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## FOREWORD

The pathogenesis and clinical course of mammary gland tumours belong to the most actual problems in the clinical and experimental oncology. Breast cancer exhausts patients both physically and emotionally. Recently (2009) 192,370 of new cases of invasive breast cancer were expected in USA, as well as an estimated 62,280 additional cases of in situ breast cancers. Approximately 40,170 women were expected to die in consequence of the disease (American Cancer Society). Only lung cancer accounts for more cancer deaths in women. Many of the known breast cancer risk factors, such as age, family history, early menarche, late menopause, age at first full-term pregnancy, and breast density, are endogenous risk factors and are not easily modifiable. However, other factors associated with increased breast cancer risk – postmenopausal obesity, use of combined oestrogen and progestin menopausal hormones, alcohol consumption, and physical inactivity are modifiable. A woman's best overall preventive health strategy is to reduce her known, above mentioned risk factors.

Chemoprevention is rapidly growing area of oncology. In the beginning of the 21st century a vision of chemoprevention as an effective tool to fight with breast cancer exists. Chemoprevention is the use of pharmacological agents that inhibit or reverse the process of carcinogenesis. Whilst chemotherapy is targeted at people with manifest disease, primary chemoprevention is directed at individuals who are healthy, although those with in high risk of the disease (e.g. family history, mutated genes carriers). As treatment (surgical, chemotherapy, radiotherapy) of breast cancer in some cases brings little or no success (depending on the stage of disease), it is necessary to be concentrated on the prevention not only from ethical but also economical reasons.

The aim of the chemoprevention trials is to find an efficient substance that can be administered for a long period with minimum adverse effects. The drugs with pleiotropic effects are very suitable for the chemoprevention. In the clinical oncology, some classes of new agents, namely selective oestrogen receptor modulators, non-steroidal anti-inflammatory drugs, and retinoids are discussed. Moreover, the drugs primary used in the treatment of any disease with anticarcinogenic side effect are also statins, antidiabetics or pineal hormone melatonin – they may be potentially profitable in the breast cancer chemoprevention. Our working group tested all above mentioned classes of drugs.

Chemopreventive animal studies play an important role in the evaluation of the effects of new drugs in oncochemoprevention projects. Rodent mammary models are widely used to study the aetiology, tumour biology, and treatment strategies of breast cancer. Mice and rats have for many decades provided unparalleled insights into cellular and molecular aspects of neoplastic transformation and tumorigenesis. Mammary carcinomas have been induced by both chemical xenobiotics and physical agents in rats and mice. In general, it is more efficient to induce these carcinomas in the rat. The most widely used chemical models to induce mammary carcinogenesis are directly acting alkylating agent N-methyl-N-nitrosourea (NMU), or the polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA). In our experiments, the chemoprevention initiated one week prior to carcinogen application and lasted remaining 15 – 25 weeks have provided results reproducible to human oncology.

In this supplement of Acta Medica Martiniana, the current clinical approaches in the breast cancer chemoprevention will be resumed. In regard to research of our group, we will summarize the chemopreventive efficacy of different classes of drugs in rat mammary carcinogenesis.

Assoc. Prof. Peter Kubatka, RNDr., PhD.  
Assoc. Prof. Martin Pěč, MD., PhD.  
Prof. Ivan Ahlers, MD., DSc.

## SELECTIVE OESTROGEN RECEPTOR MODULATORS AND RETINOIDS IN THE CHEMOPREVENTION OF BREAST CANCER

KUBATKA P.<sup>1</sup>, BOJKOVA B.<sup>2</sup>, ORENDAS P.<sup>2</sup>, KASSAYOVA M.<sup>2</sup>, PEC M.<sup>1</sup>, KAJO K.<sup>3</sup>, STOLLAROVA N.<sup>4</sup>, ZIHLAVNIKOVA K.<sup>1</sup>, AHLERS I.<sup>2</sup>, AHLERSOVA E.<sup>2</sup>

