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Mixture of *Uncaria* and *Tabebuia* Extracts are Potentially Chemopreventive in CBA/Ca Mice - A Long-term Experiment

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Experimental work

Budán et al: Uncaria and Tabebuia are Chemopreventive in Long-term Mice Experiment

Key Words: Gene expression, carcinogenesis, chemoprevention, DMBA, Uncaria, Tabebuia

Abstract.

A long-term experimental animal model was developed by our research group for the evaluation of potential chemopreventive effects. The inhibitory effects of agents on carcinogen (7,12-Dimethylbenz[a]anthracene (DMBA)) induced molecular epidemiological biomarkers, in this case the expression of key onco/suppressor genes were investigated.

Expression pattern of *c-myc*, *Ha-ras*, *Bcl-2*, *K-ras* protoonco and *p53* tumoursuppressor genes were studied to elucidate early carcinogenic and potential chemopreventive effects. Consumption of so called "CoDTM tea" (abbreviation of Claw of Dragon tea) containing bark of *Uncaria guianensis*, Cat's Claw (*Uncaria* sp. *U. tomentosa*) and Palmer trumpet-tree (*Tabebuia* sp. *T. avellanae*) was able to decrease the DMBA-induced onco/suppressor gene overexpression in a short-term animal experiment. In following study CBA/Ca mice were treated with 20 mg/bwkg DMBA intraperitoneally (*ip.*) and expression patterns of onco/suppressor genes were examined at several time intervals. According to the examined gene expression patterns in this long-term experiment the chemopreventive effect of CoDTM tea consumption could be confirmed.

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In our previous studies a unique animal test model was developed through the investigation of onco/suppressor genes expression patterns, as molecular epidemiological biomarkers (Ember *et al.*, 1998). 7,12-dimetilbenz[a]anthracene (DMBA) is a pluripotent and complete carcinogen, therefore it is applied to induce carcinomas in animal experiments. DMBA increases the expression of *c-myc*, *Ha-ras*, *Bcl-2*, *K-ras* protoonco (Gyöngyi and Somlyai, 2002) and consequently *p53* tumoursuppressor genes in the lungs and in the liver, as well (Sándor *et al.*, 1995) in several animal species and also in both sex, indicating both carcinogenic exposure and early steps of tumour formation (Ember *et al.*, 1998). The purpose was to study the early biological effect of DMBA and the protective effect of some potential chemopreventive agents in so called “CoDTM tea” (abbreviation of Claw of Dragon tea). Consumption of “CoDTM tea”, which contains extract of *Uncaria guianensis*, Cat's Claw (*Uncaria* sp. *U. tomentosa*) and Palmer trumpet-tree (*Tabebuia* sp. *T. avellanedae*), was able to decrease the DMBA-induced overexpression of the genes mentioned above, in a short-term animal experiment (Orsós *et al.*, 2007). According to pharmacological studies *Uncaria* species have cytotoxic, anti-inflammatory, antiviral, immunostimulative, antioxidant and antibacterial properties (Heitzman *et al.*, 2005). Keplinger *et al.* confirmed that the pentacyclic oxindol alkaloides (POA) are the main biological active components of *Uncaria* species, which *in vitro* activate lymphocyte-proliferation regulating factor in endothelial cells (Laus *et al.*, 1997; Keplinger *et al.*, 1999). The bark of Palmer trumpet-tree is abundant in lapachol (2-hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone) (2-7%), lapachone and xyloidone (de Santana *et al.*, 1968), which are anticarcinogenic (Ueda *et al.*, 1994) and inhibit the growth of the human keratinocyte cell line HaCaT culture *in vitro* (Müller *et al.*, 1999). Our research group supposed that CoDTM tea consumption was able to decrease the overexpression of certain key onco/suppressor genes.

In this long term study the effect of CoDTM tea was investigated on the expression of *c-myc*, *Ha-ras*, *Bcl-2*, *K-ras* and *p53* onco/suppressor genes in CBA/Ca mice test model (Varga *et al.*, 1991; Ember *et al.*, 1992; Gyöngyi and Somlyai 2000).

