

## RESEARCH ARTICLE

# Cellular DNA breakage by soy isoflavone genistein and its methylated structural analogue biochanin A

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Epidemiological studies have indicated that populations with high isoflavone intake through soy consumption have lower rates of breast, prostate, and colon cancer. The isoflavone polyphenol genistein in soybean is considered to be a potent chemopreventive agent against cancer. In order to explore the chemical basis of chemopreventive activity of genistein, in this paper we have examined the structure–activity relationship between genistein and its structural analogue biochanin A. We show that both genistein and its methylated derivative biochanin A are able to mobilize nuclear copper in human lymphocyte, leading to degradation of cellular DNA. However, the relative rate of DNA breakage was greater in the case of genistein. Further, the cellular DNA degradation was inhibited by copper chelator (neocuproine/bathocuproine) but not by compounds that specifically bind iron and zinc (desferrioxamine mesylate and histidine, respectively). We also compared the antioxidant activity of the two isoflavones against *tert*-butylhydroperoxide-induced oxidative breakage in lymphocytes. Again genistein was found to be more effective than biochanin A in providing protection against oxidative stress induced by *tert*-butylhydroperoxide. It would therefore appear that the structural features of isoflavones that are important for antioxidant properties are also the ones that contribute to their pro-oxidant action through a mechanism that involves redox cycling of chromatin-bound nuclear copper.

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## 1 Introduction

Cancer development is a dynamic and long-term process that involves many complex factors through critical steps of initiation, promotion, and progression, leading to an uncontrolled growth of cancerous cells throughout the body. It is believed that dietary constituents derived from plant sources have the ability to modify the process of carcinogenesis, thus

relating the foodstuffs, beyond their basic nutritional benefits, to disease prevention [1, 2]. Consistent with this observation are the epidemiological findings associating high soy consumption with lower incidences of breast, prostate, and colon cancer in Asian countries, particularly in Japan [3–5]. It has been suggested that the isoflavone constituents provide at least part of the protective effect of soy food [6]. Genistein, a predominant isoflavone present in soy, has been shown to have potent anticancer properties both *in vitro* and *in vivo* [7, 8]. However, genistein and some other isoflavones have also been shown to stimulate the growth of estrogen receptor positive breast cancer cells [9] and to induce endometrial stromal cell proliferation *in vitro* [10]. The 4'-O-methyl derivative of genistein, biochanin A that is a major isoflavone constituent in red clover (*Trifolium pratense*), has also been reported to possess cytotoxic properties against cancer cells [11]. Various other polyphenolic compounds such as

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**Abbreviations:** FOX, ferrous oxidation-xylenol orange; ROS, reactive oxygen species; SOD, superoxide dismutase; TBHP, *tert*-butylhydroperoxide

flavonoids, tannins, curcuminoids, gallocatechins, stilbenes, and anthocyanidins have been implicated as chemopreventive agents [12]. However, the mechanism by which these compounds inhibit proliferation and induce apoptosis in cancer cells has been the subject of considerable interest. In recent years, several reports have documented that plant polyphenolics including genistein (from soybean) induce apoptosis in various cancer cell lines [13–18]. Of particular interest is the observation that a number of these polyphenols including EGCG, gallic acid, resveratrol, and genistein induce apoptotic cell death in various cell lines but not in normal cells [14–16, 19].

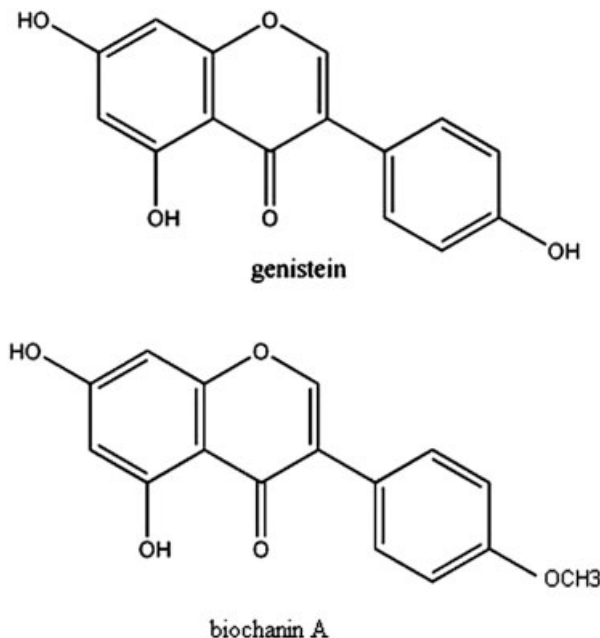
Earlier studies in our laboratory have shown that various classes of plant polyphenols including flavonoids [20–25] cause oxidative strand breakage in DNA either alone or in the presence of transition metal ions such as copper. Copper is an important metal ion present in chromatin and is closely associated with DNA bases, particularly guanine [26]. It is one of the most redox active of the various metal ions present in cells. Most of the plant polyphenols possess both antioxidant as well as pro-oxidant properties [15, 20] and we have earlier proposed that the pro-oxidant cytotoxic action of polyphenolics may be an important mechanism of their anticancer- and apoptosis-inducing properties [27, 28]. Such a mechanism for the cytotoxic action of these compounds against cancer cells would involve mobilization of endogenous copper ions and the consequent pro-oxidant action.

The chemopreventive properties of genistein are well documented [7, 8, 29]. In order to explore the chemical basis of the chemopreventive activity of genistein, in this paper, we have examined the structure–activity relationship between genistein and its structural analogue biochanin A (Fig. 1). Using a cellular system of peripheral lymphocytes isolated from human blood and alkaline single-cell gel electrophoresis (Comet assay), we show that both genistein and biochanin A are able to cause oxidative DNA breakage in cells, however, with different efficiencies. Further, we also studied the antioxidant potential of the two isoflavones against *tert*-butylhydroperoxide (TBHP)-induced oxidative stress. These studies suggest that both the pro-oxidant and antioxidant properties of isoflavones are determined by the same structural pattern.

