Regulatory Toxicology and Pharmacology 76 (2016) 94–101

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Inhibition by blueberries (bilberries) and extract from milk thistle of rat forestomach hyperplasia induced by oral smokeless tobacco (Swedish snus)

Robert Nilsson [a, *], Mileva Mićić [b], Jelena Filipović [a], Ana Valenta Šobot [a], Dunja Drakulić [a], Miloš Stanojlović [a], Gordana Joksić [a]

[a] Vinča Institute of Nuclear Sciences, Laboratory for Physical Chemistry, University of Belgrade, Vinča, Belgrade, Serbia
[b] Institute for Medical Investigation, University of Belgrade, Serbia

ARTICLE INFO

Article history:
Received 2 November 2015
Received in revised form
20 January 2016
Accepted 25 January 2016
Available online 28 January 2016

Keywords:
Smokeless tobacco
Snus
Forestomach
Hyperplasia
Bromodeoxyuridine
Phytoprotection
Blueberries
Silymarin
Milk thistle

ABSTRACT

The aim of this study was to identify palatable additives which have a significant protective action against soft tissue changes in the oral cavity caused by Swedish smokeless tobacco ("snus"), and that satisfy existing legal requirements.

Although the cancer risk from snus is extremely low, long term use may result in highly undesirable keratotic lesions and associated epithelial abnormalities in the oral cavity.

The rat forestomach, which is vulnerable to the irritative action of non-genotoxic compounds like butylated hydroxyanisole, propionic acid as well as snus, was chosen as an experimental model. Studied toxicological endpoints included histopathology and cellular proliferation based on DNA incorporation of bromodeoxyuridine.

After 6 weeks' exposure, blueberries (bilberries) and an extract from the common milk thistle were found to exert a highly significant inhibition of cell proliferation induced by snus in the rat forestomach epithelium, indicating a potential protection with respect soft tissue changes in the human oral cavity.

1. Introduction

During the last 3-4 decades male smoking-related deaths have gradually decreased in Sweden. In comparison with EU member states, the median attributable death rate is less than half. The prevalence of smokers among Swedish men is currently below 11%, as compared with more than 40% e.g. in Russia. This development can be attributed to the fact that smoking has to a large extent been replaced by nicotine delivery by a smokeless tobacco product called "snus" (Foulds et al., 2003; WHO, 2012; Ramström, 2014).

In spite of the fact that about 20% of the adult male population uses snus regularly, there is no convincing epidemiological evidence that this is associated with an elevated risk for cancers in the oral cavity, esophagus or stomach (Lewin et al., 1998; Schildt et al., 1998; Luo et al., 2007; SCENIHR, 2008, p.11; Lee, 2011). Further, it has not been possible to verify the claim, that snus causes pancreatic cancer (Lee, 2011; Bertuccio et al., 2011). The failure to detect an elevated cancer incidence in users of snus is compatible with the fact that the levels of tobacco specific nitrosamines (TSNA) are about 10 000 times lower than e.g. in some types of impure Sudanese oral tobacco (Idris et al., 1991; 1994; Österdahl et al., 2004; WHO, 2009). Further, when comparing the expected levels of promutagenic TSNA-induced DNA damages induced by snus with the "normal" background of the same lesions (mainly O^6-methylguanine, N7-methylguanine and DNA pyridyloxobutylations) (Schlöbe et al., 2008; Swenberg et al., 2011), no increase in cancer risk can be expected (Nilsson, 2011).

After long term exposure snus may induce local oral lesions known as "snuff dipper's lesions", leucoplakias, or snuff-induced keratosis, characterized by a hyperplastic epithelium with vacuolization and keratinization. Acanthosis, and slight inflammation may also be present accompanied by an increased mitotic rate (Larsson et al., 1991). It should be distinguished from smoking

* Corresponding author. Department of Physical Chemistry, Vinča Institute of Nuclear Sciences, University of Belgrade, Mike Petrovica Alasa bb Vinča, Belgrade 11350, Serbia.
E-mail address: robertn65@telia.com (R. Nilsson).

http://dx.doi.org/10.1016/j.yrtph.2016.01.017
0273-2300/

induced preneoplastic forms of leucoplakia displaying cellular atypia (dysplasia (Axéll, 1993; Axéll et al., 1976; Andersson and Axéll, 1989). These dysplastic lesions are typically induced by betel chewing, or by other impure tobacco products used in South East Asia and Sudan (Zain et al., 1999).

The smokeless tobacco keratosis induced by modern Swedish snus is reversible after cessation of exposure, and has a very low probability for malignant transformation (Roosaar et al., 2006). Nevertheless, a carcinogenic risk associated with snus lesions in some particularly susceptible individuals cannot be ruled out, although the population risk increase would be too low for detection by conventional epidemiological methods. These lesions represent a clinical problem in themselves. Snus users with leukoplakias are sometimes referred for various, unnecessary clinical work ups, including surgical biopsy.

A number of potent carcinogens induce tumors in the rodent forestomach, which is also susceptible to the irritative or corrosive action of several non-genotoxic compounds, like acrylic acid, butylated hydoxytoluene (BHA) and propionic acid. Stimulation of cell division induced by cytotoxicity and regenerative hyperplasia represents important underlying mechanisms (Clayson et al., 1991). IARC, which disregards mechanisms of action as well as levels of exposure, has classified BHA as a B2 carcinogen: Nevertheless, this additive is universally used as an antioxidant in food products like fats and oils (E321), and propionic acid (E280) is employed as an antifungal agent in bread and other commodities.

In two Swedish studies, where rats were exposed to Swedish snus for 8–22 months in a surgically produced artificial oral canal, squamous epithelial hyperplasias were detected in the forestomach. During treatment the rats had swallowed tobacco, and the effects were interpreted as due to non-specific irritation (Hirsch and Johansson, 1983; Hirsch et al., 1984). In this study the rat forestomach epithelium was chosen as a potentially useful experimental system to investigate modulation of hyperplasia induced by tobacco.

In rodents a number of constituents from plants as well as other chemicals have demonstrated inhibition of tissue lesions such as hyperplasia and tumors. However, the efforts within EU and the US to drastically limit and eliminate undesirable additives to tobacco products seriously limit the number of such agents that can be added (EU, 2015; FDA, 2015). The aim of this experimental study was to ascertain the utility of blueberries and silymarin as possible additives as potentially protective agents against soft tissue changes in the human oral cavity caused by oral tobacco.