Materials and methods

Animals and Treatments.

Infuse of CoDTM tea (D/Eu reg. numb.: PZN Deutschland: 4226037) was prepared according to the instruction of the manufacturer.

10g of the powder was suspended in 750 ml tap water and left to soak for twelve hours, and then it was heated to 80 °C for thirty minutes. After filtration and water refill, the infuse was kept in a closed bottle avoiding light at 4 °C.

250 ml extract is advised to be consumed three times a day, therefore the human dose is 0.167 g powder/kg *per os* (calculated for 60 kg mean body weight).

Calculated equivalent dose for mice is 0.0033 g powder in 3 ml tap water.

Quantity of total pentacyclic oxindol alkaloides (POA) is supposed to be 0.1-1.12%, based on the results of Csapi *et al.* (Csapi *et al.*, 2008).

Six- to eight-week-old (20±4 g) conventionally kept CBA/Ca inbred H-2^K haplotype mice (6 males and 6 females in each group) were used for this experiment according to the following arrangement: control groups, DMBA treated groups, CoDTM consuming groups and DMBA treated groups with CoDTM consumption. These setups were measured at five time intervals after the first DMBA treatment: 1 week, 1 month, 3, 6 and 12 months later.

Control groups were given tap water and treated with *ip.* corn oil. DMBA treated groups received 20 mg/bwkg DMBA. (Sigma Aldrich Budapest, Hungary) dissolved in corn oil (Teva Pharmaceutical Industries Ltd. Debrecen, Hungary) intraperitoneally. DMBA treatment was applied at the beginning of the experiment and in addition two months later.

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Gene expression investigations.

One week, one, three, six and twelve months later the actual four groups were sacrificed and their liver, lungs, kidneys and spleen were removed. 100 mg tissue samples from each organ were collected, and samples of one group were pooled.

After homogenization of the organs, total cellular RNA was isolated using TRIZOL reagent (Invitrogen, Paisley, UK). The RNA quality was checked by denaturing gel-electrophoresis, and absorption measurement was performed at 260/280 nm (A260/A280 was over 1.8). After the necessary dilution, 10 µg RNA was dot-blotted onto Hybond N⁺ nitrocellulose membrane (ECL kit, Amersham, Little Chalfont, UK) and hybridized with chemiluminescently labelled specific probes for *c-myc*, *Bcl-2*, *K-ras*, *p53* and *Ha-ras* (Professor J. Szeberényi, University of Pécs, Hungary) genes. Isolation of RNA, hybridization and detection were performed according to the instructions of the manufacturer. The membranes were rehybridized with the constitutively expressed beta-actin gene as a positive control. The chemiluminescent signals were detected on X-ray films, scanned into a computer and evaluated by Quantiscan software (Biosoft, Cambridge, UK). The results were expressed in the percentage of the positive control. (Data are shown in **Figure 1-8.**)

Results

In this study we investigated the level of certain onco/suppressor gene expressions. We found, that in the control group basal *c-myc*, *Ha-ras*, *p53*, *Bcl-2* and *K-ras* gene expressions vary both in time and in different organs. In the liver of male mice gene expression was low comparing to the beta-actin reference with a slight fluctuation (**Figure 1.**), in the lungs and kidneys (**Figure 3, 5.**) key onco/suppressor expression was higher with the same reference. Compared to the results of male mice (**Figure 1, 3, 5, 7.**) we found, that in female mice (**Figure 2, 4, 6, 8.**) gene expressions were slightly lower nearly in every organ. Gene

expression patterns of the liver were nearly the same (**Figure 1, 2.**), but in the lungs and in the kidneys expression was lower in female mice (**Figure 4, 6.**).

The effect of DMBA treatment:

DMBA caused the overexpression of the investigated key onco/suppressor genes. Male mice showed major elevations, especially in the lungs, kidneys and spleen (**Figure 3, 5, 7.**). In the lungs and kidneys these overexpressions decayed after one month but in the spleen key gene expressions remained high.

In the liver the key gene expression elevations were present - they were not as high as in other organs (**Figure 1.**). Moreover, at the 12th month control major elevations were present. In the lungs, spleen and kidneys key gene expression elevations were detected.

