

Reduced isoflavone metabolites formed by the human gut microflora suppress growth but do not affect DNA integrity of human prostate cancer cells

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Dietary isoflavones, such as genistein and daidzein, are metabolised by the human gut microflora. Case–control studies have disclosed a link between the formation of the daidzein metabolite equol and prostate cancer risk. We evaluated the effects of genistein, daidzein and five metabolites on two prostate cancer cell lines by determining DNA integrity and cell growth. LNCaP cells contain the T877A androgen receptor mutation whereas Los Angeles prostate cancer (LAPC)-4 cells express the wild-type receptor, both of which may affect responses to isoflavones. DNA integrity was determined using the comet assay. Cell growth was assessed by staining DNA with 4',6'-diamidino-2-phenylindole hydrochloride. Endogenous steroid hormones, but not isoflavones, induced DNA strand breaks. Dihydrotestosterone stimulated the growth of both cell lines. 17 β -Oestradiol increased the growth of LNCaP but not LAPC-4 cells, pointing to an involvement of the T877A androgen receptor. Isoflavones did not stimulate growth in either prostate cancer cell line. However, the growth of LNCaP and LAPC-4 cells was suppressed by genistein (inhibitory concentration 50% (IC₅₀) 39.7 μ mol/l, 37.2 μ mol/l) and by equol (IC₅₀ 53.8 μ mol/l, 35.1 μ mol/l). *O*-desmethylangolensin inhibited the growth of LAPC-4 cells (IC₅₀ 45.2 μ mol/l), but not of LNCaP cells. In conclusion, isoflavones do not damage DNA or promote growth of androgen-dependent prostate cancer cells. Several isoflavones, including the reduced daidzein metabolites equol and *O*-desmethylangolensin, suppress cancer cell growth. Taken together, these data suggest a contribution of gut-formed isoflavone metabolites to the beneficial effects of dietary isoflavones on prostate cancer risk.

Isoflavones: Prostate cancer: Cell growth: DNA damage

Incidence and mortalities of prostate cancer are low in many Asian countries but high in Western societies (Hsing *et al.* 2000; Jemal *et al.* 2002; Quinn & Babb, 2002). This has epidemiologically been attributed to the traditional Asian soya-based diet, since Asians who migrated to the USA became more likely to develop prostate cancer (Shimizu *et al.* 1991; Gomez *et al.* 2003). In three prospective cohort studies, the consumption of pulses or soya milk was associated with reduced prostate cancer risk (Dagnelie *et al.* 2004). Soya and soya products are extraordinarily rich in isoflavones (Liggins *et al.* 2000; Valsta *et al.* 2003). Asians consume up to 100 mg isoflavones/d, whereas Europeans usually consume less than 1 mg/d (van Erp-Baart *et al.* 2003). Similarly, plasma isoflavone concentrations are more than ten times higher in Asians than in Europeans or in Americans and reach up to 1 μ mol total isoflavones/l in plasma (Morton *et al.* 2002; Akaza *et al.* 2004). Single-dose administration of purified isoflavones to postmenopausal American women resulted in increased plasma levels of up to 28 μ mol genistein/l (Bloedon *et al.* 2002). Moreover, some isoflavones can accumulate in the prostate, reaching concentrations that are several times higher than the respective concentrations in plasma (Hedlund *et al.* 2005). Due to some structural similarities to steroid

hormones, isoflavones are able to bind with human oestrogen receptors and to mimic or block oestrogenic transactivation activities (Kuiper *et al.* 1997, 1998). Based on these so-called phyto-oestrogenic effects, isoflavones were thought to contribute to the chemopreventive effects of the Asian diet (Ren *et al.* 2001).

Most of the published *in vitro* work has addressed effects of the major isoflavones genistein and daidzein. However, the exposure *in vivo* is more complex. Isoflavones can undergo metabolism by the human gut microflora (Fig. 1) and those products may help to prevent the development or progression of prostate cancer. For the first time in the present study we compared the biological activities of genistein, daidzein and five of their reduced gut metabolites, detected in human body fluids (Heinonen *et al.* 1999), using human prostate cancer cells.

Beside their proposed beneficial properties, isoflavones are suspected to be genotoxic. In a number of *in vitro* studies, they induced chromosomal aberrations and DNA strand breaks in V79 Chinese hamster fibroblasts (Kulling & Metzler, 1997; Schmitt *et al.* 2003; Di Virgilio *et al.* 2004). A few studies, however, also revealed a genotoxic potential of isoflavones in human prostate (Mitchell *et al.* 2000) and colon cell lines (Pool-Zobel *et al.* 2000). In the present study we

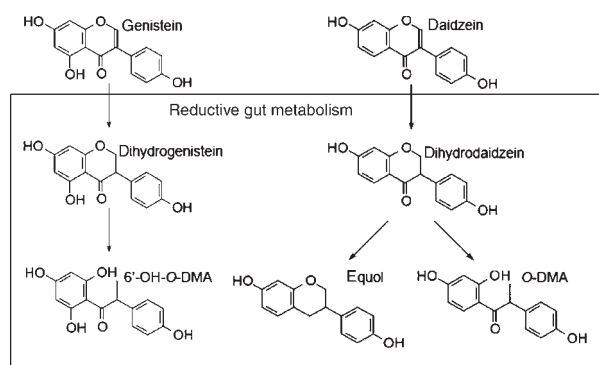


Fig. 1. Metabolism of genistein and daidzein by human gut microflora. Genistein is reduced to dihydrogenistein which undergoes further reduction to 6'-hydroxy-*O*-desmethylangolensin (6'-OH-*O*-DMA). Daidzein is reduced to dihydrodaidzein which is further reduced to either *O*-desmethylangolensin (*O*-DMA) or equol (modified according to and with permission of Heinonen *et al.* (1999)).

extended these findings to include several reduced isoflavone metabolites which have not yet been investigated in prostate cells. This new information was expected to reveal whether or not the reduction of soya isoflavones by the human gut microflora alters their genotoxic potential in prostate cells.