## 2 Materials and methods

### 2.1 Materials

Genistein, biochanin A, ascorbic acid, tannic acid, neocuproine, bathocuproine disulphonate, superoxide dismutase (SOD), agarose, low melting point agarose, RPMI 1640, Triton X-100, Trypan blue, Histopaque1077, and PBS  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade. Fresh solutions of genistein and biochanin A were prepared as a stock of 2.5 mM in absolute methanol. In all the studies (except ferrous oxidation-xylenol orange (FOX) assay), the reaction mixture



**Figure 1.** Chemical structures of isoflavone genistein and biochanin A.

contained  $\leq 2\%$  methanol (v/v). To test any effect of solvent on the DNA breakage and antioxidant studies, methanol solution was added to the cells at the final concentration of 2% v/v, which was the highest concentration of methanol used in the isoflavone-treated reaction medium. No difference was observed with or without methanol, indicating that the methanol at the tested concentrations did not influence the results. For FOX assay, the solvent control concentration varied between 1 and 12% v/v and again no influence of the solvent was observed on the results. On addition to reaction mixtures, in the presence of buffers mentioned and at concentrations used, all the polyphenols used remained in the solution. The volumes of stock solution added did not lead to any appreciable change in the pH of reaction mixtures.

### 2.2 Isolation of lymphocytes

Heparinized blood samples (2 mL) from a single healthy donor was obtained by venepuncture and diluted suitably in  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma) and the cells were finally suspended in RPMI 1640. A single donor (M. F. Ullah) donated blood for all experiments.

### 2.3 Viability assessment of lymphocytes

The lymphocytes were checked for their viability before the start and after the end of the reaction using Trypan Blue Exclusion Test [30].

## 2.4 Treatment of lymphocytes and evaluation of DNA breakage by alkaline single-cell gel electrophoresis (Comet assay)

Lymphocytes isolated from 2 mL blood were diluted to the count of  $2 \times 10^5$  cells/2 mL and suspended in RPMI 1640. Approximately 10 000 of these cells were mixed with 75  $\mu$ L of pre-warmed low melting point agarose in PBS and immediately applied to a frosted microscopic slide layered with 75  $\mu$ L of 1% standard agarose in PBS. The slides were allowed to gel at 4°C for 10 min. The layered lymphocytes on slides were then subjected to treatment with isoflavones and processed further for Comet assay as already described [31].

Treatment of lymphocytes with isoflavones was also carried out using permeabilized cells [32]. For this purpose, lymphocytes prior to treatment with isoflavones were exposed to the permeabilization solution (0.5% Triton X-100 in 0.004 M Tris-HCl, pH 7.4) for 10 min on ice. For treatment, each slide was then transferred to a rectangular dish (8 cm  $\times$  3 cm  $\times$  5 mm) that contained a reaction mixture of isoflavones and other additions as mentioned in various legends to figures and tables. The slides with the reaction mixture were incubated at 37°C for desired time periods (1 h for whole cells/30 min for permeabilized cells) and were then washed twice by placing in 0.4 M phosphate buffer pH 7.5 for 5 min at room temperature before being processed further for Comet assay.

For antioxidant study [33], the cells were preincubated with isoflavones in eppendorf tubes in a reaction volume of 1 mL. After the preincubation (for 30 min at 37°C), the reaction mixture was centrifuged at 4000 rpm, the supernatant was discarded, and the pelleted lymphocytes were re-suspended in 100  $\mu$ L of PBS ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free) and layered for further treatment with TBHP (50  $\mu$ M). The incubation period was 30 min at 37°C in dark. The other conditions remained the same as described earlier.

## 2.5 Stoichiometric titration of Cu(I) production

The stoichiometry of Cu(I) production was measured by mixing the isoflavones (25  $\mu$ M) in 10 mM Tris-HCl; pH 7.5, with varying amounts of  $\text{CuCl}_2$  (5–200  $\mu$ M) and a stock bathocuproine solution to give a final concentration of 0.4 mM in a total volume of 3 mL. Bathocuproine complexes with Cu(I) to form a  $\text{Cu}(\text{Bathocuproine})_2^+$  complex, which absorbs maximally at 480 nm. Absorbance was recorded at 480 nm after 1 h incubation at 37°C in dark [34].

## 2.6 Detection of $\text{H}_2\text{O}_2$ in the incubation medium by FOX assay

The FOX assay [35] was adapted to detect and quantify the generation of  $\text{H}_2\text{O}_2$  in the incubation medium (RPMI 1640, phosphate buffer 0.4 M, pH 7.5) by various isoflavones. The

simplified reaction sequence involves the oxidation of ferrous ( $\text{Fe}^{2+}$ ) to ferric ( $\text{Fe}^{3+}$ ) ions by  $\text{H}_2\text{O}_2$  with the subsequent binding of the  $\text{Fe}^{3+}$  ion to the ferric-sensitive dye xlenol orange, yielding an orange to purple complex, which is measured at 560 nm. The reaction mixture contained the isoflavones along with the incubation medium used in the treatment of lymphocytes. After incubation for 2 h at 37°C, an aliquot of 200  $\mu$ L was analyzed for  $\text{H}_2\text{O}_2$  formation [31].

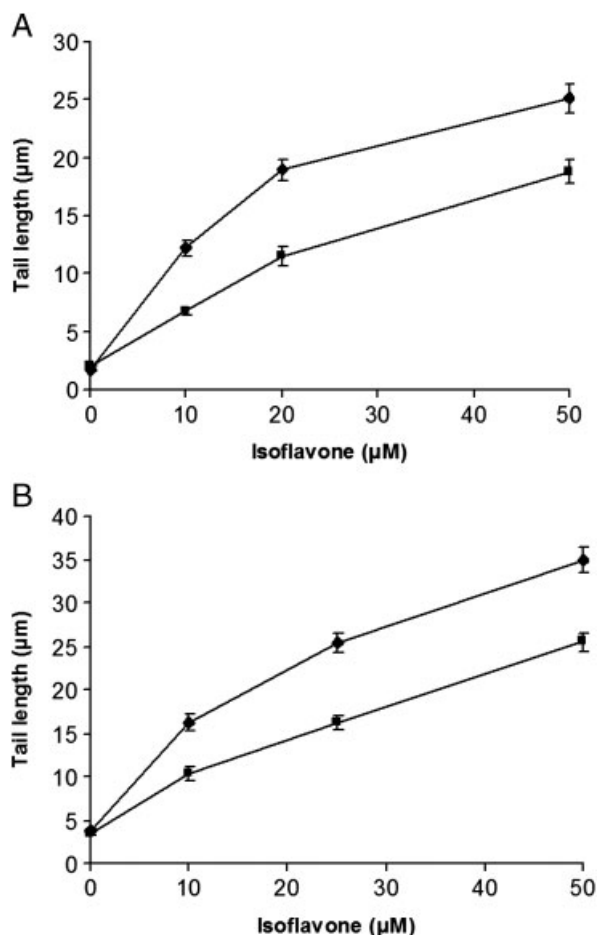
## 2.7 Statistics

The statistical analysis was performed as described by Tice *et al.* [36] and is expressed as  $\pm$  SEM of three independent experiments. A student's *t*-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. *p*-Values  $< 0.05$  were considered statistically significant.