2. Materials and methods

2.1. Substances and herbal products

2.1.1. 3-t-Butyl-4-hydroxyanisole

BHA (99%) was obtained from Sigma–Aldrich Co., USA. It was dissolved in corn oil (Sigma, Aldrich), and mixed with pulverized commercial pellet (Veterinarian Institute Subotica, Serbia) to a final concentration of 2%, and used as such.

2.1.2. Bromodeoxyuridine

(BrdU) (≥99%) – (Sigma–Aldrich Co. USA) was purchased from Uni-Chem, Belgrade. Flat-faced cylindrical matrices containing 50 mg of BrdU with a diameter of 5.6 mm were prepared at the Faculty of Pharmacy, Belgrade University, by a direct compression technique using an eccentric tablet press (Korsch EK-0, Korsch, Berlin, Germany).

2.1.3. Standardized oral tobacco

(snus "Ettan" brand) was obtained from Swedish Match AB. The level of selected impurities are given below (Table 1) (Rutqvist et al., 2011).

The levels for nitrosamines and benzo(a)pyrene (B(a)P) are in agreement with the limits recommended by WHO (2 mg/kg for NNN + NNK and 5 μg/kg for B(a)P; WHO, 2009).

The tobacco was thoroughly homogenized with distilled water in a blender, and used as such or mixed with the putative protective agent. At high concentrations the tobacco slurry separated into two phases, which was inappropriate for intubations. 10 g tobacco homogenized in 100 ml of H_2O was found to be appropriate.

2.1.4. Blueberries

(*Vaccinium myrtillus*) - A freeze dried powder from wild Swedish blueberries produced by Superfruit, Stockholm, Sweden, was used. The product was distributed by the Stockholm University under the EU 7th Framework Programme Collaborative project with Department of Biotechnology, New Delhi, "*Impact of agents with potential use in functional foods on biomarkers for induction of age related diseases*" (Grant Agreement, 245030, completed in 2014).

0.15 g Blueberry powder was mixed with 100 ml distilled water and used in the current study.

2.1.5. Extract from milk thistle

(*Silybum marianum*) – An extract from milk thistle seeds was obtained from Favora Belupo, Croatia. In violation of the EU Directives on Traditional Herbal Medicinal Products, the producer refused to reveal the concentration of active principles. According to UPLC analysis carried out at Vinča, the content of silymarin was 52%, and the relative proportions of active ingredients were comparable to an extract standardized to 50–60% silymarin from Indena S.p.A., Milano, Italy (44% vs, 46.5% silybinin in the Italian product, 12% vs. 16.3% isosilybinin and 44% vs. 37.2% silydianin plus silychristin). According to EFSA (2010) the Indena product was adequately characterized.

Each animal was administered 0.06 g of the Favora product corresponding to 0.03 g of silymarin.

2.2. Animals and treatments

Seven weeks old male inbred Wistar rats (250 ± 10 g pilot experiment; 300 ± 19 g main experiment) from separate litters obtained from a colony kept at the Vinča breeding facility, were maintained under the standard conditions, group-housed (4 per cage) on chip bedding with free access to food (pelleted commercial diet from Veterinarian Institute Subotica, Serbia) and tap water, regular 12 h light/12 h dark cycle and constant temperature (21 ± 2 °C) and humidity. Body weights were measured 3 times per week, and the animals observed daily for signs of toxicity and behavioral changes.

In the pilot, as well as in the main study, volumes of 1.5 ml containing the tested agents at levels given below were intragastrically intubated 3 times a week (9–10 AM), using reusable stainless steel feeding needles, 3 mm ball diameter (Cadence Inc. USA).

Table 1
Impurities in Swedish snus. Average observed levels in Swedish Match's products 2009.

Component	Content	Component	Content
Nitrite	2.0 (mg/kg)	Cadmium	0.6 (mg/kg)
TSNA total	1.6 (mg/kg)	Lead	0.3 (mg/kg)
NDMA*)	0.7 (μg/kg)	Arsenic	0.1 (mg/kg)
B(a)P	1.1 (μg/kg)	Nickel	1.3 (mg/kg)

*) NDMA, N-nitrosodimethylamine.

- 10 g Swedish snus homogenized in 100 ml distilled water (≈0.5 g/kg bw).
- A homogenized water suspension of 10 g freeze dried blueberry powder in 100 ml distilled water (≈0.5 g/kg bw).
- 4 g Extract (52% silymarin) dissolved in 100 ml distilled water (≈0.1 g/kg bw).
- A slurry of 10 g snus plus 10 g blueberries in 100 ml distilled water (≈0.5 g/kg bw for each agent).
- 10 g Snus plus 4 g extract (52% silymarin) in 100 ml distilled water (≈0.5 g/kg bw snus and ≈0.1 g/kg bw silymarin).

The Wistar rats refused to consume a diet containing 2% of BHA as described for Fischer 344 rats by Cantoreggi et al. (1993). For this reason, BHA was mixed with pulverized food pellets, and administered by gavage.

All procedures for treatment of the animals were approved by the Ethical Committee for the Use of Laboratory Animals of the Vinča Institute of Nuclear Sciences, University of Belgrade, according to the guidelines issued by the EU registered Serbian Laboratory Animal Science Association implementing the European Communities Council Directive of 24 November 1986 (86/609/EEC) as well as the rules for good laboratory practice established by EU and OECD. All animal experiments are under the guidance of an authorized veterinarian specialized in the conduction of animal experiments, and the principal investigators and technicians are authorized to perform experiments in animals.

2.2.1. Pilot study

Animals were randomly divided into groups of four rats (including controls) and used to establish the following experimental conditions.

1) Effects of BHA, positive controls (4 and 8 weeks' exposure).
2) Determination of the appropriate length of exposure for inducing lesions in the forestomach by snus (4 and 8 weeks).
3) Optimal dose and dosing frequency for exposure to the tested agents.
4) Optimization of BrdU uptake.
5) Optimization of histological and immunohistochemical techniques.
6) Standardizing the measurement of oxidative stress parameters in plasma.