In addition to their ability to bind with oestrogen receptors, isoflavones were recently discovered to bind with the androgen receptor as well, but with weak affinity (Beck *et al.* 2003). Mutations in the gene encoding the androgen receptor are common events during prostate carcinogenesis, which may affect the binding capacity of the receptor (Suzuki *et al.* 2003). Therefore we investigated the activities of isoflavones in two prostate cancer models with different receptor phenotypes. The androgen receptor of LNCaP cells contains a well-characterised alteration of the amino acid sequence in its ligand-binding domain (T877A), resulting in an increased affinity to 17 β -oestradiol while the affinity to dihydrotestosterone declines (Suzuki *et al.* 2003). Furthermore, even non-steroids, such as the anti-androgen flutamide, were found to act as receptor agonists (Taplin *et al.* 1999; Sack *et al.* 2001). The Los Angeles prostate cancer (LAPC)-4 cell line, in contrast, expresses the wild-type androgen receptor (Klein *et al.* 1997). On account of these different phenotypes, we proposed different responses to isoflavones between the cell lines.

First, we evaluated the effects of isoflavones on cell growth. This endpoint is regulated with high complexity and therefore appears to be suitable to compare the general biological activities of the tested compounds. An inhibition of cell turnover may decrease the vulnerability to carcinogens and suppress the progression of malignant foci (Hedlund *et al.* 2003). In contrast, an isoflavone-mediated stimulation of cell growth would promote cancer progression.

Materials and methods

Materials

Genistein and daidzein were purchased from TCI Tokyo Kasai (Tokyo, Japan); dihydrogenistein, dihydrodaidzein and *O*-desmethylangolensin (*O*-DMA) were obtained from Plantech Ltd (Reading, Berks, UK); equol and 4',6'-diamidino-2-phenylindole hydrochloride were from Fluka (Buchs, Switzerland).

Dihydrotestosterone, 17 β -oestradiol and SybrGreen were purchased from Sigma (Seelze, Germany). All test compounds were dissolved in dimethyl sulfoxide and stored at -20°C . Cell culture media and supplements were purchased from Gibco (Karlsruhe, Germany), except the charcoal-stripped fetal calf serum (Biological Industries, Beit Haemek, Israel) and mibolerone (Perkin Elmer Life Sciences, Boston, MA, USA).

The genistein metabolite 6'-hydroxy-*O*-DMA was synthesised at the Department of Organic Chemistry, University of Helsinki (Finland). In brief, 4',7-dihydroxyisoflavone was reduced by lithium aluminium hydride (5.5 equivalents) in refluxing tetrahydrofuran. The reaction mixture was quenched into saturated ammonium chloride and neutralised with hydrochloric acid and extracted with ethyl acetate. After evaporation of the solvent the crude product was purified by flash chromatography, giving a good yield of 6'-*O*-DMA. The product was characterised by proton and carbon NMR, low-resolution MS and high-resolution MS.

Cell lines and cell culture procedures

The LNCaP cell line was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum, L-glutamine (2 mmol/l) and 1% penicillin–streptomycin. In order to avoid interference of phenol red or steroid hormones from the serum, medium was replaced by phenol red-free RPMI-1640 supplemented with 10% charcoal-stripped fetal calf serum, L-glutamine (2 mmol/l) and 1% penicillin–streptomycin, 24 h before treatment. The LAPC-4 cell line was a generous gift of Professor C. L. Sawyers, Department of Medicine, University of California (Los Angeles, CA, USA). These cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, L-glutamine (2 mmol/l), 1% penicillin–streptomycin and mibolerone (2 nmol/l). Likewise, 24 h before treatment, medium was replaced by phenol red-free Iscove's modified Dulbecco's medium supplemented with 10% charcoal-stripped fetal calf serum, L-glutamine (2 mmol/l), 1% penicillin–streptomycin but no mibolerone. Steroid hormone depletion of the charcoal-stripped serum was ascertained by analyses of dihydrotestosterone (RIA-kit; DSL, Sinsheim, Germany) and 17 β -oestradiol (AVIDA Centaur ACS; Bayer HealthCare, Leverkusen, Germany) performed at our department of clinical chemistry (< 26 pmol 17 β -oestradiol/l and 219 pmol dihydrotestosterone/l).

Comet assay

As a general laboratory standard, we minimise carry-over of the solvent in order to avoid interference during the experiments. In the present study, all samples contained a final dimethyl sulfoxide concentration of 0.1%. To achieve this, stock solutions of the tested compounds (100 mmol/l) were first serially diluted in dimethyl sulfoxide and subsequently dissolved (1:1000) in cell culture medium to yield the required final compound concentrations. By applying this procedure, we noticed precipitation of some compounds at 100 $\mu\text{mol/l}$. Thus, compounds were only tested up to 80 $\mu\text{mol/l}$.

a maximum concentration at which precipitation was not observed.