# 3 Results

## 3.1 DNA breakage by isoflavones in cellular and permeabilized lymphocytes as measured by Comet assay

Single-cell gel electrophoresis (Comet assay) is a sensitive technique to quantify DNA damage in cellular system. In the original version of the assay, cells embedded in agarose microscopic slides were lysed and subjected to electrophoresis under neutral conditions enabling the detection of DNA double-strand breaks [37]. A later modification using alkaline conditions made it possible to detect DNA single-strand breaks and alkaline-labile sites in DNA [38]. Further, in case two single-strand breaks are closely opposed, they show up as a double strand break. We have previously shown that the various classes of polyphenols on incubation with human peripheral lymphocytes are able to degrade cellular DNA and that such degradation can be measured by Comet assay [25, 39]. Studies from other laboratories such as by Cemeli *et al.* have also used Comet assay to demonstrate that estrogen-like compounds such as diethylstilbestrol, daidzein, and genistein induce DNA breakage in lymphocytes that is mediated by reactive oxygen species (ROS) [40]. In the present experiment, increasing concentrations of genistein and biochanin A (10, 20, and 50  $\mu$ M) were tested for DNA breakage in isolated lymphocytes using alkaline single-cell gel electrophoresis (Comet assay). As can be seen in Fig. 2A, there is a dose-dependant increase in the DNA breakage induced by genistein and biochanin A with clear tail lengths indicative of significant breakage of cellular DNA. We further tested the effect of these isoflavones on permeabilized lymphocytes at the same concentrations (Fig. 2B). Use of permeabilized lymphocytes allows the direct interaction of isoflavones with cell nuclei at physiological pH, eliminating the need to first



**Figure 2.** A comparison of DNA breakage induced by genistein (◆) and biochanin A (■) in (A) intact lymphocytes and (B) permeabilized lymphocytes as a function of comet tail lengths. Values reported are  $\pm$  SEM of three independent experiments.  $p < 0.01$  by comparison with control (untreated cells).

lyse the cells at alkaline pH and then re-suspending them at neutral pH as we have done previously [31]. Thus, considerably greater DNA breakage should be observed in permeabilized lymphocytes as compared with the intact cells. Figure 2B in comparison to Fig. 2A shows that the rate of tail formation induced by genistein and biochanin A is greater in the case of permeabilized lymphocytes suggesting that isoflavones are able to interact with the nuclei when a permeabilized system is used. However, in both intact and permeabilized cells, the rate of DNA breakage is greater in the case of genistein relative to biochanin A.

### 3.2 Effect of reactive oxygen scavengers on the isoflavone-induced DNA breakage in permeabilized lymphocytes

It is previously established from our work that the plant polyphenol-mediated DNA cleavage is inhibited to signifi-

cant degrees by various scavengers of ROS [31, 39]. Table 1 gives the result of an experiment where the effect of catalase, SOD, and thiourea were tested on isoflavone-induced DNA degradation in permeabilized lymphocytes. All three caused significant inhibition of DNA breakage as evidenced by the decreased tail length of comets. Catalase and SOD remove  $H_2O_2$  and superoxide, respectively, and thiourea removes hydroxyl radicals. From the data, we conclude that the isoflavone-induced cellular DNA degradation is the result of the formation of ROS of which superoxide anion and the hydroxyl radical are the proximal DNA damaging agents. It is to be noted that the generation of superoxide anion may spontaneously lead to the formation of  $H_2O_2$ , which in turn causes the formation of hydroxyl radical through oxidation of reduced transition metals such as copper (Fenton reaction) [41]. However, it may be mentioned that due to the site-specific nature of the reaction of hydroxyl radicals with DNA, it is difficult for any trapping molecule to intercept them completely [32].

### 3.3 Effect of metal-specific chelators on the isoflavone-induced DNA breakage in cellular and permeabilized lymphocytes

In the experiment shown in Table 2, we have used various metal-specific chelators, which selectively bind to copper, iron, and zinc, to study their effect on isoflavone-induced DNA degradation in whole lymphocytes as well as in permeabilized cells. In whole cells, a clear inhibition of DNA degradation was observed with neocuproine (a cell membrane-permeable Cu(I)-specific chelator). Further, no such inhibition was seen with bathocuproine (a water-soluble membrane-impermeable analogue of neocuproine), desferrioxamine mesylate (an Fe(II)-specific chelator), and histidine (a zinc-specific chelator). However, in permeabilized lymphocytes, both neocuproine and bathocuproine were found to inhibit the DNA breakage while iron and zinc

**Table 1.** Effect of scavengers of reactive oxygen species on isoflavone-induced DNA breakage in permeabilized lymphocytes

Dose	Tail length (μm)	Inhibition (%)
Untreated	02.53 $\pm$ 0.17	
<i>Genistein</i> (50 μM)	35.24 $\pm$ 2.82 <sup>#</sup>	
Catalase (100 μg/mL)	24.39 $\pm$ 2.14 <sup>*</sup>	30.72
SOD (100 μg/mL)	20.62 $\pm$ 1.65 <sup>*</sup>	41.48
Thiourea (1 mM)	14.67 $\pm$ 1.18 <sup>*</sup>	58.37
<i>Biochanin A</i> (50 μM)	27.19 $\pm$ 2.31 <sup>#</sup>	
Catalase (100 μg/mL)	17.05 $\pm$ 1.23 <sup>*</sup>	37.29
SOD (100 μg/mL)	15.28 $\pm$ 1.17 <sup>*</sup>	43.80
Thiourea (1 mM)	10.12 $\pm$ 1.01 <sup>*</sup>	62.78

<sup>\*</sup> $p < 0.05$  as compared with control (#). Data represent  $\pm$  SEM of three independent experiments