2.2.2. Main study

7 groups of 4 rats were treated for 6 weeks as described above under 2.2 *Animals and treatments*, with the inclusion of BHA and an untreated control group. Sacrifice on day 43.

2.3. Autopsy and tissue preparation

The animals were sacrificed by decapitation and the stomachs with the parts of upper small intestine were removed. The forestomachs were separated (near the limiting ridge) and cut along the minor curvature. During target tissue separation, the animals' abdominal and thoracic organs were inspected for any pathological changes.

After separation, forestomachs were washed in 0.9% aqueous sodium chloride, spread on paraffin substrate with pins and covered with 10% neutral buffered formalin for one week's fixation. Tissue blocks of the forestomach were cut transversely into serial Sections 5 μm in thickness from the forestomach-esophageal junction, middle region and forestomach-glandular junction.

Two consecutive sections, with ten levels, in the range of 250 μm between each level, were taken for analysis. The tissue sections were stained with hematoxylin and eosin for histological analysis and immunohistochemically for the incorporation of BrdU into DNA for analysis of cell proliferation. The analysis was done on light microscope Olympus AX70 with the objective magnification 10x and 20x.

2.4. Cell proliferation study

After deparaffinization in xylene, dehydration with a series of decreasing concentrations of ethanol, and rehydration in distilled water, the forestomach sections were stained immunohistochemically (Pharmingen™ BD Biosciences®, San Jose, CA, USA). Basically, the procedure involves the use of a monoclonal antibody against BrdU (anti-BrdU murine IgG), which is detected with a secondary antibody (biotinylated goat anti-mouse IgG) linking the primary antibody to a label (streptavidin-HRP) as the detection system, and 3,3′-diaminobenzidine as chromogen for visualization of BrdU incorporation (BD Biosciences, 2014). The forestomach tissue sections incubated with normal serum instead of the primary antiserum were used for control staining. The sections were counterstained with Mayer's hematoxylin (Merck) for analysis under light microscope.

The number of immunoreactive cells in the squamous stratified epithelium was determined using a computer-supported imaging system connected to a light microscope with an objective magnification of 10x. BrdU positive cells were expressed per mm^2 of epithelium. The area of the epithelium was calculated using the following expression,

$$p = p \times d^2 \Big/ 10^6$$

Here, P is the surface area, p is the number of grid points that lie in the epithelium, and d is the size of the square network at a magnification of 200 (10x objective). The number of proliferating ($BrdU^+$) cells was expressed per mm^2 of epithelium, and calculated according to the expression

$$N = n/P$$

where n is the number of BrdU positive cells on the presented surface, and P is the examined surface area of epithelium. At least 10 tissue sections per animal were counted in the forestomach-esophageal junction, middle region and forestomach-glandular junction. In addition to the number of BrdU positive cells/mm^2, the ratio between dividing cells and the total number of cells per unit area (proliferation index), expressed as percentage is given. This enabled a comparison to be made between the two manners of presentation.

2.5. Statistical analysis

The results are expressed as the mean ± SE. Values were compared using the nonparametric Mann–Whitney U test in the program SPSS 10 for Windows. Differences at $p < 0.05$ were accepted as the level of significance.

3. Results

3.1. Pilot study

3.1.1. Optimization of BrdU uptake

The results from optimization of BrdU exposure are shown in Fig. 1. An increase of dose from 50 to 100 mg resulted in more than a doubling of labeling efficacy, and 200 mg gave an additional enhancement of some 20%. A further elevation of dose had a

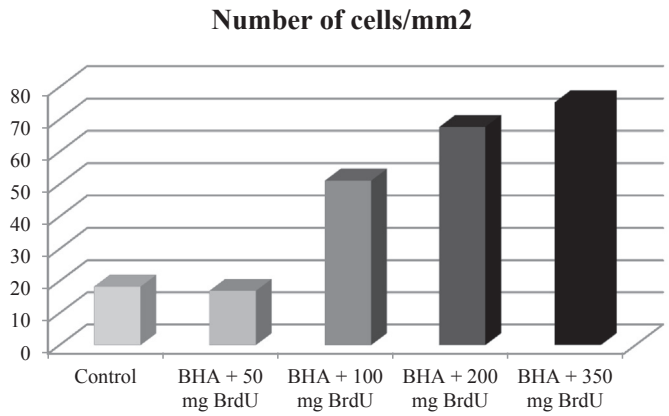

Fig. 1. Levels of BrdU positive cells in the rat forestomach in controls and in animals administered a BHA containing diet (2%) by intragastric intubation for 4 weeks, and where matrices with a different total content of BrdU were inserted subcutaneously in the dorsal neck region 2 days prior to sacrifice.

relatively minor impact. We therefore concluded 200 mg to represent an appropriate BrdU dose level.

3.1.2. Histopathological findings

Effects from dietary exposure to BHA or Swedish snus was seen already after 4 weeks of treatment, and consisted of dilation of blood vessels in the submucosa. These changes were more apparent after 8 weeks. In comparison with controls, there was a thickening of the basal region of squamous epithelium forestomach as a result of enlargement due to proliferation of cells of the basal layer, while the thickness of the surface keratin was similar (Fig. 2b).

3.1.3. Cell proliferation

A significant increase in the number of proliferating BrdU positive cells ($BrdU^+$) was found in the basal and granulose layers of the squamous epithelium from the rats exposed to BHA as well as in animals administered snus for 4 as well as 8 weeks. Proliferating cells were mainly localized in the basal layer of epithelium, although they could be seen in the spinous epithelial layer, especially in animals treated with snus of BHA for eight weeks where the number of $BrdU^+$ cells was significantly higher than for the shorter treatments ($p < 0.01$). In Table 2 the number of BrdU positive cells as well as the proliferation index are presented for controls, exposure to BHA or snus after 4 respective 8 weeks in three regions of the forestomach. Marked cell proliferation was seen in all analyzed forestomach regions.

Fig. 2 demonstrates the incorporation of BrdU in the rat forestomach epithelium in controls (a) and after treatment with snus for 8 weeks (b).

3.2. Main study

3.2.1. Body weight gain

After 6 weeks' exposure, there were no statistically significant divergences between groups with respect to the rate of body weight increase, although there was a (non-significant) tendency towards a lower weight increase for the snus group (Fig. 3). There were no significant differences ($p < 1.0$) vs. controls in the average group body weights at the end of the treatments.