A total of 7.5×10^6 cells were seeded in 75 cm² culture flasks and allowed to attach for 48 h. Medium was changed as described earlier. After 24 h, cells were harvested, washed with PBS, sampled to 1.0×10^6 and incubated with test compounds (80 µmol/l) for 60 min at 37°C and 450 rpm. Thereafter, cells were washed and viability was determined by the trypan blue exclusion assay using a Neubauer haemocytometer. The following comet assay procedure has been described previously (Schaeferhenrich *et al.* 2003). In brief, incubated cells were placed on agarose-coated microscopic slides and covered with 0.7% low-melting agarose dissolved in PBS. Slides were exposed to a lysis solution (tri(hydroxymethyl)-aminomethane (Tris)-HCl (10 mmol/l), Na₂EDTA (100 mmol/l), NaCl (2.5 mol/l), 10% dimethyl sulfoxide, 1% Triton X-100, pH 10) for 60 min and subsequently incubated with alkaline electrophoresis buffer (Na₂EDTA (1 mmol/l), NaOH (300 mmol/l), pH 13) for 20 min. Thereafter, electrophoresis was carried out for 20 min (25 V, 300 mA). Slides were washed with neutralisation buffer (Tris-HCl (4.2 mol/l), Tris-Base (0.08 mol/l), pH 7.2) three times for 5 min. DNA was then stained with SybrGreen. The rate of DNA strand breaks was measured as 'percentage DNA in comet tail' (tail intensity). Samples were investigated in triplicate and, for each of the slides, fifty cells were evaluated using a Zeiss Axiolab HB50 microscope, COHU high performance CCD camera and the Komet 4.0 imaging and analysis system (Kinetic Imaging, Nottingham, Notts, UK). The means of one experiment were used to calculate the means of at least three independent reproductions.

Cell growth assay

Cells were seeded in ninety-six-well plates at a density of 8000 cells per well and allowed to attach for 48 h. Thereafter, medium was changed as described earlier; cells were incubated with the test compound (0.001–80 µmol/l). After 96 h, medium was removed and cells were fixed with methanol. Cell growth was determined from the content of residual DNA by 4',6'-diamidino-2-phenylindole hydrochloride staining and fluorimetric detection at 360/465 nm using Tecan GENios. Results are expressed as percentage of cell growth compared with solvent control. To assure an appropriate growth of control cells, we first determined doubling times of both cell lines using these experimental conditions. LNCaP cells had a doubling time of 43 (SEM 5) h (*n* 3). LAPC-4 cells had a doubling time of 57 (SEM 7) h (*n* 3).

Statistical analyses

Results from the comet assay analysis are shown in Tables 1 and 2 and represent arithmetic means with their standard errors. The *P* values were obtained by a two-tailed unpaired *t* test. Results from growth experiments are shown in the Figs. and represent arithmetic means with their standard errors. Significance was established by one-way ANOVA and Dunnett's *post hoc* test. All statistical analyses were performed with GraphPad Prism software version 4.0 (GraphPad, San Diego, CA, USA).

Results

Induction of DNA strand breaks

Comet assay analysis revealed that 17β-oestradiol and dihydrotestosterone significantly increased DNA strand breaks in LAPC-4 cells from 5.6% to 9.6% tail intensity (*P*=0.029) and 7.6% (*P*=0.013) tail intensity, respectively. In LNCaP cells, 17β-oestradiol elevated the tail intensity from 5.0% to 8.9% (*P*<0.001). While there was a tendency for dihydrotestosterone to increase DNA strand breaks in LNCaP cells (*P*=0.051), this effect did not reach statistical significance (Table 1). In contrast, the incubation with isoflavones had no effect on the rate of DNA strand breaks in either cell line (Table 2). Neither steroid hormones nor isoflavones affected cell viability during the comet assay experiments, as determined by trypan blue exclusion (data not shown).

Modulation of prostate cell growth

The experimental set up of our growth assay and the sensitivity of cells lines were verified by the responses to steroid hormones. Fig. 2 (A) shows biphasic effects of dihydrotestosterone in both cell lines after 96 h incubation. However, their magnitudes of effects differed markedly. The growth of LAPC-4 cells increased by 86%, whereas the growth of LNCaP cells increased by 36%. Higher concentrations decreased the growth of both cell lines, which was significant for LNCaP cells at 40 and 80 µmol/l. In contrast, 17β-oestradiol increased the growth of LNCaP cells by about 90% but had no stimulating effects in LAPC-4 cells. 17β-Oestradiol between 20 and 80 µmol/l decreased the growth of LAPC-4 cells (Fig. 2 (B)).

Genistein decreased the growth of LNCaP and LAPC-4 cells with inhibitory concentration 50% (IC₅₀) values of 39.7 and 39.3 µmol/l, respectively (Fig. 3 (A)). Dihydrogenistein had no effect on cell growth (Fig. 3 (B)), whereas 6'-hydroxy-*O*-DMA at 80 µmol/l decreased the growth of LNCaP cells (Fig. 3 (C)). Neither genistein nor its metabolites induced growth of either cell line.