<sup>1</sup>Department of Medical Biology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic, <sup>2</sup>Department of Animal Physiology, Institute of Biological and Ecological Sciences, Faculty of Science, P.J.Safarik University, Kosice, Slovak Republic, <sup>3</sup>Department of Pathological Anatomy, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic and BB Biocyt, Diagnostic Centre, Ltd., Banska Bystrica, Slovak Republic, <sup>4</sup>Department of Biology and Ecology, Faculty of Education, Catholic University, Ruzomberok, Slovak Republic

### Abstract

Mammary gland cancer is a serious worldwide medical problem. Chemoprevention is rapidly growing area of oncology. The strategy of chemoprevention seems to be promising for reducing cancer incidence both in well-defined high-risk individuals and also in the general population. Based on mechanism of action, two classes of agents – selective oestrogen receptor modulators and retinoids – are effective in the suppression of breast cancer initiation and progression. Several clinical trials have shown that selective oestrogen receptor modulators significantly decrease breast cancer incidence in high risk women. In premenopausal women, fenretinide have shown a durable trend towards reducing second breast malignancies. Clinical data suggest that fenretinide could be useful for breast cancer chemoprevention in young women, perhaps in combination with tamoxifen. This review summarizes the results of preclinical and clinical breast cancer research with selective oestrogen receptor modulators and retinoids and discusses possible role of these drugs in the prevention and treatment of the disease.

**Key words:** chemoprevention, breast cancer, selective oestrogen receptor modulators, retinoids, rexinoids

### INTRODUCTION

In the beginning of 21st century pathogenesis and clinical course of mammary gland tumours belong to the most pressing problems in the clinical and experimental oncology. After colorectal cancer, breast cancer is the second most frequent cancer in Europe. There were an estimated 421,000 new cases (13.1% of all cancers) of breast cancer and 129,000 deaths (7.5%) from this malignity in 2008 (1). The chemoprevention of breast cancer was often discussed in last years and presents a serious and practical approach to the control of cancer disease. Chemoprevention has been defined as the use of nontoxic nutrients or pharmacologic agents which enhance physiologic mechanisms that protect organism against development and progression of mutant clones to malignant cancer (2). Various groups of subjects could attribute to the selection of the tumour chemoprevention. According to Decensi and Costa (3), there are: a) subjects with high genetic risk for some types of tumours. Mutated forms of BRCA 1 and BRCA 2 genes represent the genes of susceptibility for malignant breast cancer forms (in women as well as in men) and for ovarian carcinomas and they conform the inherited forms of breast cancer with a frequency about 5%; b) subjects with well-defined precancerous diseases (e.g. leukoplakias in oral cavity); c) subjects with cured form of the disease for the prevention of the disease occurrence on the other place (contralateral breast in breast cancer); d) subjects exposed to well-defined risk factors.

Address for correspondence:

Assoc. Prof. RNDr. Peter Kubatka, PhD., Department of Medical Biology, Jessenius Faculty of Medicine, Comenius University, Malá Hora 4, 037 54 Martin, Slovakia,  
Phone: 043/4131425, Fax: 043/413 63 32, e-mail: kubatka@jfmed.uniba.sk

Despite the fact that carcinogenesis is primary driven by mutations of some genes, there are also many epigenetic factors, particularly those relating to the action of autocrine, paracrine and endocrine regulatory molecules. These molecules can be important determinants during the latent period of cancer disease. Pharmacologic modulation of these regulatory cell pathways along with the use of drugs and micronutrients which could effectively block mutagenesis may offer the strategy for cancer prevention. Selective oestrogen receptor modulators and retinoids, which demonstrate several mechanisms of anticarcinogenic action in the cell, have been shown to be very effective agents for prevention of breast cancer in women at different degrees of risk. In this review, the current clinical approaches in the breast cancer chemoprevention using the above mentioned groups of drugs will be resumed. We will summarize results of our working group – the experiments of chemoprevention of rat mammary carcinogenesis using the oestrogen receptor modulators and retinoids.