3.2.2. Gross pathology

Except for one rat in the milk thistle extract group, where upon autopsy pathological changes in the liver were detected, there were

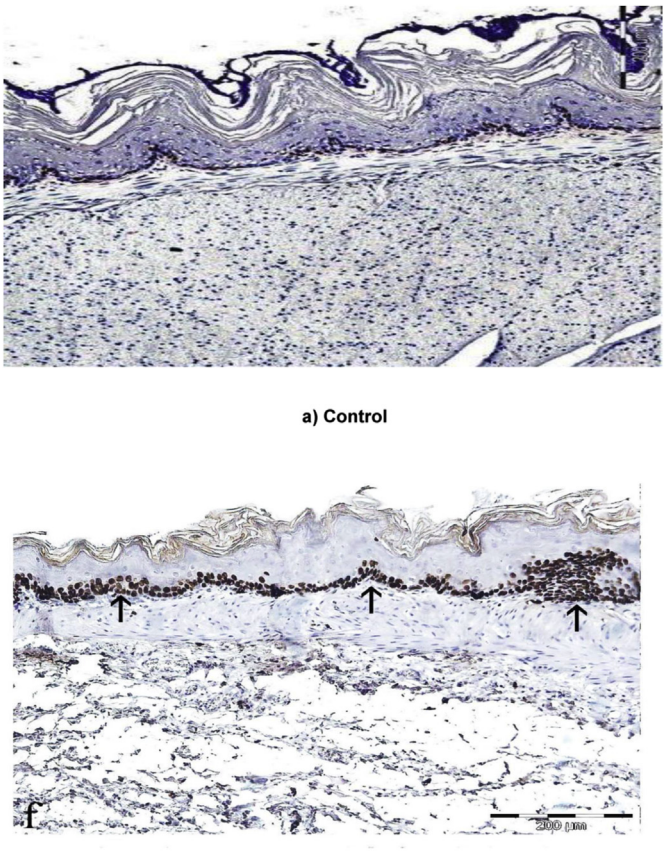

Fig. 2. Incorporation of BrdU in the rat forestomach epithelium. a) Control (10x magnification); b) Snus 8 weeks Arrows in b) indicate BrdU positive cells in the granulose layer (10x magnification) Scale bars bottom right.

no significant gross pathological alterations in controls or in the treated animals. The liver lesions of the single, aforementioned animal consisted of focal liver fibrosis in the left and middle liver lobes, with numerous eosinophils in the forestomach. The cause for these findings remain unclear. Surveillance to detect bacterial or parasitic infections of the rat colonies has been maintained quarterly, and samples for microbial analysis sent to the Veterinary Institute in Belgrade. For the target litter of rats microbiological findings have been negative.

3.2.3. Cell proliferation

Treatment with snus for 6 weeks led to a significant increase ($p < 0.001$) in $BrdU^+$ labeled cells in the forestomach epithelium. The cells were distributed through several layers in the basal epithelium, moreover, their presence in the stratum granulosum was observed (Fig. 4). Focal accumulation of a large number of $BrdU^+$ cells penetrating into the forestomach epithelium was also detected. In comparison with treatment with snus only, combined administration with blueberries or extract from milk thistle decreased the number of proliferating cells significantly by 36–44% (Table 3).

4. Discussion

When the tobacco specific nitrosamines 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone and N'-nitrosonornicotine were swabbed in the oral cavity of the rat, with and without an extract from US oral tobacco, the yield of oral tumors

Table 2
Number of BrdU positive cells/mm^2 and proliferative indices (%) in three regions of the forestomach in rats treated for 4 or 8 weeks with BHA or snus.

Treatments	Forestomach region					
	Forestomach-esophageal junction		Middle region		Forestomach-glandular junction	
	Number of BrdU positive cells/mm^2	Index of proliferation – IP (%)	Number of BrdU positive cells/mm^2	Index of proliferation – IP (%)	Number of BrdU positive cells/mm^2	Index of proliferation – IP (%)
Control	18,83 ± 2,04	25,32 ± 2,6	18,58 ± 1.3	25,9 ± 1,77	16,46 ± 1,57	22,52 ± 2,2
BHA 4 weeks	68,85 ± 0.3***	77,99 ± 0,7***	78,67 ± 5,7***	77,14 ± 1,65***	44,03 ± 1,1***	51,8 ± 1,1***
BHA 8 weeks	91,46 ± 0.4***	87,71 ± 0,9***	117,97 ± 6,8***	91,17 ± 1,4***	89,00 ± 8,4***	76,26 ± 5,5***
Snus 4 weeks	42,79 ± 3,8**	44,86 ± 1,3**	56,55 ± 3,9**	57,76 ± 3,1**	30,42 ± 0,6**	49,66 ± 0,8**
Snus 8 weeks	71,56 ± 2,1***	64,7 ± 2,4***	81,00 ± 5,4***	64,03 ± 1,5***	66,93 ± 3,05***	79,78 ± 0,36***

The results are presented as average ± SE.
***p < 0.001, **p < 0.01, *p < 0.05, control vs. animals treated with BHA or Snus.

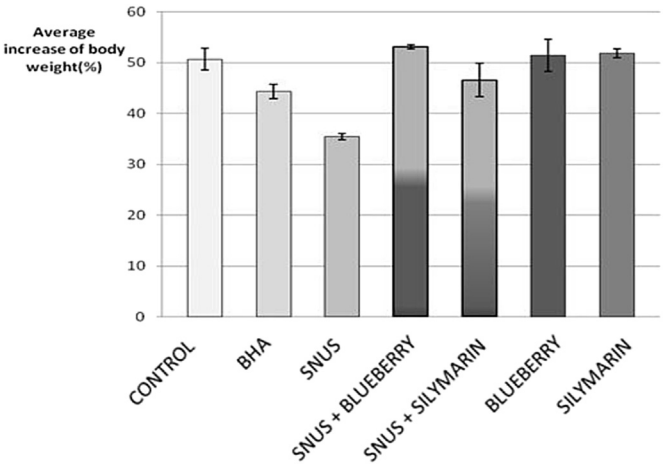

Fig. 3. Average increase of body weights of treated animals, treatment for 6 weeks.