**Table 2.** Effect of various metal chelators on DNA degradation in lymphocytes induced by isoflavones

Dose	Whole lymphocytes		Permeabilized lymphocytes	
	Tail length	% of Control	Tail length	% of Control
Genistein (50 $\mu$ M)	26.32 $\pm$ 2.13*	–	35.76 $\pm$ 2.67**	–
Neocuproine (100 $\mu$ M)	17.16 $\pm$ 0.78	65.19 $\pm$ 4.53	24.84 $\pm$ 1.29	69.49 $\pm$ 5.21
Bathocuproine (100 $\mu$ M)	26.04 $\pm$ 1.80	98.93 $\pm$ 6.85	20.42 $\pm$ 0.69	57.12 $\pm$ 3.41
Histidine (100 $\mu$ M)	25.59 $\pm$ 1.63	97.26 $\pm$ 6.37	36.20 $\pm$ 2.81	101.25 $\pm$ 7.79
Desferioxamine mesylate (100 $\mu$ M)	26.70 $\pm$ 2.01	101.48 $\pm$ 7.28	35.24 $\pm$ 2.30	98.57 $\pm$ 6.53
Biochanin A (50 $\mu$ M)	20.76 $\pm$ 1.82*	–	26.94 $\pm$ 2.21**	–
Neocuproine (100 $\mu$ M)	09.34 $\pm$ 0.27	44.99 $\pm$ 2.93	15.24 $\pm$ 0.56	56.57 $\pm$ 3.68
Bathocuproine (100 $\mu$ M)	20.80 $\pm$ 1.52	100.24 $\pm$ 7.32	10.58 $\pm$ 0.32	39.30 $\pm$ 2.98
Histidine (100 $\mu$ M)	21.53 $\pm$ 2.10	103.75 $\pm$ 9.77	26.17 $\pm$ 2.00	97.17 $\pm$ 7.65
Desferioxamine mesylate (100 $\mu$ M)	20.65 $\pm$ 1.72	99.51 $\pm$ 8.37	26.46 $\pm$ 2.20	98.21 $\pm$ 8.32

The values shown represent isoflavone-induced DNA breakage in whole lymphocytes and permeabilized lymphocytes in the presence of metal chelators measured as a percentage of the control (DNA breakage induced by isoflavone in the absence of any chelator). Data represent  $\pm$ SEM of three independent experiments.

\* $p < 0.05$  as compared with untreated whole lymphocytes (tail length =  $2.30 \pm 0.13$ ).

\*\* $p < 0.05$  as compared with untreated permeabilized lymphocytes (tail length =  $2.72 \pm 0.17$ ).

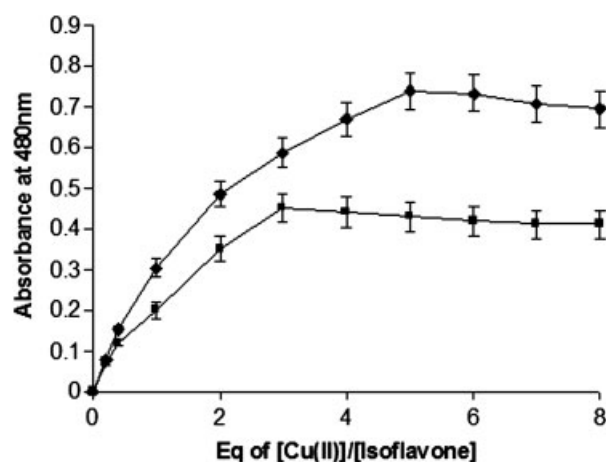
chelators were still ineffective in causing such inhibition. Bathocuproine disulphonic acid, which is impermeable to cell membrane, may freely traverse through permeabilized cells to directly interact with the cell nuclei. Further, it may be noted that the nuclear pore complex is permeable to small molecules. Therefore, we take these results to suggest that isoflavones mobilize chromatin-bound copper, leading to oxidative DNA breakage.

### 3.4 Stoichiometry of Cu(II) reduction by genistein and biochanin A

It has been previously suggested that the redox cycling of Cu(II)/Cu(I) is an essential element in the induction of copper-assisted oxidative DNA breakage [27]. To assess the relative efficiency of reduction of Cu(II) by genistein and biochanin A, stoichiometric studies were carried out. Job plots of absorbance *versus* [Cu(II)]/[Isoflavone] show that genistein and biochanin A reduce Cu(II) to Cu(I) with an approximate stoichiometry of 1:5 and 1:3, respectively (Fig. 3). It would appear from the results that both genistein and biochanin A can bind and reduce Cu(II). However, it can be inferred from the figure that genistein is more efficient reducer of Cu(II) than biochanin A.

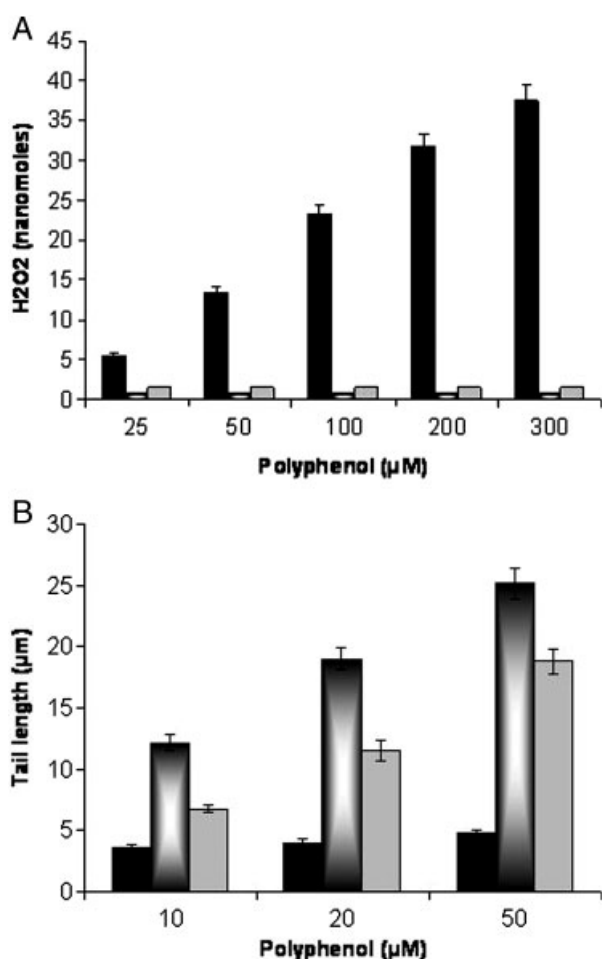
### 3.5 H<sub>2</sub>O<sub>2</sub> generation by isoflavones in the incubation medium

It is well known that polyphenols auto-oxidize in cell culture media to generate H<sub>2</sub>O<sub>2</sub> and quinone that can enter cells/nuclei causing damage to various macromolecules [42]. This may lead to extraneous production of ROS that could also account for cellular DNA breakage. In order to examine this



**Figure 3.** Detection of stoichiometry of isoflavone–Cu(II) interaction. The concentration of genistein (◆) and biochanin A (■) were 25  $\mu$ M in the presence of 0.4 mM bathocuproine. The absorbance of samples at 480 nm is plotted *versus* equivalents of Cu(II) per molar equivalent of compounds. All points represent triplicate samples and the mean values are plotted.