### SELECTIVE OESTROGEN RECEPTOR MODULATORS

Oestrogens have an important role in the process of breast cancer initiation and progression. It is more than 100 years since Beatson (4) removed ovaries of a 33-year old patient with advanced breast cancer. This woman responded to the treatment, what led to the general acceptance of this form of therapy. All the effects of oestrogens are mediated through their binding to nuclear proteins called oestrogen receptors (ER) (5). Through this action oestrogens have an important role in the treatment of oestrogen-dependent breast tumours. Some natural compounds and synthetic drugs are also capable of binding to the ER. Some of these drugs mimic the effects of oestrogen, others have more antioestrogen properties. In the 1960s, the triphenylethylene tamoxifen was synthesized and demonstrated apparent antiproliferative effects in the breast. The anti-oestrogenic activity of tamoxifen in the breast has established it as the “gold standard” for the treatment of all stages of breast cancer. Later, it was discovered that tamoxifen have many oestrogenic qualities. Oestrogen-like effects were observed in the increase of bone density; decrease of serum cholesterol and of low-density lipoproteins and in the endometrium (6). These dual activities provide several advantages for women receiving tamoxifen. For instance, there is inhibition of proliferation in breast epithelium, but these women have benefit by both maintenance of bone density and reduction of cholesterol. Tamoxifen and other similar drugs with mixed oestrogenic/antioestrogenic properties, which are species-, tissue-, cell- and gene-dependent, are collectively called selective oestrogen receptor modulators (SERMs).

**Tamoxifen** given for different durations in an adjuvant setting has been associated with a reduction of the risk of both contralateral and metastatic cancer. Adjuvant studies of tamoxifen demonstrated a 30-50% decrease in the incidence of contralateral tumours in tamoxifen treated women (7). Tamoxifen was well tolerated with potential benefits on postmenopausal bone mineral density and lipid profile. These data supported the prospective evaluation of tamoxifen in the prevention of cancer in women at a high risk from the disease (Table 1).

In 1998, a randomized trial of National **Surgical Adjuvant Breast and Bowel Project P-1 (NSABP P-1)** assessed more than 13.000 healthy women at a risk for the development of invasive breast cancer demonstrated, than tamoxifen could reduce the risk of invasive and in situ ER-positive breast cancer in these women (8). Women were considered at high risk if they were 60 and more years old, were between the ages of 35-59 with a 5-year predicted risk for breast cancer, or had a history of LCIS. After a median follow-up of 54.6 months, tamoxifen decreased the overall risk of invasive breast cancer by 49% and non-invasive breast cancer by 50%. Tamoxifen decreased the risk of breast cancer incidence in women with LCIS by 56% and atypical ductal hyperplasia by 86%. The breast cancer risk reduction was limited to the reduction of ER-positive breast cancer.

Tamoxifen reduced the incidence of ER-positive breast cancer by 69%, but no difference was observed in the occurrence of ER-negative tumours. Moreover, a 19% decrease in bone fractures was observed in women treated with tamoxifen (8). Oestrogen-like effects were also observed in the decrease of serum cholesterol and of low-density lipoproteins. Based on results from NSABP P-1 trial, tamoxifen has Food and Drug Administration (FDA) approval for the prevention of breast cancer in high-risk women.

The interim analysis of the **Royal Marsden Hospital chemoprevention trial (RMH)** with administered tamoxifen (20 mg/day) to healthy women at increased risk of breast cancer (family history) was realized from 1986-1996 (9). With a median follow-up of 70 months, no difference in the breast cancer incidence was noted between compared groups. Thirty four of 1238 tamoxifen-treated women developed mammary gland cancer vs. 36 of 1233 patients with placebo. In 1992, Italian investigators (**Italian study**) initiated a trial of tamoxifen (20 mg/day for 5 years) vs. placebo in healthy 35-70 years old women with a hysterectomy (10). At a median of 46 months, no differences were found in the incidence of breast cancer in the placebo (22 cases) or the tamoxifen arm (19 cases). A statistically significant reduction of breast cancer in tamoxifen treated women who also used hormone replacement therapy during the trial was recorded. The RMH and Italian study did not show a statistically significant risk reduction effect of tamoxifen, but a trend toward lower risk after treatment was observed in both trials.