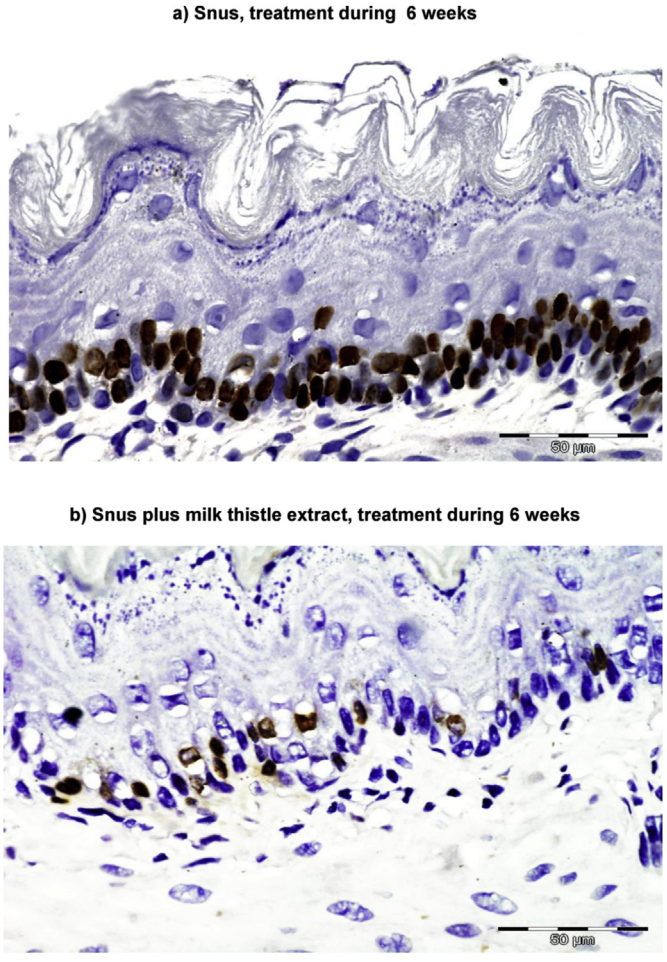

Fig. 4. Modulation by blueberries and milk thistle extract of BrdU Incorporation induced by snus in the rat forestomach epithelium. Treatment for 6 weeks. a) Snus; b) Snus + milk thistle extract. 40x magnification. Scale bars bottom right.

was drastically reduced in presence of the extract (Hecht et al., 1986), findings, that are in line with the presence of potent anti-carcinogens in plant materials. In humans, retinol, beta-carotene and canthaxanthin exerted a protective action with respect to the cytotoxic and genotoxic action of betel chewing (Stich and Tsang, 1989; Stich et al., 1984).

In our investigations blueberries (bilberries) and an extract from milk thistle containing high levels of silymarin were used. Blueberries are rich in antioxidants, and prevent induction of cancers in the rat esophagus by N-nitrosomethylbenzylamine (Stoner et al., 2010). Blueberries also seem to reduce base line levels of micronuclei in lymphocytes from humans exposed to high levels of PAHs in food (Joksić, unpublished results). The mechanisms underlying chemoprotection by fruits and berries have been extensively discussed (Surh, 2003). In the hamster pouch inhibition of carcinogenesis by 7,12-dimethylbenz[a]anthracene by these berries was evidently mediated by induction of Nrf2-mediated cytoprotection, antioxidant, detoxification and DNA repair enzymes (Kavitha et al., 2013).

Silymarin is a low toxicity flavonoid complex derived from milk thistle seeds, and contains the flavonolignans silibinin, isosilibinin, silidianin, and silicristin. It has been used in traditional medicine, and because of a potential anticarcinogenic antiproliferative and antidiabetic activity, it has been the subject of a number of experimental as well as clinical investigations (Hahn et al., 1968; Flaig et al., 2007; Davis-Searles et al., 2005). The antiproiferative effect of silibinin in vivo has been ascribed to G1 cell cycle arrest associated with a marked decrease in the kinase activity of cyclin-dependent kinases (CDKs) and associated cyclins (Zi and Agarwal, 1999).

Humans do not have a functional analogue to the rodent forestomach, but this rodent tissue could provide useful indications of modulation of hyperplasia in the human oral cavity and esophagus

Table 3
The effects of blueberries or milk thistle extract on the levels of BrdU positive cells and proliferative index (%) in three regions of the rat forestomach epithelium after administration of snus or BHA for 6 weeks.

Treatments	Forestomach region					
	Forestomach-esophageal junction		Middle region		Forestomach-glandular junction	
	Number of BrdU positive cells/mm^2	Index of proliferation – IP (%)	Number of BrdU positive cells/mm^2	Index of proliferation – IP (%)	Number of BrdU positive cells/mm^2	Index of proliferation – IP (%)
Control	19,53 ± 1,8	24,2 ± 2,7	18,76 ± 1,6	25,6 ± 2,4	15,67 ± 0.9	23,2 ± 3,1
Blueberry	21,83 ± 2,5	24,57 ± 1,9	15,04 ± 1,6	17,3 ± 1,5**	15,94 ± 0,9	19,52 ± 1,4
Milk Thistle Extract	20,42 ± 0,1	19,66 ± 0,9	20,91 ± 1,5	23,11 ± 1,9	17,69 ± 1,5	20,12 ± 1,8
BHA 6 weeks	68,4 ± 1,6***	70,1 ± 0.9***	71,22 ± 2,1***	74,62 ± 1,3***	70,02 ± 2,4***	72,31 ± 2,7***
Snus 6 weeks	39,75 ± 2,6***	38,97 ± 1,9***	43,59 ± 1,7***	39,62 ± 1,3***	39,44 ± 2,9***	35,03 ± 2,4***
Snus + Blueberry	24,61 ± 2,6**,a	32,5 ± 2,8	29,12 ± 2,1***,b	34, 19 ± 1,9*,c	21,51 ± 1,8***,b	24,45 ± 1,7**,a
Snus + Milk Thistle Extract	14,92 ± 2,6***,b	19,34 ± 2,3***,b	16,44 ± 1,2***,b	21,5 ± 1,3***,b	11,79 ± 1,3***,b	15,17 ± 1,2***,b

The results are expressed as average ± SE.
*p < 0.05; **p < 0.01; ***p < 0.001/control vs. snus, control vs. blueberry or milk thistle extract.
[a]p<0.01; [b]p < 0.001; [c]p < 0.05/snus vs. snus + blueberry or snus vs. snus + milk thistle extract.

caused by chemicals. However, certain histological differences should be pointed out. Thus, the mucosa of the rat forestomach is lined with a stratified squamous keratinized epithelium (Proctor et al., 2007), whereas the aforementioned human tissues are usually non-keratinized, or minimally keratinized.