possibility we have determined the formation of H<sub>2</sub>O<sub>2</sub> by isoflavones in the incubation medium and compared it with a known generator of H<sub>2</sub>O<sub>2</sub>, namely tannic acid [43]. In the results given in Fig. 4A, we show that the rate of formation of H<sub>2</sub>O<sub>2</sub> by tannic acid in the incubation medium is considerably greater whereas that by isoflavones is relatively negligible. Further, we have also compared the comet tail length formation as a function of increasing concentrations of isoflavones and tannic acid. As seen in Fig. 4A, whereas isoflavones show significant tail formation in a dose-dependant manner, tannic acid is not effective in causing DNA breakage. These results indicate that cellular DNA

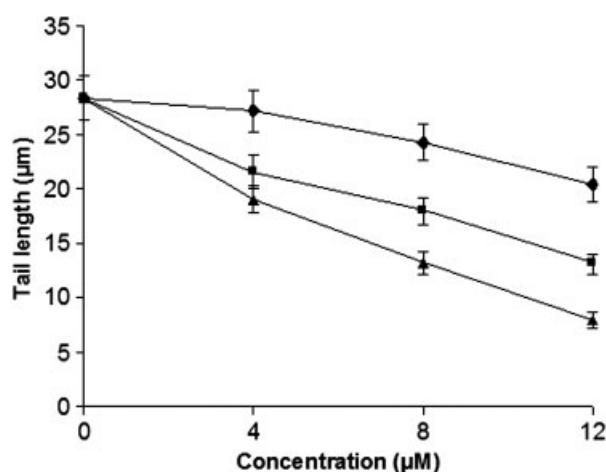


**Figure 4.** (A) A comparison of the rate of H<sub>2</sub>O<sub>2</sub> formation by tannic acid (■), genistein (▨), and biochanin A (□) in the incubation medium as determined by FOX assay. (B) A comparison of DNA breakage induced by tannic acid (■), genistein (▨), and biochanin A (□) in human peripheral lymphocytes as a function of comet tail lengths. Values reported are  $\pm$ SEM of three independent experiments.

breakage by isoflavones observed in our studies is not the result of extracellular production of H<sub>2</sub>O<sub>2</sub> as there exists no correlation between relative H<sub>2</sub>O<sub>2</sub> production by tannic acid and isoflavones and their ability to cause DNA breakage.

### 3.6 Antioxidant activity of isoflavones against TBHP-induced oxidative stress in lymphocytes

TBHP is a well-known inducer of ROS-mediated oxidative stress that results in DNA damage [44, 45]. In the present study, we have evaluated the antioxidant potential of genistein and biochanin A in providing protection to lymphocytes against TBHP-induced oxidative injury. Ascorbic acid (vitamin C), a known antioxidant, has been used as a positive control in the experiments. Figure 5 shows that whereas all the three were able to inhibit the TBHP-induced lymphocyte



**Figure 5.** A comparison of antioxidant activities of ascorbic acid (▲), genistein (■) and biochanin A (◆) as a function of decreasing tail length of comets against TBHP-induced oxidative DNA breakage in human peripheral lymphocytes as assessed by Comet assay.  $p < 0.05$  by comparison with TBHP-treated positive control. Values reported are  $\pm$ SEM of three independent experiments.

DNA degradation, their relative antioxidant activities were different and appeared in the following order; ascorbic acid > genistein > biochanin A. The results indicate that genistein is a more effective antioxidant as compared to biochanin A.

## 4 Discussion

In order to examine the significance of the structural features of isoflavones toward their chemopreventive effects, we have studied the structure–activity relationship between two related isoflavones, genistein and biochanin A. As can be inferred from our results, genistein is a more efficient pro-oxidant as well as antioxidant relative to biochanin A. It has been reported earlier that both the pro-oxidant and the antioxidant activities of a flavonoid depend on the number of hydroxyl substitutions in its backbone structure [46]. Our results are in agreement with these findings and we believe that hydroxyl groups are essential features responsible for the biological activities of isoflavones. Further, it has also been shown that modification of the hydroxyl groups may alter the biological activity of phenolics. For example, it has been reported that the methylation of the 3-hydroxyl group in protocatechuic acid to form vanillic acid caused significant reduction of the radical scavenging capacity [47]. It has also been reported that the formation of 3,4'-dimethylether or 3,4',7-trimethylether from kaempferol (3,4',5,7-tetrahydroxyflavone) inactivated the pro-oxidant activity of the flavone [46]. Similarly, Liu *et al.* have reported that of various synthetic structural analogues of resveratrol, 3,5,4'-trimethoxy-*trans*-stilbene was ineffective in both antioxidant as well as pro-oxidant activities [48]. The

oxidation of flavonoid to yield stable phenoxyl radical is facilitated through electron delocalization. It is believed that the presence of electron donating or withdrawing groups at the aromatic system strongly influences the redox potential of phenols. The presence of electron-donating groups such as methyl group makes the aromatic structure rich in electrons. This confers a higher degree of instability to the phenoxyl radical, which in turn reduces the redox efficiency of the process [48, 49]. Furthermore, we have shown that apart from inducing greater DNA breakage, genistein is also a more efficient reducer of Cu(II) than biochanin A. The correlation between the DNA cleavage activity and the reducing potential of the compounds indicates that the electron transfer between isoflavones and endogenous copper may play a crucial role in the pro-oxidant activity of the isoflavones. This is also in agreement with the finding where genistein was shown to possess a lower IC<sub>50</sub> for apoptosis induction than biochanin A in various cancer cell lines [50]. Thus, the structural feature responsible for a greater chemopreventive and anticancer activity of genistein is the presence of a free 4'-OH group. It would thus appear that the structural features of isoflavones that are important for the antioxidant activity are also the same that contribute to their pro-oxidant potential through a mechanism that involves redox cycling of chromatin-bound nuclear copper.