Daidzein decreased the growth of both cells lines, but without reaching the IC₅₀ level (Fig. 4 (A)). Dihydrodaidzein had no effect on cell growth (Fig. 4 (B)). *O*-DMA was unique among the tested isoflavones, since it only suppressed the growth of LAPC-4 cells (IC₅₀ 45.2 µmol/l) but not of LNCaP cells (Fig. 4 (C)). Equol decreased the growth of both LNCaP and LAPC-4 cells, with IC₅₀ values of 53.8 and 35.1 µmol/l, respectively (Fig. 4 (D)). Neither daidzein nor its metabolites increased the growth of LNCaP and LAPC-4 cells.

Discussion

In the present study we investigated the isoflavones genistein, daidzein and their reduced metabolites formed by the human gut microflora for effects on DNA integrity and growth of prostate cancer cells. We did not find any genotoxic potential of the tested isoflavones but confirmed reports showing growth-inhibiting effects of equol in prostate cells (Mitchell *et al.* 2000; Hedlund *et al.* 2003). Furthermore, we revealed that *O*-DMA is another gut-formed daidzein metabolite with potential to suppress prostate cancer cell growth.

Table 1. Effects of human steroid hormones (80 $\mu\text{mol/l}$) on the rate of DNA strand breaks in LNCaP and Los Angeles prostate cancer (LAPC)-4 cells
(Mean values with their standard errors)

Hormone	LNCaP			LAPC-4		
	Tail intensity (%)†			Tail intensity (%)†		
	Mean	SEM	<i>P</i> *	Mean	SEM	<i>P</i> *
Control‡	5.0	0.4	–	5.6	0.1	–
17 β -Oestradiol	8.9	0.1	<0.001	9.6	1.4	0.029
Dihydrotestosterone	7.9	1.1	0.051	7.6	0.6	0.013

* *P* values from unpaired *t* tests (two-tailed) by comparison of the individual hormones with control (*n* 4).

† Percentage migrated DNA as determined by comet assay after 60 min treatment.

‡ Solvent control (0.1% dimethyl sulfoxide).

Genotoxic activities of the test compounds were studied at high concentrations of 80 $\mu\text{mol/l}$, which have never been reached *in vivo*. In one study with postmenopausal women, supplementation of purified soya isoflavones (up to 16 mg genistein/kg body weight) resulted in plasma peak concentrations of up to 28.1 μmol total genistein/l and up to 9.0 μmol total daidzein/l (Bloedon *et al.* 2002). Thus, total soya-isoflavone plasma concentrations of 40 $\mu\text{mol/l}$ might be possible. Another study in men revealed that isoflavone concentrations in prostate fluid are several times higher than in plasma (daidzein, 5.1-fold; dihydrodaidzein, 5.6-fold; *O*-DMA, 2.5-fold; equol, 21.7-fold), pointing to an accumulation of isoflavones in the prostate (Hedlund *et al.* 2005). Thus, when taking the possibility of isoflavone accumulation in the prostate tissue into account, the *in vitro* concentrations of up to 80 μmol isoflavones/l used in the present study appear to be reasonable.

Several isoflavones have previously been shown to have genotoxic activities. The treatment of Chinese hamster V79 lung fibroblasts with genistein, daidzein and equol resulted in the induction of micronuclei but not of DNA strand breaks (Di Virgilio *et al.* 2004). Genistein was also moderately mutagenic in these cells by causing hypoxanthine–guanine

phosphoribosyl transferase mutations (Kulling & Metzler, 1997). Equol and *O*-DMA, as well as two oxidative daidzein metabolites, induced micronuclei in L5174Y mouse lymphoma cells (Schmitt *et al.* 2003). We previously detected DNA strand breaks in HT29 human colon cells, following treatment with 100 μmol genistein/l and *O*-DMA (Pool-Zobel *et al.* 2000). In addition, genistein (≥ 10 $\mu\text{mol/l}$) was reported to induce DNA strand breaks in the human prostate cancer cell lines LNCaP and PC-3 (Mitchell *et al.* 2000). The present study with prostate cells, however, does not point to genotoxic activities. This discrepancy might be due to differences in treatment. While the present study was addressed to determine direct genotoxic effects of the isoflavones after 60 min incubation, Mitchell *et al.* (2000) favoured 24 h incubation. The latter time span presumably allows isoflavone modifications to occur, such as through oxidation. In human subjects, oxidation of isoflavones was demonstrated *in vitro* and *in vivo* (Kulling *et al.* 2001). The emerging metabolites have not to date been evaluated in prostate cells. Such oxidised isoflavones, however, induced DNA strand breaks in MCF-7 human breast cells, whereas their plant-derived precursors had no effect (Murata *et al.* 2004). Thus, one may propose that isoflavones are metabolically activated to genotoxic intermediates in prostate cells (for example, by cytochrome P450 enzymes) which could account for the demonstrated DNA damage after 24 h. Therefore, the present study indicates that the tested isoflavones are not directly genotoxic in prostate cells. However, we demonstrated that dihydrotestosterone and 17 β -oestradiol do induce DNA strand breaks in the cell lines studied here, which points to an enhancement of prostate cancer progression by these endogenous hormones.