Initial results from the first **International Breast Cancer Intervention Study (IBIS-1)** found that tamoxifen (20 mg/day) reduced the risk of invasive ER-positive tumours by 31% in women at increased risk for breast cancer. After a median follow-up of 96 months after randomization, 142 breast cancers were diagnosed in the 3579 women in the tamoxifen group and 195 in the 3575 women in the placebo group. The prophylactic effect of tamoxifen was fairly constant for the entire follow-up period, and no decreased benefit was observed for up to 10 years after randomization (11).

Since results from the tamoxifen prevention trials suggest that tamoxifen may only reduce the risk of ER-positive disease. Differences among the study populations in the proportion of women at risk for developing ER-positive breast cancer could alter the observed tamoxifen risk reduction effects. All above mentioned breast cancer prevention trials differed in terms of sample size, eligibility regiments, breast cancer risk of participants, and concomitant use of hormone therapy. Any of these factors may explain why no overall risk reduction was observed in RMH and Italian trials. The study population of the RMH trial was chosen primarily on the basis of family history, therefore it might be assumed that a lower proportion of women were at risk for developing ER-positive breast cancer (9). The lack of overall risk reduction effects of tamoxifen in the RMH and Italian trials may also be due to smaller trial population.

Tamoxifen breast cancer prevention trials have provided relevant information regarding side effects. The oestrogen agonist effects of tamoxifen on the uterus and liver, which result in an increased incidence of uterine cancer and thromboembolic phenomena, keep it from being considered an ideal SERM. Incidence of thromboembolic disease and endometrial cancers increased approximately threefold when tamoxifen was used for 5 years. The most frequently reported side effects were also hot flushes in postmenopausal women. The gynaecologic side effects of tamoxifen are diverse and reflect the complexity of its mechanism of action, with agonistic and antagonistic effects on various tissues, depending on the ambient oestradiol concentration and hence menopausal status of the patient (12). Although reducing the risk of breast cancer may outweigh the toxicity of tamoxifen for women at high risk for breast cancer and a low risk for side effects, a compound with a superior benefit risk profile would be preferable for a broader spectrum of healthy postmenopausal women. Raloxifene may be such a compound.

**Table 1.** Characteristics and results of chemopreventive trials with tamoxifen.

\* (tamoxifen vs placebo), DCIS – ductal carcinoma in situ, # – incl. DCIS

| Trial                                                     | Population                              | Number of women | Breast cancers* |           |          | ER status* |           |
|-----------------------------------------------------------|-----------------------------------------|-----------------|-----------------|-----------|----------|------------|-----------|
|                                                           |                                         |                 | totally         | invasive  | DCIS     | +          | -         |
| <b>NSABP P-1</b> <sup>8</sup><br>(USA, Canada)            | Healthy women with high risk            | 13.388          | 124 vs 244      | 89 vs 175 | 35 vs 69 | 41 vs 130  | 38 vs 31  |
| <b>RMH</b> <sup>9</sup><br>(UK)                           | High risk, family history, women 30-70y | 2.494           | 62 vs 75        | 54 vs 64  | 7 vs 7   | 31 vs 44   | 17 vs 10  |
| <b>Italian study</b> <sup>10</sup>                        | Normal or low risk                      | 5.408           | 34 vs 45        | 28 vs 40  | 5 vs 4   | 19 vs 30#  | 14 vs 12# |
| <b>IBIS-1</b> <sup>11</sup><br>(UK, Australia, N. Zeland) | ≥2-fold risk women 35-70y               | 7.152           | 69 vs 101       | 64 vs 85  | 5 vs 16  | 44 vs 63   | 19 vs 19  |

**Raloxifene**, a derivative of benzothiophene, represents the second generation of SERMs. Originally it was developed for treatment and prevention of postmenopausal osteoporosis. However, its oestrogen-antagonistic properties in other tissues of the organism were also observed. Clinical studies have shown oestrogen-like effects in the bone density increase and total serum cholesterol and low density lipoproteins decrease in postmenopausal women (13).