When evaluating chemical exposure by the oral route, the tissue dose in humans is obviously not equivalent to that in the forestomach of the rat. While there is an extended period of exposure in this rodent organ to an agent in food or delivered by gavage, oral contact and passage through the mouth and esophagus are rapid in humans. On the other hand, for users of oral tobacco the exposure of the oral cavity and esophagus would be comparable to the situation in the rodent forestomach given tobacco by gavage.

In some older studies the cheek pouch of hamsters were exposed to tobacco of varying degree of purity with scant evidence of induced histopathological lesions (Grasso and Mann, 1998). In one of the best documented, acanthosis of the pouch epithelium was the only significant finding after 26 weeks of exposure (Summerlin et al., 1992). Because of a lower sensitivity, the hamster was found to be less suitable for our purposes.

After administration for 2 years, 2% BHA in the diet has been shown to be carcinogenic to the rat forestomach (Ito et al., 1993). The fact that lesions induced by Swedish snus have been shown to be reversible after cessation of exposure (Larsson et al., 1991), represents an important feature when trying to replicate the effects in an animal model. After 24 weeks of treatment with 2% BHA in the diet, exophytic epithelial proliferation (simple hyperplasia or papilloma) in the rat forestomach was found to be reversible, whereas basal cell hyperplasia proved more persistent. However, a second study demonstrated, that after withdrawal of BHA also basal cell hyperplasia regressed (Masui et al., 1987; Tatematsu et al., 1991). These findings suggest that the rat forestomach lesions induced by snus may prove to be reversible.

In the investigation conducted with BHA in Zurich (Cantoreggi et al., 1993), a treatment period of 4 weeks was chosen with good results. However, this study involved continuous exposure to BHA with food by gavage which results in a more exact dosing. This treatment was performed 3 times per week, and resulted in a 40% lower exposure in comparison with the Swiss study, and the duration of treatment was therefore extended to 6 weeks. In addition, the unknown treatment time required for exposure to tobacco had to be determined in the pilot study.

The pathological changes recorded in this study for BHA were clearly less severe than those described by Cantoreggi et al. (1993), where differences in strain sensitivity could have played a role. Likewise, the epithelial changes induced by snus were mild, and less pronounced than those described by the group of Hirsch et al. (1984), where the time of exposure was considerably longer (8–22 months).

As demonstrated in this study, incorporation of BrdU in replicating DNA represents a sensitive method for monitoring cell proliferation. IP injection of BrdU has sometimes been employed, but there are few reports on oral application, and in one study where BrdU was administered in drinking water, inconsistent results were obtained with respect to labeling of proliferating cells in rat bone marrow (Jecker et al., 1997).

The proliferation marker Ki-67 represents an alternative to BrdU. Quantitative assessment of BrdU and Ki-67-positive cells gave higher numbers of the latter. In view of the fact, that BrdU is incorporated into DNA only during the S-phase, whereas Ki-67 is expressed during all active phases of the cell cycle, this is to be expected. However, experimentally increased cell proliferation, or reduction (by irradiation), resulted in parallel changes in BrdU and Ki-67 signals. It has been convincingly demonstrated that BrdU incorporation is equally useful (Oliver et al., 2000; Kee et al., 2002). To deliver BrdU. Cantoreggi et al. (1993) employed an osmotic minipump implanted s.c. in the upper back of rats. In this study we employed subcutaneously inserted BrdU pellets which provide a slow and uniform delivery, a method that has proved to give reliable results in mice (Allen et al., 1978; King et al., 1982) and rats (Van Kesteren-van Leeuwen and Natarajan, 1980). The turnover of cells in the forestomach epithelia is rapid, and exposure to BrdU for two days before sacrifice provides an adequate picture of the dynamics for label incorporation.

The impact of BrdU *per se* on cellular proliferation and genotoxicity has been studied in mouse femoral bone marrow after subcutaneous implantation of BrdU pellets containing 20–53 mg BrdU. There was a small, but statistically significant dose-dependent increase of sister chromatid exchanges, whereas the frequency of chromosome aberrations and mitotic index were unaffected (Wilmer and Soares, 1980). We conclude that the BrdU treatment would not be expected to significantly affect our results. In our study expressing cell proliferation as number of dividing cells per unit area and as the proliferation index give comparable results.

5. Conclusions

In spite of a relatively short time of exposure, the marked inhibition by blueberries and milk thistle extract on cellular proliferation induced by Swedish snus in the rat forestomach epithelium indicates a possible approach for achieving protection against the soft tissue changes in the human oral cavity caused by smokeless

tobacco.

Given the reversibility of snus-induced oral lesions in humans, the effects of cessation of exposure with respect to the tobacco induced effects in the epithelia of this rodent model deserves investigation.

Acknowledgments

The preparation of the BrdU tablets by Dr. Bojan Čalija, Faculty of Pharmacy, University of Belgrade, is gratefully acknowledged. We thank A.T. Natarajan, professor emeritus, Leiden University, for his valuable suggestions. This study was supported by a grant from Swedish Match AB. The sponsor had no role in study design, the collection, analysis and interpretation of data, writing of the report or in the decision to submit the article for publication.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2016.01.017

References

Allen, J.W., Shuler, C.F., Latt, S.A., 1978. Bromodeoxyuridine tablet methodology for in vivo studies of DNA synthesis. Somat. Cell Genet. 4, 393–405.

Andersson, G., Axéll, T., 1989. The clinical appearance of lesions associated with the use of loose and portion-bag packed Swedish moist snuff, a comparative study. J. Oral Pathol. Med. 18, 2–7.

Axéll, T., 1993. Oral mucosal changes related to smokeless tobacco usage, research findings in Scandinavia. Oral Oncol. Eur. J. Cancer 29B, 299–302.

Axéll, T., Mörnstad, H., Sundström, B., 1976. The relation of the clinical picture to the histopathology of snuff dipper's lesions in a Swedish population. J. Oral Pathol. 5 (4), 229–236.