In vivo, genistein administration did not induce DNA strand breaks in mice splenocytes, even though plasma concentrations increased up to 9.2 $\mu\text{mol/l}$ (Record *et al.* 1995). Within a human intervention study, supplementation of isoflavones increased concentrations of genistein in plasma individually up to 27 $\mu\text{mol/l}$, which did not affect DNA integrity of primary lymphocytes (Miltyk *et al.* 2003). Thus, these studies do not support the hypothesis that isoflavones are genotoxic *in vivo*. Further *in vitro* and *in vivo* work is needed to fully evaluate the genotoxic potential of isoflavones.

We also determined effects on cell growth and demonstrated that there were different responses to steroid hormones. Dihydrotestosterone stimulated the growth of both cell lines.

Table 2. Effects of isoflavones (80 $\mu\text{mol/l}$) on the rate of DNA strand breaks in LNCaP and Los Angeles prostate cancer (LAPC)-4 cells
(Mean values with their standard errors)

Isoflavone	LNCaP			LAPC-4		
	Tail intensity (%)†			Tail intensity (%)†		
	Mean	SEM	<i>P</i> *	Mean	SEM	<i>P</i> *
Control‡	4.8	0.4	–	4.3	0.3	–
Genistein	7.3	2.3	0.343	5.9	1.7	0.410
Dihydrotestosterone	6.2	1.4	0.396	5.1	0.7	0.324
6'-OH- <i>O</i> -DMA	6.1	1.7	0.521	4.4	0.5	0.873
Daidzein	4.6	0.5	0.770	4.1	0.6	0.762
Dihydrotestosterone	4.5	0.5	0.726	3.9	0.4	0.510
<i>O</i> -DMA	4.9	0.7	0.884	4.5	0.5	0.715
Equol	5.3	0.3	0.383	4.4	0.4	0.745

6'-OH-*O*-DMA, 6'-hydroxy-*O*-desmethylangolensin; *O*-DMA, *O*-desmethylangolensin.

* *P* values from unpaired *t* tests (two-tailed) by comparison of the individual isoflavones with control (*n* 3).

† Percentage migrated DNA as determined by comet assay after 60 min treatment.

‡ Solvent control (0.1% dimethyl sulfoxide).

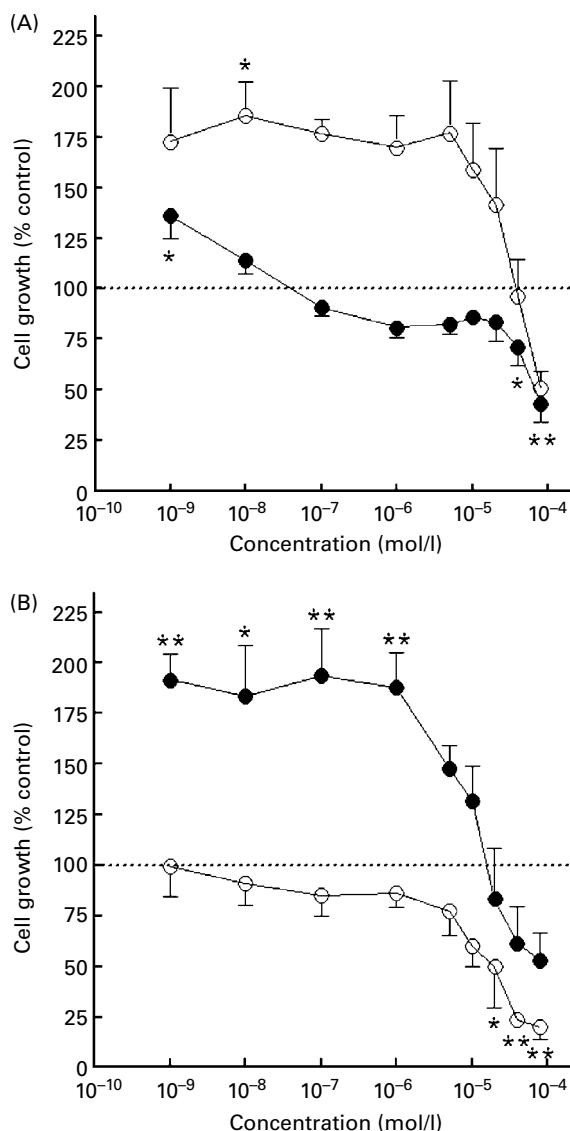


Fig. 2. Modulation of prostate cell growth by human steroid hormones. LNCaP (●) and Los Angeles prostate cancer (LAPC)-4 (○) cells were treated for 96 h and growth was determined from the content of residual DNA by 4',6'-diamidino-2-phenylindole hydrochloride staining. (A) Dihydrotestosterone increased growth of LNCaP cells by 36% at 0.001 $\mu\text{mol/l}$ but decreased growth between 40 and 80 $\mu\text{mol/l}$ (n 5). In LAPC-4 cells, dihydrotestosterone at 0.01 $\mu\text{mol/l}$ increased growth by 86% (n 3). (B) 17 β -Oestradiol increased growth of LNCaP cells at 0.001–1 $\mu\text{mol/l}$ by about 90% (n 4) whereas growth of LAPC-4 cells decreased between 20 and 80 $\mu\text{mol/l}$ (n 4). Data are mean values of relative cell growth compared with control (0.1% dimethyl sulfoxide), with their standard errors represented by vertical bars. Mean values were significantly different from control: * P < 0.05, ** P < 0.01 (one-way ANOVA and Dunnett's *post hoc* test).