**The Multiple Outcomes of Raloxifene Evaluation (MORE)**, the study initiated in 1994, aimed at osteoporosis prevention in postmenopausal women (n=7.705), investigate also the possibilities of raloxifene (60 or 120 mg/day) to decrease breast carcinoma incidence. Analysis of the MORE trial found that 4 years of raloxifene therapy decreased the incidence of all breast cancer by 62%, invasive breast cancer by 72% and invasive ER-positive breast cancer by 84% among postmenopausal women with osteoporosis compared with placebo (14). Investigators conducted the **Continuing Outcomes Relevant to Evista (CORE)** trial to examine the effect of 4 additional years of raloxifene (60 mg/day) therapy on the incidence of invasive breast cancer in women in MORE who agreed to continue in CORE. During the CORE trial, the 4-year incidence of total breast cancers were reduced by 50%, invasive breast cancer was reduced by 59%, and ER-positive invasive breast cancer was reduced by 66% in the group with raloxifene compared to placebo. Similar to MORE, there was no reduction in DCIS and ER-negative invasive breast cancer for women randomly assigned to raloxifene (15). Over the 8 years of both trials, the incidence of invasive breast cancer was reduced by 66% and ER-positive breast cancer by 76% among raloxifene treated women compared to placebo. In the raloxifene group, the increased relative risk of thromboembolism in CORE trial (2.17), also observed in MORE trial, persisted over the 8 years of both trials.

A study comparing the effectiveness of the 2 drugs – **The Study of Tamoxifen and Raloxifene (STAR)** found that raloxifene (60 mg/day) decreased the risk of invasive breast cancer to the same degree as tamoxifen (20 mg/day), although it did not have the same protective effects against in situ cancer (16). There were more cases of uterine cancer with tamoxifen than with raloxifene. Thromboembolic events occurred less often in the raloxifene group and there were fewer cataracts and cataract surgeries in the women taking raloxifene (17,18). The STAR trial has shown that raloxifene is as effective as tamoxifen in reducing the risk of invasive breast cancer and has a lower risk of adverse events but a non-statistically significant higher risk of non-invasive breast cancer. The risk of other cancers, fractures, ischemic heart disease and stroke was similar for both drugs.

The potential cardioprotective effects of raloxifene in 10,000 postmenopausal women are being examined in **the Raloxifene Use for the Heart (RUTH)** trial. The primary end point is the incidence of myocardial infarction, as secondary outcomes will be examined

the incidence of breast cancer and osteoporotic fractures. Preliminary results from this trial pointed to the fact that postmenopausal women treated with raloxifene had an increased risk of venous thromboembolic events compared with placebo. Concomitant use of aspirin or nonaspirin antiplatelet agents along with raloxifene did not change this risk (19).

Tamoxifen – the first U. S. FDA approved agent for reducing breast cancer risk, did not gain wide acceptance for prevention, largely because it increased incidence of endometrial cancer and thromboembolic events. The FDA approved the raloxifene for breast cancer risk reduction in high risk women based on its demonstrated effectiveness in preventing invasive breast cancer in the STAR trial. The results from all tamoxifen and raloxifene prevention trials have important public health implications and clarify that both raloxifene and tamoxifen are good preventive choices for postmenopausal women with higher risk for breast cancer (18).