In male mice highly increased key gene expression levels were measured in the lungs, kidneys and spleen (**Figure 3, 5, 7.**). In female mice similar - but milder - gene expressions elevations were detected in the organs mentioned above (**Figure 4, 6, 8.**).

In female mice major gene expression elevations were present at the 12th month in every organ (**Figure 2, 4, 6, 8.**).

The effect of CoDTM on DMBA treatment:

We compared the results of CoDTM tea consumption on DMBA treated mice to the DMBA induced overexpressed cases (positive control). In case of the DMBA we arbitrary chose more than 100% gene expression increase as overexpression, and in case of the CoDTM treatment we chose more than 100% key onco/suppressor gene expression decrease as major decrease in comparison to the positive control.

In **Table 1.** (male) and **Table 2.** (female) we highlighted the organs - and also the overexpressed genes - where major decrease were present.

Comparing to the DMBA treated group, CODTM tea consumption decreased onco/suppressor gene expression in the following cases: checking the one week control in the kidneys all of

the key onco/suppressor gene levels decreased (**Figure 5, 6.**). In the lungs *c-myc*, *Ha-ras*, *p53*, *K-ras* showed major decrease (**Figure 3, 4.**) and in the spleen *c-myc*, *Ha-ras* and *K-ras* declined at the same time point (**Figure 7, 8.**). In case of liver, the kidneys and spleen at one year, results showed decreasing of *c-myc* and *p53* expression (**Figure 1, 2, 5, 6, 7, 8.**). *Ha-ras* also decreased in the lungs, the kidneys and spleen (**Figure 3, 4, 5, 6, 7, 8.**). There was decrease of *Bcl-2* in liver and the kidneys (**Figure 1, 2, 5, 6.**) and *K-ras* in liver, the lungs and spleen (**Figure 1, 2, 3, 4, 7, 8.**).

In female mice we found fewer differences than in male mice, the definite decrease of onco/suppressor gene expressions are shown in **Table 2.**

We highlight the kidney (**Figure 6.**) at the one week control, in which *c-myc*, *Ha-ras*, *p53* and *K-ras* onco/suppressor gene expression levels were decreased. In the lungs, kidneys and spleen almost every onco/suppressor gene expression was dropped at the 12th month (**Figure 4, 6, 8.**).

Discussion

The increase of *c-myc*, *Bcl-2*, *K-ras*, *Ha-ras* and *p53* gene expression indicates early carcinogenic effects (Gyöngyi and Somlyai, 2002) and their overexpression is characteristic in certain manifest tumours. The amplification and overexpression of *c-myc* gene was found to exert an initiator role in human and in mice breast carcinogenesis as well (Dickinson *et al.*, 1992). The activation and overexpression of *Ha-ras* protooncogene through point mutations was detected in colorectal carcinomas, as well (Csontos *et al.*, 2008). Moreover *Bcl-2* is an antiapoptotic gene, which is capable of causing immortalisation of tumour cells, especially if it is overexpressed (Norton and Atherton, 1998). The overexpression and activation of *K-ras* contribute to the formation of metastasis (Smakman *et al.*, 2005) and enhances initiation and progression as well.

The carcinogenic effect of DMBA in the lungs is well known (Stowers and Anderson, 1985), and correlates to our finding, because due to the treatment, all of the examined genes had increased expression in comparison to the control in both sexes. According to the gene expression patterns, CoDTM tea consumption in the lungs of male group seems to exert tumour formation inhibitory effect, usually as the results of several anticancer effects, such as anti-inflammatory, antioxidant (Heitzman *et al.*, 2005) and DNA topoisomerase II partial inhibitory (Esteves-Souza *et al.*, 2007) effects, as well.

Complete (both initiator and promoter) chemical carcinogens activate and overexpress *Ha-ras* proto-oncogene (Dickinson *et al.*, 1992; Perjési *et al.*, 2006), so the *Ha-ras* overexpression caused by DMBA correlates to these facts. Furthermore, CoDTM tea consumption decreased the overexpression of onco/suppressor genes induced by DMBA in the kidneys of male mice, presumably due to the result of antitumour properties of lapachone (Ueda *et al.*, 1994) and inhibitory effects of POA on cancer cell proliferation (Lee *et al.*, 2000).