BD Biosciences, 2014. BrdU*In-situ* Detection Kit. Instruction.Manual. http://www.bdbiosciences.com/ds/ab/others/550803_551321%20Book_Website.pdf.

Bertuccio, P., La Vecchia, C., Silverman, D.T., Petersen, G.M., Bracci, P.M., Negri, E., et al., 2011. Cigar and pipe smoking, smokeless tobacco use and pancreatic cancer, an analysis from the international pancreatic cancer case–control consortium (PanC4). Ann. Oncol. 22, 1420–1426.

Cantoreggi, S., Dietrich, D.R., Lutz, W.K., 1993. Induction of cell proliferation in the forestomach of F344 rats following subchronic administration of styrene 7,8-oxide and butylated hydroxyanisole. Cancer Res. 53, 3505–30508.

Clayson, D.B., Iverson, F., Nera, E.A., Lok, E., 1991. Early indicators of potential neoplasia produced in the rat forestomach by non-genotoxic agents, the importance of induced cellular proliferation. Mutat. Res. 248, 321–331.

Davis-Searles, P.R., Nakanishi, Y., Kim, N.C., Graf, T.N., Oberlies, N.H., Wani, M.C., Wall, M.E., Agarwal, R., Kroll, D.J., 2005. Milk thistle and prostate cancer, differential effects of pure flavonolignans from Silybum marianum on antiproliferative end points in human prostate carcinoma cells. Cancer Res. 65, 4448–4457.

EFSA, 2010. Scientific opinion on the substantiation of a health claim related to silymarin BIO-C® and increase in production of breast milk after delivery pursuant to article 13(5) of regulation (EC) no 1924/2006, 2010 EFSA J. 8 (9), 1774.

EU, 2015. Health and Food Safety, Directorate C, Public Health, Scientific Committee on Emerging and Newly Identified Health Risks, Additives Used in Tobacco Products, 15 July, 2015. http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_051.pdf.

FDA, 2015. FDA Warns Tobacco Manufacturers about 'natural' Labeling. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm459840.htm.

Flaig, T.W., Gustafson, D.L., Su, L.-J., Zirrolli, J.A., Crighton, F., Harrison, G.S., Pierson, A.S., Agarwal, R., Glodé, L.M., 2007. A phase I and pharmacokinetic study of silybin-phytosome in prostate cancer patients. Investig. New Drugs 25, 139–146.

Foulds, J., Ramström, L., Burke, M., Fagerström, K., 2003. Effect of smokeless tobacco (snus) on smoking and public health in Sweden. Tob. Control 12, 349–359.

Grasso, P., Mann, A.H., 1998. Smokeless tobacco and oral cancer, an assessment of evidence derived from laboratory animals. Food Chem. Toxicol. 36, 1015–1029.

Hahn, G., Lehmann, H.D., Kürten, M., Uebel, H., Vogel, G., 1968. On the pharmacology and toxicology of silymarin, an antihepatotoxic active principle from Silybum marianum (L.) gaertn. Arzneimittelforsch 18, 698–704.

Hecht, S.S., Rivenson, A., Braley, J., DiBello, J., Adamas, J.D., Hoffmann, D., 1986. Induction of oral cavity tumors in F344 rats by tobacco-specific nitrosamines and snuff. Cancer Res. 46, 4162–4166.

Hirsch, J.-M., Johansson, S.L., 1983. Effect of long-term application of snuff on the oral mucosa — an experimental study in the rat. J. Oral Pathol. 12, 187–198.

Hirsch, J.-M., Johansson, S.L., Thilander, H., Vahlne, A., 1984. Effect of long-term application of snuff and herpes simplex virus 1 on rat oral mucosa. Possible association with development of oral cancer. IARC Sci. Publ. 57, 829–836.

Idris, A.M., Nair, J., Oshima, H., Friesen, M., Brouet, I., Faustman, E.M., Bartsch, H., 1991. Unusually high levels of carcinogenic nitrosamines in Sudan snuff (toombak). Carcinogenesis 12, 1115–1118.

Idris, A.M., Prokopczyk, B., Hoffmann, D., 1994. Toombak, a major risk factor for cancer of the oral cavity in Sudan. Prev. Med. 23, 832–839.

Ito, N., Hirose, M., Takahashi, S., 1993. Cell proliferation and forestomach carcinogenesis. Environ. Health Perspect. 5 (101 Suppl. l), 107–110.

Jecker, P., Beuleke, A., Dressendörfer, I., Pabst, R., Westermann, J., 1997. Long-term oral application of 5-bromo-2-deoxyuridine does not reliably label proliferating immune cells in the LEW rat. J. Histochem Cytochem 45 (3), 393–401.

Kavitha, K., Thiyagarajan, P., Rathna Nandhini, J., Mishra, R., Nagini, S., 2013. Chemopreventive effects of diverse dietary phytochemicals against DMBA-induced hamster buccal pouch carcinogenesis via the induction of Nrf2-mediated cytoprotective antioxidant, detoxification, and DNA repair enzymes. Biochimie 95, 1629–1639.

Kee, N., Sivalingam, S., Boonstra, R., Wojtowicz, J.M., 2002. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J. Neurosci. Methods 115 (1), 97–105.

King, M.T., Wild, D., Gocke, E., Eckhardt, K., 1982. 5-Bromodeoxyuridine tablets with improved depot effect for analysis in vivo of sister-chromatid exchanges in bone-marrow and spermatogonial cells. Mutat. Res. 97, 117–129.

Larsson, A., Axéll, T., Andersson, G., 1991. Reversibility of snuff dippers' lesion in Swedish moist snuff users – a clinical and histologic follow-up study. J. Oral Pathol. Med. 20, 258–264.

Lee, P.N., 2011. Summary of the epidemiological evidence relating snus to health. Regul. Toxicol. Pharmacol. 59, 197–214.

Lewin, F., Norell, S.E., Johansson, H., Gustavsson, P., Wennerberg, J., Björklund, A., Rutqvist, L.E., 1998. Smoking tobacco, oral snuff, and alcohol in the etiology of squamous cell carcinoma of the head and neck. A population based case-referent study in Sweden. Cancer 82, 1367–1375.