### **TAMOXIFEN AND RALOXIFENE IN EXPERIMENTAL MAMMARY CARCINOGENESIS**

Experimental rat mammary carcinogenesis induced by chemocarcinogens is a widely used model for the study of oestrogen and antioestrogen role in the process of breast cancer initiation. N-methyl-N-nitrosourea (NMU) and 7,12-dimethylbenz(a)anthracene (DMBA) are the most frequently used chemical substances in mammary tumours induction. Our group (Ahlers et al.) evaluated chemopreventive effects of SERMs tamoxifen and raloxifene in NMU- and DMBA-induced mammary gland cancer in female Sprague-Dawley rats. Moreover the effects of chemopreventives on body and uterine weight, food and water intake were recorded. In our experiments, both agents – tamoxifen and raloxifene demonstrated high antineoplastic activity in carcinogen-induced mammary cancer in rats. Total mammary tumour growth suppression was registered in group with tamoxifen of both experiment with NMU and DMBA (20). In NMU-induced mammary carcinogenesis, tumour incidence was decreased by 67% ( $P<0.001$ ), tumour frequency per group was reduced by 90% ( $P<0.0002$ ) and latency period lengthened by 27 days in raloxifene group compared to control group (21). In DMBA-induced mammary carcinogenesis, raloxifene decreased tumour incidence by 80% ( $P<0.01$ ) and tumour frequency per group by 84% ( $P<0.01$ ) in comparison with the control group (22). The investigation of mammary tumours confirmed relative stable relation between adenocarcinomas and fibroadenomas (rate about 70:30 %). The decrease in body weight accompanied with apparent reduction of white adipose tissue suggested the oestrogen-like effects of tamoxifen and raloxifene in regulation of energy metabolism in rats. On the other hand, uterine atrophy suggested the oestrogen-antagonistic effects of tamoxifen and raloxifene on this organ in the rat. The important finding in our experiments is marked decrease in water intake in tamoxifen- and raloxifene- treated rats, which was not accompanied by changes in food intake. As there is an obvious connection in food and water intake typical for rodents, we can declare dissociation in food and water intake after tamoxifen and/or raloxifene treatment. High antineoplastic effects of tamoxifen and raloxifene in chemically-induced rat mammary carcinogenesis have been also confirmed by other investigators (23,24).

### **RETINOIDS**

Retinoids, a family of compounds structurally related to vitamin A or retinol and include the all-trans-, 9-cis- and 13-cis-retinoic acids (ATRA, 9-cis-RA, 13-cis-RA). These agents are presumed to exert their activity through binding of two distinct classes of retinoid receptors – retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (25), each of which comprise three isotypes designated  $\alpha$ ,  $\beta$ ,  $\gamma$  as well as several isoforms generated by alternative splicing or promoter use. ATRA selectively binds to RARs, but 9-cis-RA binds to both RARs and RXRs, albeit with differing affinities. 13-cis-RA gets

isomerised to ATRA, and thus, is functionally equivalent. RARs and RXRs are transcription factors that act predominantly as RAR-RXR heterodimers, positively or negatively modulating specific genetic programmes. Binding of retinoids to these receptors leads to regulation of several cellular processes. Retinoids have been shown to suppress neoplastic growth in a variety of experimental models (26). The positive effects of retinoids on chemically induced mammary carcinogenesis were reported by Moon et al. already in 1976 (27). Two experiments investigating the effects of retinyl acetate were performed at our laboratory. In both of them, female Sprague-Dawley rats bred in the same conditions were used, but in the first experiment mammary carcinogenesis was induced by DMBA (28) and in the second by NMU (29). In the first experiment retinyl acetate significantly reduced the incidence and frequency of tumours and lengthened the latency compared to control group. In the second experiment retinoid lengthened latency by 16 days; an additive effects of combined administration of retinyl acetate and melatonin in this experiment were observed in tumour incidence and latency period of tumours, this combination of drugs demonstrably inhibited tumour burden in rats.

Retinoids can act both as anti-initiators and anti-promoters; alter carcinogen metabolism, cell division and differentiation, as well as expression of oncogenes and biosynthesis of polyamines and prostaglandins (30). Retinoids can induce apoptosis, carcinogenesis could be influenced by their immunostimulative activities – they activate cell-mediated cytotoxicity and increase of natural killers (31). Several retinoids are able to inhibit the activator protein-1 (AP-1) transcription pathway, which is involved in breast cancer cell proliferation and transformation (32). Retinoids have been shown to inhibit the growth of ER-positive (33) and ER-negative breast cancer (34).

The application of retinoids to decrease cancer development has a long history. Early clinical trials evaluated ATRA, 9-cis-RA and 13-cis-RA in individuals at increased risk of cancer. These trials have established proof of principle for human cancer chemoprevention and their aim was to show the potential utility of retinoids in this regard. Based on these trials, widespread use of above mentioned retinoids for long-term administration in cancer chemoprevention is unsuitable because of apparent adverse effects such as headaches and mucocutaneous toxicity (30). It is hoped that increased understanding of retinoid receptor biology has facilitated the discovery of selective modulators of the RXRs and thus will open new opportunities for the development of novel retinoids with an enhanced therapeutic profile with decreased toxicity. The potential utility of selective RXR agonists may evolve from their ability to induce apoptosis (35) or possibly from regulation through heterodimer formation with RARs (36), peroxisome proliferator-activated receptors (PPARs) (37), thyroid hormone receptor (38) and vitamin D receptor (39). Specifically, ligands selective for the RXR have been developed, the so-called rexinoids, which seems to afford equivalent or greater protection against cancer with substantially diminished toxicity.