We have earlier proposed that an important mechanism for the cytotoxic action of plant-derived polyphenolic compounds against cancer cells could be the mobilization of endogenous copper ions and the consequent pro-oxidant action [27, 28]. This is based on several lines of indirect evidence in literature and our own studies where we have shown that plant polyphenols are able to mobilize endogenous copper ions leading to oxidative breakage of cellular DNA [31, 39]. In further support of our hypothesis, we have used a lysed version of Comet assay and have demonstrated the direct interaction of polyphenols with cell nuclei that leads to oxidative DNA breakage [31]. However, since the lysed version required the preparation of cell nuclei at an alkaline pH, there existed a possibility that the DNA might undergo structural changes, which may be responsible for the enhanced DNA breakage. In order to rule out the possibility of such a structural change in cellular DNA, we have carried out permeabilization of cells at neutral pH [32], thereby eliminating the need for pretreatment of cells at an alkaline pH and allowing the isoflavones to interact with cell nuclei at physiological pH. The results obtained with Cu(I)-specific chelators (neocuproine and bathocuproine) are the same as in the previous study [31]. Further, using either whole cells or permeabilized cells, iron- and zinc-specific chelators did not cause inhibition of isoflavone-induced cellular DNA degradation (Table 2). It may be mentioned that copper and zinc are the major metal ions present in the nucleus [51]. Thus, our result strongly indicates that genistein- and biochanin A-induced cytotoxic action involves mobilization of nuclear copper. We have earlier suggested that the preferential cytotoxicity of plant polyphenols toward cancer cells is explained by the observation made several decades earlier which showed that

serum [52, 53], tissue [54], and intracellular [55] copper levels in cancer cells are significantly enhanced in various malignancies. Since cancer cells contain elevated levels of copper, they may be more subject to electron transfer with polyphenols [48] to generate ROS. Thus, because of higher intracellular copper levels in cancer cells, it may be predicted that the cytotoxic concentrations of polyphenols required would be lower in these cells as compared with normal cells. Such lower cytotoxic concentrations of polyphenols against cancer cells have been demonstrated [56, 57]. It may be mentioned that some plant polyphenols including genistein have been shown to cause regression of tumors in animal models [58–60]. Thus, the mechanism proposed by us would be an alternative, non-enzymatic, and copper-dependent pathway for the cytotoxic action of these compounds that are capable of mobilizing and reducing endogenous copper. Indeed such a common mechanism better explains the anti-cancer effects of polyphenols with diverse chemical structures as also the preferential cytotoxicity toward cancer cells. As such this would be independent of Fas- and mitochondria-mediated programmed cell death. Several studies have indicated that apoptosis induction by several polyphenols and other anti-cancer agents is independent of caspases and mitochondria [61, 62] and is accompanied by an increase in the intracellular levels of ROS [63–65]. This is also consistent with our hypothesis where we propose that plant polyphenols mobilize chromatin-bound copper, which is redox cycled and which in turn leads to the formation of ROS.

Further the question of bioavailability of genistein in mammalian systems also needs to be addressed. Genistein and its metabolites have been detected in plasma, prostatic fluid, breast aspirate, cyst fluid, urine, and feces [66–69]. Studies have reported that the plasma levels of genistein in people having a soy-rich diet could reach up to 5  $\mu$ M [66]. A study targeting phase I pharmacokinetic and pharmacodynamic analysis following administration of unconjugated soy isoflavones (containing 43 and 90% genistein, respectively) to individuals with cancer found plasma concentration of genistein closer to concentrations associated with anticancer activity *in vitro* [70]. It is to be noted that in our studies the concentrations of genistein used (such as 10  $\mu$ M in Fig. 2 and 4–12  $\mu$ M in Fig. 5) are fairly close to the concentrations achieved in plasma on consumption of soy-rich diet [66].

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## 5 References

- [1] Rogers, A.-E., Zeisel, S.-H., Groopman, J., Diet and carcinogenesis. *Carcinogenesis* 1993, 14, 2205–2217.