Luo, J., Ye, W., Zendehdel, K., Adami, J., Adami, H.O., Boffetta, P., Nyrén, O., 2007. Oral use of Swedish moist snuff (snus) and risk for cancer of the mouth, lung, and pancreas in male construction workers, a retrospective cohort study. Lancet 369 (9578), 2015–2020.

Masui, T., Asamoto, M., Hirose, M., Fukushima, S., Ito, N., 1987. Regression of simple hyperplasia and papillomas and persistence of basal cell hyperplasia in the forestomach of F344 rats treated with butylated hydroxyanisole. Cancer Res. 47, 5171–5174.

Nilsson, R., 2011. The molecular basis for induction of human cancers by tobacco specific nitrosamines. Regul. Toxicol. Pharmacol. 60, 268–280.

Oliver, R.J., MacDonald, D.G., Felix, D.H., 2000. Aspects of cell proliferation in oral epithelial dysplastic lesions, 2000 Feb J. Oral Pathol. Med. 29 (2), 49–55.

Österdahl, B.-G., Jansson, C., Paccou, A., 2004. Decreased levels of tobacco-specific N-nitrosamines in moist snuff on the Swedish market. J. Agric. Food Chem. 52, 5085–5088.

Proctor, D.M., Gatto, N.M., Hong, S.J., Allamneni, K.P., 2007. Mode-of-action framework for evaluating the relevance of rodent forestomach tumors in cancer risk assessment. Toxicol. Sci. 98, 313–326.

Ramström, L., 2014. In: Low Mortality Attributable to Tobacco Among Men in Sweden Compared with Other European Countries, an Analysis of WHO Data. SRNT Meeting, Seattle, February 6, 2014. http,//nicotinepolicy.net/lars-ramstrom/929-mortality.

Roosaar, A., Johansson, A.L., Sandborgh-Englund, G., Nyrén, O., Axéll, T., 2006. A long-term follow-up study on the natural course of snus-induced lesions among Swedish snus users. Int. J. Cancer 119, 392–397.

Rutqvist, L.E., Curvall, M., Hassler, T., Ringberger, T., Wahlberg, I., 2011. Swedish snus and the GothiaTek standard. Harm Reduct. J. 8, 11. http://www.harmreductionjournal.com/content/8/1/11.

SCENIHR, 2008. Scientific Committee on Emerging and Newly Identified Health Risks, "Health Effects of Smokeless Tobacco Products". EU Health and Consumer Protection Directorate-General. http,//ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_013.pdf.

Schildt, E.-B., Eriksson, M., Hardell, L., Magnusson, A., 1998. Oral snuff, smoking habits, and alcohol consumption in relation to oral cancer in a Swedish case-control study. Int. J. Cancer 77, 341–346.

Schlöbe, D., Hölzle, D., Hatz, D., von Meyer, L., Tricker, A.R., Richter, E., 2008. 4-Hydroxy-1- (3-pyridyl)-1-butanone (HPB) releasing DNA adducts in lung, esophagus and cardia of sudden death victims. Toxicol 245, 154–161.

Stich, H.F., Tsang, S.S., 1989. Promoting activity of betel quid ingredients and their inhibition by retinol. Cancer Lett. 45, 71–77.

Stich, H.F., Stich, W., Rosin, M.P., Vallejera, M.O., 1984. Use of the micronucleus test to monitor the effect of vitamin A, beta-carotene and canthaxanthin on the buccal mucosa of betel nut/tobacco chewers. Int. J. Cancer 34, 745–750.

Stoner, G.D., Wang, L.S., Seguin, C., Rocha, C., Stoner, K., Chiu, S., Kinghorn, A.D., 2010. Multiple berry types prevent N-nitrosomethylbenzylamine-induced esophageal cancer in rats. Pharm. Res. 27, 1138–1145.

Summerlin, D.J., Dunipace, A., Potter, R., 1992. Histologic effects of smokeless tobacco and alcohol on the pouch mucosa and organs of the Syrian hamster. J. Oral Pathol. Med. 21, 105–108.

Surh, Y.J., 2003. Cancer chemoprevention with dietary phytochemicals. Nat. Rev. Cancer 3 (10), 768–780.

Swenberg, J.A., Lu, K., Moeller, B.C., Gao, L., Upton, P.B., Nakamura, J., Starr, T.B., 2011. Endogenous versus exogenous DNA adducts, their role in carcinogenesis, epidemiology, and risk assessment. Toxicol. Sci. 120 (Suppl. 1), S130–S145.

Tatematsu, M., Ogawa, K., Mutai, M., Aoki, T., Hoshiya, T., Ito, N., 1991. Rapid

regression of squamous cell hyperplasia and slow regression of basal cell hyperplasia in the forestomach of F344 rats treated with N-methyl-N′-nitro-N-nitrosoguanidine and/or butylated hydroxyanisole. Cancer Res. 51, 318–323.
Van Kesteren-van Leeuwen, A.C., Natarajan, A.T., 1980. Localisation of 7-12 dimethyl benz(a)anthrane induced chromosome breaks and sister chromatid exchanges in chromosome 1 and 2 of bone marrow cells of rat in vivo. Chromosoma 81, 373–381.
WHO, 2009. Report on the Scientific Basis of Tobacco Product Regulation, Third Report of a WHO Study Group. WHO Study Group on Tobacco Product Regulation. WHO technical report series. 955, Geneva.
WHO, 2012. World Health Organization. WHO Global Report, Mortality Attributable to Tobacco. Geneva. http,//who.int/tobacco/publications/surveillance/rep_mortality_attibutable/en/index.html.
Wilmer, J.L., Soares, E.R., 1980. Sister chromatid exchange in vivo in mice, I. The influence of increasing doses of bromodeoxyuridine. Environ. Mutagen 2, 35–42.
Zain, R.B., Ikeda, N., Gupta, P.C., Warnakulasuriya, S., van Wyk, C.W., Shrestha, P., Axéll, T., 1999. Oral mucosal lesions associated with betel quid, areca nut and tobacco chewing habits: consensus from a workshop held in Kuala Lumpur, Malaysia, november 25-27, 1996. J. Oral Pathol. Med. 28, 1–4.
Zi, X., Agarwal, R., 1999. Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: implications for prostate cancer intervention. Proc. Natl. Acad. Sci. U. S. A. 96 (13), 7490–7495.