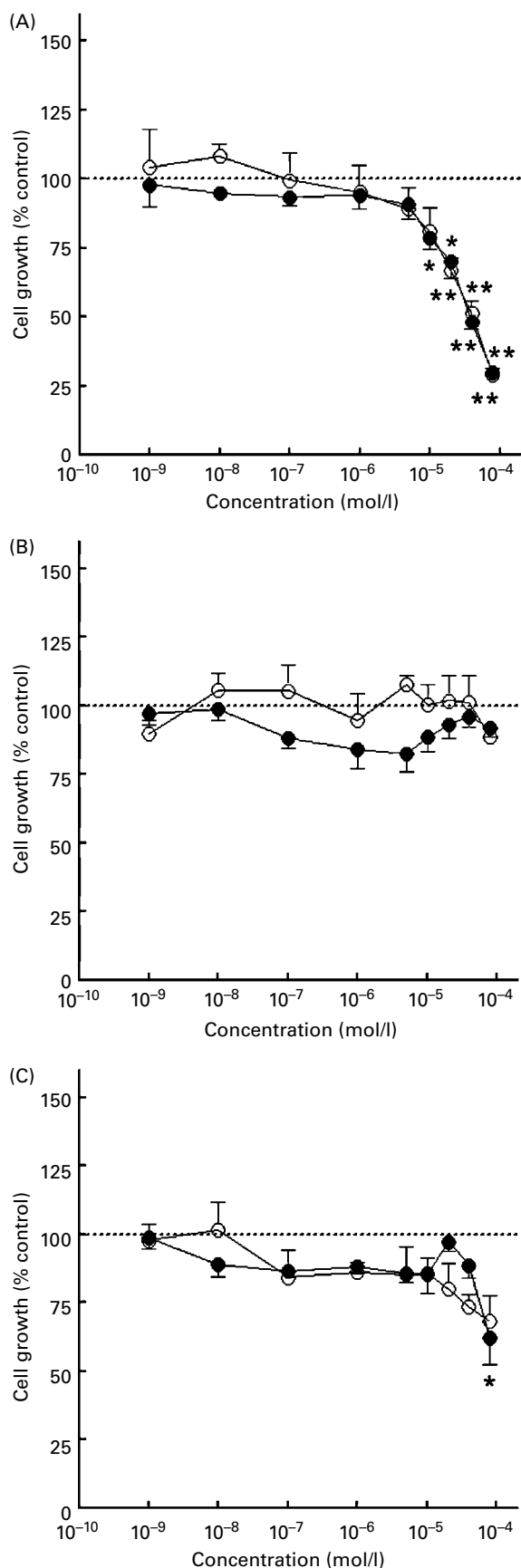
However, stimulation of LNCaP cells was rather weak, pointing to decreased androgen responsiveness. The androgen-responsiveness of this cell line was reported to decline over time, which probably reflects a continuous shift to an androgen-refractory tumour state (Igawa *et al.* 2002). Higher concentrations of dihydrotestosterone (≥ 40 $\mu\text{mol/l}$) reduced the growth of LNCaP cells, possibly by arresting proliferation, as has been reported previously (Lee *et al.* 1995). In contrast, 17 β -oestradiol accelerated the growth of LNCaP cells, which

agrees with several reports from the literature (Maggiolini *et al.* 2002; Arnold *et al.* 2005), but did not stimulate the growth of LAPC-4 cells. Together, these results indicate that the androgen receptor phenotype of LNCaP cells mediates growth-stimulation through 17 β -oestradiol, as has been proposed in the literature (Arnold *et al.* 2005).

The androgen receptor was previously found to have weak affinities for isoflavones, which bind to the receptor only at very high concentrations exceeding those found under physiological conditions (Beck *et al.* 2003). The mutated androgen receptor of LNCaP cells, however, has promiscuous binding affinities and accepts a broader range of ligands (Suzuki *et al.* 2003). Thus it is plausible that isoflavones could induce growth of these cells, as previously found with 17 β -oestradiol (Schuurmans *et al.* 1991). However, this was not observed in the present study. We conclude that isoflavones do not promote growth of androgen-dependent prostate cancer cells, regardless of whether the wild-type or the T877A androgen receptor is expressed.

In part, the present results contradict the work of Maggiolini *et al.* (2002), who reported that genistein stimulates LNCaP cell growth via the mutated T877A androgen receptor. By adding 1% charcoal-stripped fetal calf serum to their cell medium, Maggiolini *et al.* (2002) applied more stringent experimental conditions than those used in the present study, which may account for these differences.