- [2] Thomasset, S.-C., Berry, D.-P., Garcea, G., Marczylo, T. *et al.*, Dietary polyphenolic phytochemicals—promising cancer chemopreventive agents in humans? A review of their clinical properties. *Int. J. Cancer* 2007, 120, 451–458.
- [3] Adlercreutz, H., Phytoestrogens: epidemiology and possible role in cancer prevention. *Environ. Health Perspect.* 1995, 103, 103–112.
- [4] Adlercreutz, H., Goldin, B.-R., Gorbach, S.-L., Hockerstedt, K. *et al.*, Soybean phytoestrogens intake and cancer risk. *J. Nutr.* 1995, 125, 757S–770S.
- [5] Park, O.-J., Sur, Y.-J., Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies. *Toxicol. Lett.* 2004, 150, 43–56.
- [6] Barnes, S., Peterson, G., Grubbs, C., Setchell, K., Potential role of dietary isoflavones in the prevention of cancer. *Adv. Exp. Med. Biol.* 1994, 354, 135–147.
- [7] Barnes, S., Effect of genistein on *in vitro* and *in vivo* models of cancer. *J. Nutr.* 1995, 125, 777S–783S.
- [8] Sarkar, F.-H., Li, Y., Soy isoflavones and cancer prevention. *Cancer Invest.* 2003, 21, 744–753.
- [9] Hsieh, C.-Y., Santell, R.-C., Haslam, S.-Z., Helferich, W.-G., Estrogenic effects of genistein on the growth of estrogen-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. *Cancer Res.* 1998, 58, 4851–4857.
- [10] Kayisli, U.-A., Aksu, C.-A., Berkkanoglu, M., Arici, A., Estrogenicity of isoflavones on human endometrial stromal and glandular cells. *J. Clin. Endocrinol. Metab.* 2002, 87, 5539–5544.
- [11] Hsu, J.-T., Hung, H.-C., Chen, C.-J., Hsu, W.-L., Ying, C., Effects of the dietary phytoestrogen biochanin A on cell growth in the mammary carcinoma cell line MCF-7. *Nutr. Biochem.* 1999, 10, 510–517.
- [12] Pan, M.-H., Ghai, G., Ho, C.-T., Food bioactives, apoptosis and cancer. *Mol. Nutr. Food Res.* 2008, 52, 43–52.
- [13] Jaruga, E., Salvioli, S., Dobrucki, J., Apoptosis like reversible changes in plasma membrane asymmetry and permeability and transient modifications in mitochondrial membrane potential induced by curcumin in rat hepatocytes. *FEBS Lett.* 1998, 433, 287–293.
- [14] Clement, M., Hirpara, J.-L., Chawdhury, S.-H., Pervaiz, S., Chemopreventive agent resveratrol, a natural product derived from grapes, triggers CD95 signalling dependant apoptosis in human tumor cells. *Blood* 1998, 92, 996–1002.
- [15] Inoue, M., Suzuki, R., Koide, T., Sakaguchi, N. *et al.*, Antioxidant, gallic acid induces apoptosis in HL 60 R cells. *Biochem. Biophys. Res. Commun.* 1994, 204, 898–904.
- [16] Ahmad, N., Feyes, D.-K., Nieminen, A.-L., Agarwal, R., Mukhtar, H., Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J. Natl. Cancer Inst.* 1997, 89, 1881–1886.
- [17] Hou, D.-X., Ose, T., Lin, S., Harazoro, K. *et al.*, Anthocyanidins induce apoptosis in human promyelocytic leukemia cells: structure–activity relationship and mechanisms involved. *Int. J. Oncol.* 2003, 23, 705–712.
- [18] Chodon, D., Ramamurthy, N., Sakthisekaran, D., Preliminary studies on induction of apoptosis by genistein on HepG2 cell line. *Toxicol. In Vitro* 2007, 21, 887–891.
- [19] Chang, K.-L., Cheng, H.-L., Huang, L.-W., Hsieh, B.-S. *et al.*, Combined effects of terazosin and genistein on a metastatic, hormone-independent human prostate cancer cell line. *Cancer Lett.* 2008, 276, 14–20.
- [20] Ahmad, M.-S., Fazal, F., Rahman, A., Hadi, S.-M., Parish, J.-H., Activities of flavonoids for the cleavage of DNA in the presence of Cu(II): correlation with the generation of active oxygen species. *Carcinogenesis* 1992, 13, 605–608.
- [21] Khan, N.-S., Hadi, S.-M., Structural features of tannic acid important for DNA degradation in the presence of Cu(II). *Mutagenesis* 1998, 13, 271–274.
- [22] Ahsan, H., Hadi, S.-M., Strand scission in DNA induced by curcumin in the presence of Cu(II). *Cancer Lett.* 1998, 24, 23–30.
- [23] Malik, A., Azam, S., Hadi, N., Hadi, S.-M., DNA degradation by water extract of green tea in the presence of copper ions: implications for anticancer properties. *Phytother. Res.* 2003, 17, 358–363.
- [24] Ahmad, A., Asad, S. F., Singh, S., Hadi, S.-M., DNA breakage by resveratrol and Cu(II): reaction mechanism and bacteriophage inactivation. *Cancer Lett.* 2000, 154, 29–37.
- [25] Hanif, S., Shamim, U., Ullah, M.-F., Azmi, A.-S. *et al.*, The anthocyanidin delphinidin mobilizes endogenous copper ions from human lymphocytes leading to oxidative degradation of cellular DNA. *Toxicology* 2008, 249, 19–25.
- [26] Kagawa, T. F., Geierstanger, B.-H., Wang, A.-H.-J., Ho, P.-S., Covalent modification of guanine bases in double stranded DNA: the 1:2-AZ-DNA structure of dc(CACACG) in the presence of CuCl<sub>2</sub>. *J. Biol. Chem.* 1991, 266, 20175–20184.
- [27] Hadi, S.-M., Asad, S.-F., Singh, S., Ahmad, A., A putative mechanism for anticancer and apoptosis inducing properties of plant-derived polyphenolic compounds. *IUBMB Life* 2000, 50, 1–5.
- [28] Hadi, S.-M., Bhat, S.-H., Azmi, A.-S., Hanif, S. *et al.*, Oxidative breakage of cellular DNA by plant polyphenols: A putative mechanism for anticancer properties. *Semin. Cancer Biol.* 2007, 17, 370–376.
- [29] Lamartiniere, C.-A., Protection against breast cancer with genistein: a component of soy. *Am. J. Clin. Nutr.* 2000, 71, S1705–S1707.
- [30] Renner, H.-W., Schmezer, P., Assessment of genotoxic effects of lindane. *Food Chem. Toxicol.* 1993, 31, 184–191.
- [31] Shamim, U., Hanif, S., Ullah, M.-F., Azmi, A.-S. *et al.*, Plant polyphenols mobilize nuclear copper in human peripheral lymphocytes leading to oxidatively generated DNA breakage: implications for an anticancer mechanism. *Free Radic. Res.* 2008, 42, 764–772.
- [32] Czene, S., Tiback, M., Ringdahl, M.-H., pH-dependant DNA cleavage in permeabilized human fibroblasts. *Biochem. J.* 1997, 323, 337–341.
- [33] Dipti, K.-P., Sharma, S.-K., Sairam, M., Ilavazhagan, G. *et al.*, Flavonoids protect U-937 macrophages against tert-butylhydroperoxide induced oxidative injury. *Food Chem. Toxicol.* 2006, 44, 1024–1030.
- [34] Asad, S.-F., Singh, S., Ahmad, A., Khan, N.-U., Hadi, S.-M., Prooxidant and antioxidant activities of bilirubin and its metabolic precursor biliverdin: a structure-activity study. *Chem. Biol. Interact.* 2001, 137, 59–74.