Generally speaking, in male animals CoDTM tea consumption seemed to exert higher chemopreventive effect on carcinogen increased onco/suppressor gene expression, than in female groups. This could be explained with the noncompetitive, concentration-dependent oestrogen receptor binding property, and therefore oestrogen inhibitory effect of *U. tomentosa*'s. But -according to the literature- in long-term experiment, tamoxifen like paradox oestrogen enhancer effects were found (Salazar and Jayme, 1998).

Oestrogen receptor beta (ER β) is supposed to be effected (Bukulmez *et al.*, 2008) by the oestrogen receptor activating effect of *Uncaria* extracts.

Anti-inflammatory activity of *Uncaria* extracts (Heitzman *et al.*, 2005) is able to silence COX-2, thereby aromatase expression as well (Sirianni *et al.*, 2009), leading to oestrogen level decreasing. Oestrogen level decreasing might slow the proliferation of certain sensitive cells (Sirianni *et al.*, 2009). However, oestrogen increased the expression of *Ha-ras* - and

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consequently other oncogenes (Sándor *et al.*, 1995) - in endometrial cancer cells *via* increasing tyrosine kinase (TK) activity (Fujimoto *et al.*, 1995). Aromatase synthesize oestrogen in males dominantly, thus the inhibition of it underpins the higher efficacy of CoD™ tea in male groups, than in observed in female groups.

According to the expression patterns of the examined molecular epidemiological key onco/suppressor biomarker genes, in this long-term experiment the chemopreventive effect of CoD™ tea consumption could be confirmed.

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	Liver	Lungs	Spleen	Kidneys
1 week	x	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>K-ras</i>	x	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>Bcl-2</i> , <i>K-ras</i>
1 month	<i>c-myc</i>	<i>K-ras</i>	<i>K-ras</i>	<i>c-myc</i> , <i>p53</i> , <i>K-ras</i>
3 months	<i>c-myc</i>	x	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>Bcl-2</i> , <i>K-ras</i>	<i>c-myc</i> , <i>Ha-ras</i> , <i>Bcl-2</i>
6 months	<i>Ha-ras</i> , <i>p53</i>	x	x	x
12 months	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>Bcl-2</i> , <i>K-ras</i>	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>K-ras</i>	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>K-ras</i>	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>Bcl-2</i>

Table 1. The effect of CoDTM on key onco/suppressor genes in male CBA/Ca mice

	Liver	Lungs	Spleen	Kidneys
1 week	<i>p53</i> , <i>K-ras</i>	<i>Ha-ras</i> , <i>p53</i> , <i>K-ras</i>	x	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>K-ras</i>
1 month	x	<i>Ha-ras</i> , <i>Bcl-2</i> , <i>K-ras</i>	x	<i>Bcl-2</i>
3 months	<i>p53</i>	x	x	x
6 months	x	x	x	<i>Bcl-2</i>
12 months	<i>c-myc</i> , <i>p53</i> , <i>Bcl-2</i> , <i>K-ras</i>	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>K-ras</i>	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>Bcl-2</i>	<i>c-myc</i> , <i>Ha-ras</i> , <i>p53</i> , <i>Bcl-2</i>

Table 2. The effect of CoD™ on key onco/suppressor genes in female CBA/Ca mice

Figure 1. The gene expression profile of liver of male CBA/Ca mice

Figure 2. The gene expression profile of liver of female CBA/Ca mice

Figure 3. The gene expression profile of the lungs of male CBA/Ca mice

Figure 4. The gene expression profile of the lungs of female CBA/Ca mice

Figure 5. The gene expression profile of the kidneys of male CBA/Ca mice

Figure 6. The gene expression profile of the kidneys of female CBA/Ca mice

Figure 7. The gene expression profile of spleen of male CBA/Ca mice

Figure 8. The gene expression profile of spleen of female CBA/Ca mice