We also evaluated isoflavones for growth-suppressing activities, since a decrease of cell turnover may lower the vulnerability to carcinogens and suppress the progression of already existing malignant foci (Hedlund *et al.* 2003). In our experiments, the growth-inhibiting effects of genistein clearly exceeded the effects of daidzein, a finding which is consistent with several reports for LNCaP (Onozawa *et al.* 1998; Mitchell *et al.* 2000; Hedlund *et al.* 2003) and LAPC-4 cells (Hedlund *et al.* 2003). However, genistein and daidzein can be reduced by the gut microflora and the resulting metabolites might have different potentials to affect the growth of prostate cancer cells. We confirmed the work of Hedlund *et al.* (2003) showing that equol suppresses the growth of LNCaP and LAPC-4 cells more potently than its parent isoflavone daidzein. Equol concentrations in plasma are much lower than those of daidzein (Hedlund *et al.* 2005). However, equol possibly accumulates in the prostate, reaching peak concentrations of 13.5 $\mu\text{mol/l}$ in the prostate fluid (Morton *et al.* 1997). In the present study, equol at 5 $\mu\text{mol/l}$ significantly decreased LAPC-4 cell growth by 32%, which is half of the equol concentration found to reduce LAPC-4 cell growth in a study of Hedlund *et al.* (2003). Moreover, that study revealed that equol treatment at 1 $\mu\text{mol/l}$ decreases growth of benign prostatic epithelial primary cells, making those cells less vulnerable to cancer initiation (Hedlund *et al.* 2003). In the present study, equol at 10 $\mu\text{mol/l}$ also reduced growth of LNCaP cells, which confirms previous reports (Mitchell *et al.* 2000; Hedlund *et al.* 2003). However, the concentration of 5 $\mu\text{mol/l}$ has also been shown to decrease LNCaP growth, as reviewed by Adlercreutz (2002). Thus, equol seems to exert the most potent growth-inhibiting activity in comparison with the other tested compounds. Recently reported *in vivo* data also indicated chemopreventive activities of equol. Three case-control studies disclosed that prostate cancer patients are less likely to produce this reduced daidzein metabolite than healthy controls (Akaza *et al.* 2002, 2004; Ozasa *et al.* 2004).



We further observed that *O*-DMA had growth-suppressing activities in LAPC-4 cells, but not in LNCaP cells. We believe that distinct capabilities to metabolise *O*-DMA, for example, conjugation and elimination, are responsible for the observed differences. Our current work is directed to prove this assumption.

Wiseman *et al.* (2004) determined isoflavone concentrations in plasma following a 10-week consumption of a soya-rich diet (104 mg isoflavones/d) and found about 200 nmol *O*-DMA/l and 170 nmol equol/l (values approximated from figures). Only 35% of the subjects were capable of producing equol ('equol-producers'). This subset had mean plasma concentrations of 364 nmol/l (Wiseman *et al.* 2004). There are also considerable interindividual variations in the production of *O*-DMA; accordingly the term '*O*-DMA-producers' seems to be reasonable as well (Watanabe *et al.* 1998). In a recent study by Hedlund *et al.* (2005) about 95% of the subjects were found to be capable of producing *O*-DMA. After consuming a soya beverage (50 mg isoflavones/d) for 1 week, concentrations of *O*-DMA individually increased up to 367 nmol/l in plasma and 1229 nmol/l in prostate fluid (Hedlund *et al.* 2005).

Hence, the present study disclosed growth-suppressing activities of *O*-DMA at concentrations, which have not to date been determined *in vivo*. Further, we did not find any epidemiological data, for example, case-control studies, supporting a possible relationship between the human *O*-DMA exposure and the risk of prostate cancer. Therefore, our work might be seen as a first hint of chemopreventive properties of *O*-DMA in prostate cells. Additional studies are needed to further elucidate the biological activities of this compound.

We should note that the present study was designed to compare the biological activities of soya isoflavones and their reduced gut metabolites. The observed suppressive effects on cell growth, however, could result from various mechanisms, such as inhibition of cell proliferation or induction of apoptosis, which we have not analysed in detail. The present study has disclosed that this group of soya-related compounds possesses a number of interesting properties in prostate cells. For one, the tested isoflavones do not directly induce DNA damage in prostate cells. Isoflavones also do not appear to promote the growth of prostate cancer cell foci, regardless of whether those cells express the mutated T877A or the wild-type androgen receptor. This is important, since prostate cancer is often unrecognised for years due to a lack of clinical symptoms. The reductive gut metabolism of daidzein enhances its growth-suppressive activities in prostate cancer cells. Despite the potent growth-suppressive activities of genistein, its reduced metabolites have only little effect.

Fig. 3. Modulation of prostate cell growth by genistein and its reduced gut metabolites. LNCaP (●) and Los Angeles prostate cancer (LAPC)-4 (○) cells were treated for 96 h and growth was determined by quantifying residual DNA with 4',6'-diamidino-2-phenylindole hydrochloride staining. (A) Genistein decreased growth of LNCaP by ≥21% from 10 μmol/l (*n* 4) and of LAPC-4 cells by ≥34% from 20 μmol/l (*n* 4). (B) Dihydrogenistein did not affect growth of prostate cells (*n* ≥3). (C) 6'-Hydroxy-*O*-desmethylangolensin decreased growth of LNCaP at 80 μmol/l by 32% (*n* 4) whereas it had no effect in LAPC-4 cells (*n* 3). Data are mean values of relative cell growth compared with control (0.1% dimethyl sulfoxide), with their standard errors represented by vertical bars. Mean values were significantly different from control: * *P* < 0.05, ** *P* < 0.01 (one-way ANOVA and Dunnett's *post hoc* test).

