed not only a strong anti-initiation effects but also a strong antitumor promotion effects, i.e. on DMBA-induced hyperplasia.

Discussion

The current study was designed to further characterize the effects of GSE, ELA, RES, URA, LYC and NAC on critical events in skin tumor initiation and promotion. We found that the tested compounds exhibited scavenging activity for reactive oxygen species with variable efficiency. Several of the scavenging effects are reported for the first time in this study. ELA was found to be active against all tested oxygen radicals at the micromolar concentrations. The others (except NAC) were very effective in peroxyl and superoxide radicals scavenging.

According to our investigations, GSE, RES, ELA and URA are potent inhibitors of skin cells growth. The inhibiting effects observed at the lowest concentrations were visible for URA and RES for all cell lines representing different stages of skin tumorigenesis. On the other hand, ELA was a more effective growth inhibitor for the carcinoma cell line; the concentration necessary for 50% inhibition was 25 μM lower than that required for papilloma and non-tumorigenic cell lines. At the same time, ELA and GSE were the most effective toward eliminating free radicals.

The integrity of DNA is vital to cell division and oxidative alterations can disrupt transcription, translation, DNA replication and can give rise to mutations, cell senescence and death. DNA damage may either cause cell death or initiate several error-free or -prone repair pathways (45). To evaluate the extent of oxidative DNA damage induced by hydrogen peroxide in the skin cancer cell lines and the potential protective activity of different phytochemicals, DNA damage was evaluated by alkaline single-cell gel electrophoresis (comet assay). Hydrogen peroxide is a standard agent to induce DNA damage and it induces mainly oxidative modifications to the DNA bases. To examine the resistance of DNA to H_2O_2-induced DNA strand breaks, the Ca3/7 cells were exposed to hydrogen peroxide at 2 μM for 10 min. All the tested compounds showed very strong protective effects against DNA damage caused by hydrogen peroxide.

The initiation of a cascade of cysteine proteases family (caspases) plays a pivotal role in apoptosis. The results suggest that URA and GSE may exert their antiproliferative effects through the activation of caspases. ELA and NAC also showed marginal effects. There are

several reported studies showing that the compounds tested can induce apoptosis in different cell lines, i.e. ELA in cervical carcinoma (CaSki) cells (46), RES in human monocytic leukemia cells lines (47) or NAC in rat and human smooth muscle cells (48). On the other hand, they can also prevent apoptosis, like NAC in lymphocytes, neurons or vascular endothelial cells (49,50) or RES that can prevent ethanol-associated apoptosis (51).

In mouse skin, polycyclic aromatic hydrocarbons have been extensively studied for their carcinogenic potency and it is clear that they possess a wide range of carcinogenic activities in this tissue. The bioactivation of DMBA to mutagenic and carcinogenic metabolites involves the formation of both syn- and anti-7,12-dimethylbenz[*a*]anthracene-3,4-diol-1,2-epoxides (DMBADEs), which can bind to both deoxyadenosine and deoxyguanosine residues in DNA (52). Both the syn- and the anti-DMBADE are tumor initiators in mouse epidermis (53). CYP1A1 seems to be the major murine P450 family member involved in the formation of anti-DMBADE–DNA adducts, whereas CYP1B1 is the predominant murine P450 family member involved in formation of syn-DMBADE–DNA adducts (43). Unlike CYP1A1 that is crucial in the bioactivation of benzo[*a*]pyrene in mouse epidermis, both CYP1A1 and CYP1B1 are involved in the metabolic activation of DMBA in mouse epidermis (54). CYP1B1 also plays an important role in anticancer drug resistance because metabolism of various anticancer agents by CYP1B1 causes suppression of the pharmacological effects of drugs (55).

The metabolism of carcinogenic compounds, such as BaP or DMBA, depends on the relative expression of bioactivating and detoxifying enzymes. Thus, the induction or inhibition of CYPs may alter the balance between activation and detoxification. One of the goals of this study was to investigate the inhibitory effect of the tested compounds on CYP1A1 and CYP1B1 activity. Decreased metabolic activation of carcinogens via direct inhibition of CYPs activity or through modulation of their expression pathway is believed to be an important mechanism of cancer prevention (11). In the present study, we demonstrated that GSE, RES, LYC and ELA but not URA or NAC were strong inhibitors of human CYP1 enzymes.

In conclusion, the phytochemicals selected for the present study were found to be good protective agents against free radicals' and CYPs' activity. Some of them (URA and GSE) also induced apoptosis. All tested compounds protected genomic DNA from oxidative damage. Not all the test compounds decreased proliferation of non-tumorigenic and tumorigenic keratinocytes but some of them (RES and ELA) showed a better effect on cell proliferation when used in combination. In *in vivo* studies, we have found that all the selected compounds caused marked decreases of epidermal thickness (antitumor promotion effect) compared with DMBA-treated group. Also, all selected compounds (except RES) reduced percentages of mice with mutation in 61 codon of Ha-*ras* (anti-initiation effect). In fact, differential effects of the selected phytochemicals and their derivatives on events and processes critical for the growth inhibition of keratinocytes *in vitro* and *in vivo* indicate the need to use combinations of these agents to counteract detrimental effects of carcinogenesis and tumor promotion. The present study and future *in vitro* studies of the tested compounds may indicate which combinations of compounds are able to give the most promising effects in animal experiments. Of great importance would be identification of meaningful interactions and a comprehensive analysis of interactive effects of chemopreventive agents using established methods for evaluating additivity or synergy, such as the median effect analysis. A comprehensive analysis of interactive effects of phytochemicals is carried out in our next *in vivo* study using the median effect equation as described by Chou *et al.* (56). Synergy, when it can be convincingly established, is an effective strategy for the development of novel test compounds combinations. We are currently working on evaluation of the interactions between the different phytochemicals able to lower initiation and promotion/progression steps.

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