- [35] Long, L.-H., Clement, M.-V., Halliwell, B., Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin, (–)-epigallocatechingallate, (+)-catechin and quercetin to commonly used cell culture media. *Biochem. Biophys. Res. Commun.* 2000, 273, 50–53.
- [36] Tice, R.-R., Agurell, E., Anderson, D., Burlinson, B. *et al.*, Single cell gel electrophoresis/Comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.* 2000, 35, 206–221.
- [37] Ostling, O., Johansson, K.-J., Microelectrophoretic study of radiation-induced DNA damages in individual cells. *Biochem. Biophys. Res. Commun.* 1984, 123, 291–298.
- [38] Singh, N.-P., McCoy, M.-T., Tice, R.-R., Schneider, E.-L., A simple technique for quantification of low levels of DNA damage in individual cells. *Exp. Cell. Res.* 1988, 175, 184–191.
- [39] Azmi, A.-S., Bhat, S.-H., Hanif, S., Hadi, S.-M., Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: a putative mechanism for anticancer properties. *FEBS Lett.* 2006, 580, 533–538.
- [40] Cemeli, E., Schmid, T.-E., Anderson, D., Modulation by flavonoids of DNA damage induced by estrogen-like compounds. *Environ. Mol. Mutagen.* 2004, 44, 420–426.
- [41] Badwey, A., Karnovsky, M. L., Active oxygen species and the functions of phagocytic leukocytes. *Ann. Rev. Biochem.* 1980, 49, 695–726.
- [42] Long, L.-H., Clement, M.-V., Halliwell, B., Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin, (–)-epigallocatechin gallate, (+)-catechin and quercetin to commonly used cell culture media. *Biochem. Biophys. Res. Commun.* 2000, 273, 50–53.
- [43] Bhat, R., Hadi, S.-M., DNA breakage by tannic acid–Cu(II): sequence specificity of the reaction and involvement of Cu(II). *Mutat. Res.* 1994, 313, 39–48.
- [44] Dubuisson, M.-L., De Wergifosse, B., Trouet, A., Baguet, F. *et al.*, Antioxidative properties of natural coelenterazine and synthetic methyl coelenterazine in rats hepatocytes subjected to tert-butylhydroperoxide induced oxidative stress. *Biochem. Pharmacol.* 2000, 60, 471–478.
- [45] Suzuki, Y., Apostolova, M.-D., Cherian, M.-G., Astrocyte cultures from transgenic mice to study the role of metallothionein in cytotoxicity of tert-butylhydroperoxide. *Toxicology* 2000, 145, 51–62.
- [46] Simic, A., Manojlovic, D., Segan, D., Todorovic, M., Electrochemical behavior and antioxidant and prooxidant activity of natural phenolics. *Molecules* 2007, 12, 2327–2340.
- [47] Cao, G., Sofic, E., Prior, R.-L., Antioxidant and prooxidant behaviour of flavonoids : structure-activity relationship. *Free Radic. Biol. Med.* 1997, 22, 749–760.
- [48] Zheng, L.-F., Wei, Q.-Y., Cai, Y.-J., Fang, J.-G. *et al.*, DNA damage induced by resveratrol and its synthetic analogs in the presence of Cu(II) ions: mechanism and structure-activity relationship. *Free Radic. Biol. Med.* 2006, 41, 1807–1816.
- [49] Majo, D.-D., Giammanco, M., Guardia, M.-L., Tripoli, E. *et al.*, Flavanones in citrus fruits: structure-antioxidant activity relationships. *Food Res. Int.* 2005, 38, 1161–1166.
- [50] Su, S.-J., Chow, N.-H., Kung, M.-L., Hung, T.-C., Chang, K.-L., Effects of soy isoflavones on apoptosis induction and G2-M arrest in human hepatoma cells; involvement of caspase-3 activation, Bcl-2 and bcl-XL downregulation, and Cdc2 kinase activity. *Nutr. Cancer* 2003, 45, 113–123.
- [51] Bryan, S.-E., *Metal Ions in Biological Systems*, Marcel Dekker, New York 1979.
- [52] Ebadi, M., Swanson, S., The status of zinc, copper and metallothionein in cancer patients. *Prog. Clin. Biol. Res.* 1988, 259, 61–67.
- [53] Margalioth, E.-J., Udassin, R., Cohen, C., Maor, J., Serum copper level in gynaecologic malignancies. *Am. J. Obstet. Gynaecol.* 1987, 157, 93–96.
- [54] Yoshida, D., Ikeda, Y., Nakazawa, S., Quantitative analysis of copper, zinc and copper/zinc ratio in selective human brain tumors. *J. Neurooncol.* 1993, 16, 109–115.
- [55] Ebara, M., Fukuda, H., Hanato, R., Saisho, H. *et al.*, Relationship between zinc, copper and metallothionein in hepatocellular carcinoma and its surrounding liver parenchyma. *J. Hepatol.* 2000, 33, 415–422.
- [56] Chen, Z.-P., Schell, J.-B., Ho, C.-T., Chen, K.-Y., Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett.* 1998, 129, 73–179.
- [57] Lu, J., Ho, C.-T., Ghai, G., Chen, K.-Y., Differential effects of theaflavin monogallates on cell growth, apoptosis, and Cox-2 gene expression in cancerous *versus* normal cells. *Cancer Res.* 2000, 60, 6465–6471.
- [58] Gupta, S., Hastak, K., Ahmad, N., Lewin, J.-S., Mukhtar, H., Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols. *Proc. Natl. Acad. Sci. USA* 2001, 98, 10350–10355.
- [59] Orsolic, N., Terzic, S., Sver, L., Basic, I., Honey-bee products in the prevention and therapy of murine transplantable tumors. *J. Sci. Food Agric.* 2005, 85, 363–370.
- [60] Wang, Y., Eltoum, I.-E., Lamatiniere, C.-A., Genistein chemoprevention of prostate cancer in TRAMP mice. *J. Carcinogenesis* 2007, 6, 3–9.
- [61] Piwocka, K., Zablocki, K., Weickowski, M.-R., Skierski, J., A novel apoptosis like pathway, independent of mitochondria and caspases, induced by curcumin in human lymphoblastoid T (Jurkat) cells. *Exp. Cell. Res.* 1999, 249, 299–307.
- [62] Leist, M., Jaattela, M., Four deaths and a funeral: from caspases to alternative mechanisms. *Nat. Rev. Mol. Cell. Biol.* 2001, 2, 589–598.
- [63] Yoshino, M., Haneda, M., Naruse, M., Htay, H.-H. *et al.*, Prooxidant activity of curcumin: copper-dependent formation of 8-hydroxy-2'-deoxyguanosine in DNA and induction of apoptotic cell death. *Toxicol. In Vitro* 2004, 18, 783–788.
- [64] Noda, C., He, J., Takano, T., Tanaka, C. *et al.*, Induction of apoptosis by epigallocatechin-3-gallate in human lymphoblastoid B cells. *Biochem. Biophys. Res. Commun.* 2007, 362, 951–957.
- [65] Heiss, E.-H., Schilder, Y.-D.-C., Dirsch, V.-M., Chronic treatment with resveratrol induces redox stress- and

- ATM-dependent senescence in p53-positive cancer cells. *J. Biol. Chem.* 2007, 282, 26759–26766.
- [66] Adlercreutz, H., Markkanen, H., Watanabe, S., Plasma concentrations of phyto-oestrogens in Japanese men. *Lancet* 1993, 342, 1209–1210.
- [67] Mills, P.-K., Beeson, W.-L., Phillips, R.-L., Fraser, G.-E., Cohort study of diet lifestyle and prostate cancer in Adventist men. *Cancer* 1989, 64, 598–604.
- [68] Knight, D.-C., Eden, J.-A., A review of the clinical effects of phytoestrogens. *Obstet. Gynecol.* 1996, 87, 897–904.
- [69] Zava, D.-T., Dollbaum, C.-M., Blen, M., Estrogen and progestin bioactivity of foods, herbs, and spices. *Proc. Soc. Exp. Biol. Med.* 1998, 217, 369–378.
- [70] Takimoto, C.-H., Glover, K., Huang, X., Hayes, S.-A. *et al.*, Phase I pharmacokinetic and pharmacodynamic analysis of unconjugated soy isoflavones administered to individuals with cancer. *Cancer Epidemiol. Biomarkers. Prev.* 2003, 12, 1213–1221.