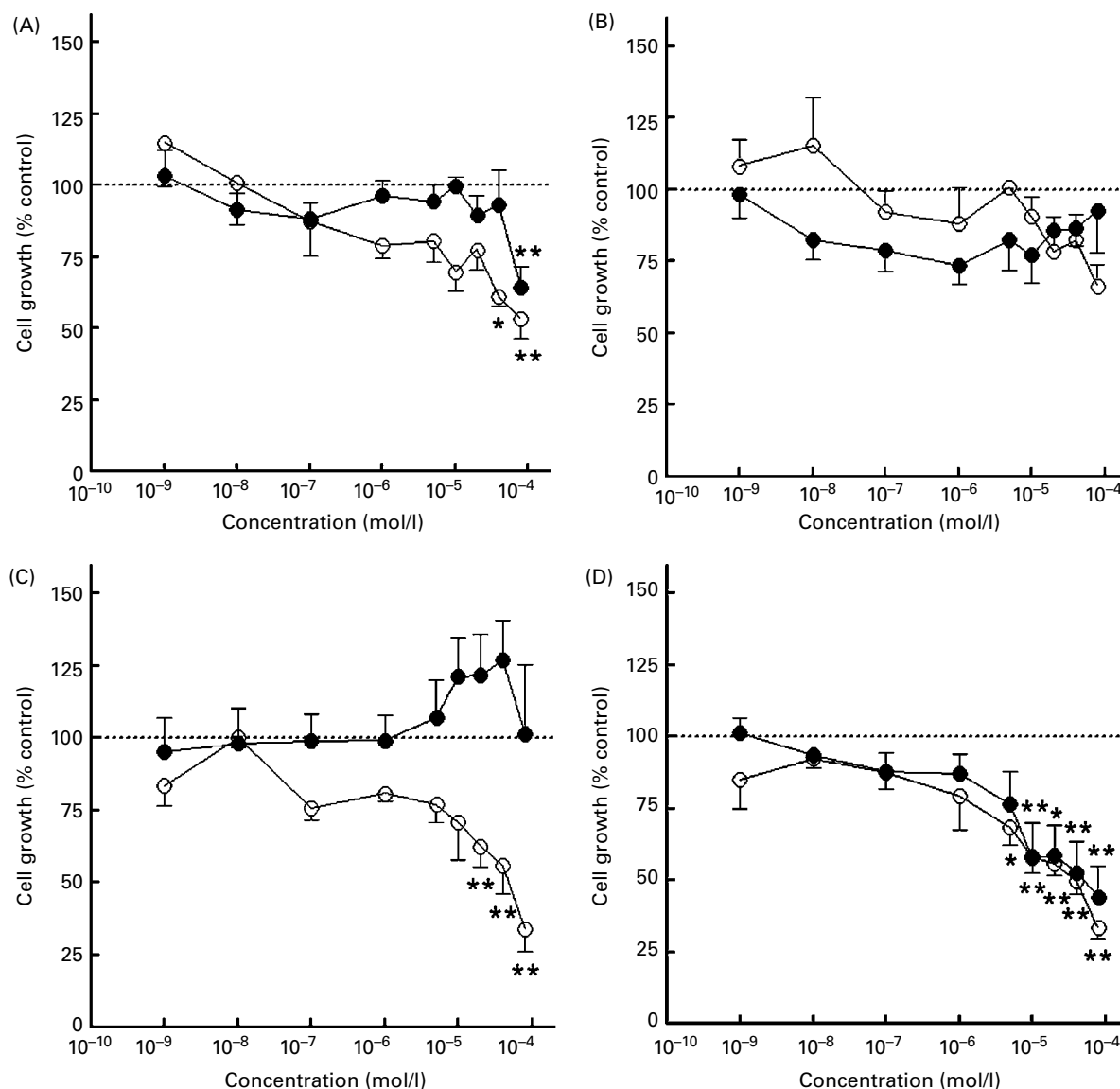


Fig. 4. Modulation of prostate cell growth by daidzein and its reduced gut metabolites. LNCaP (●) and Los Angeles prostate cancer (LAPC)-4 (○) cells were treated for 96 h and growth was determined from the content of residual DNA by 4',6'-diamidino-2-phenylindole hydrochloride staining. (A) Daidzein decreased growth of LNCaP by 36% at 80 $\mu\text{mol/l}$ (n 4) and of LAPC-4 cells by $\geq 39\%$ from 40 $\mu\text{mol/l}$ (n 3). (B) Dihydrodaidzein did not affect growth of prostate cells (n ≥ 3). (C) *O*-Desmethylangolensin had no effect on LNCaP cell growth (n 3) but decreased growth of LAPC-4 cells by $\geq 37\%$ from 20 $\mu\text{mol/l}$ (n 3). (D) Equol decreased growth of LNCaP by 42% from 10 $\mu\text{mol/l}$, and of LAPC-4 cells by $\geq 32\%$ from 5 $\mu\text{mol/l}$. Data are mean values of relative cell growth compared with control (0.1% dimethyl sulfoxide), with their standard errors represented by vertical bars. Mean values were significantly different from control: * P < 0.05, ** P < 0.01 (one-way ANOVA and Dunnett's *post hoc* test).

Taken together, the present *in vitro* data support a possible preventive role of soya-based isoflavones in the progression of prostate cancer. Our findings also point to a possible contribution of the reduced daidzein gut metabolites, equol and possibly *O*-DMA, by inhibiting growth of malignant foci. The growth-suppressing activities of genistein and equol occur at physiologically relevant concentrations and therefore are probably of significance *in vivo*.

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