### **FENRETINIDE FOR BREAST CANCER CHEMOPREVENTION**

The retinoid most widely studied in chemoprevention clinical trials is the synthetic amide of retinoic acid N-(4-hydroxyphenyl)retinamide (4-HPR), or fenretinide. Sporn et al. (40) demonstrated preferential accumulation of this drug in the breast rather than in the liver. This agent has activity in both ER-positive and ER-negative breast cancer cells, although there tends to be a more effective in ER-positive cells (41). Fenretinide has been described to inhibit chemically induced mammary carcinogenesis in rats (42). Early clinical studies with this drug demonstrated its promising toxicity profile. The most remarkable adverse effect was an increase in nyctalopia, which was successfully managed with a 3-day drug holiday every 28 days (43).

The Istituto Nazionale dei Tumori in Milan started in 1987 a multicentre phase III randomized trial. Women (n=2972) ages 30 to 70 with stage I breast cancer were ran-

domized to group with fenretinide (200 mg daily for 25 days followed by a 3-day drug holiday) and control group without intervention (44). The trial primary end point was the occurrence of contralateral breast cancer as the first malignant event. The secondary end point was the incidence of ipsilateral breast cancer reappearance, defined as local occurrence in the same quadrant or occurrence of a second breast malignancy in a different quadrant from the primary tumour. After a median follow-up duration of 97 months, the results from this trial did not show a difference in breast cancer incidence between women with fenretinide vs. without intervention. Fenretinide showed no effect on contralateral breast cancer occurrence and a non-significant 17% reduction in ipsilateral breast tumour reappearance. However, a different trend was noted when the analysis was stratified by menopausal status; there was a beneficial trend in premenopausal women on both contralateral and ipsilateral breast cancer. No effect of fenretinide treatment was observed in distant metastases and death. The frequency of second primary tumours was comparable between the fenretinide and control group. Moreover, potential important trial outcome is the finding about significant lower incidence of ovarian cancer after 5-year fenretinide treatment compared to control group (6 vs. 0 cases). Further clinical trials are necessary to clarify this effect of the drug. Epidemiologic studies have observed an increase in serum concentrations of insulin-like growth factor 1 (IGF-1) in premenopausal and postmenopausal breast cancer women (45). It has been reported that IGF-1 is a key regulator of proliferation and apoptosis of both normal and malignant cells including mammary gland cancer cells (46). Torrisi et al., (47) have reported that fenretinide decreases serum concentrations of IGF-1 and its main binding protein IGFBP-3 in premenopausal women. Thus, modulation of IGF-1 could be a supposed biological mechanism, which may explain the menopausal interaction with fenretinide and contralateral breast cancer incidence in Milan study (44).

Detailed analysis of the trial reported some adverse effects in women. The most common were diminished dark adaptation (19%) and dermatological disorders (18.6%) – such as skin and mucosal dryness, pruritus and urticaria. Less common events were gastrointestinal symptoms and alterations of the ocular surface (48). The findings of the phase III trial support the role of fenretinide as a preventive agent in mammary gland carcinogenesis in women but indicate its lack of efficacy on the progression to a more malignant phenotype.

It is well known, that combination of agents with different mechanism of action may increase the efficacy and minimize adverse effects. In preclinical model, combined administration of fenretinide and tamoxifen has proven additive or synergistic effects (49,50). The effectiveness and the safety of the combination of fenretinide and tamoxifen has been evaluated in breast cancer clinical trials both in metastatic patients (51) and in the adjuvant setting (52), as well as in healthy women at increased risk (53).

In a pilot study involving 32 healthy women at high risk for developing breast cancer, fenretinide was administered for four cycles at a daily oral dose of 200 mg for 25 out of 28 days (53). Tamoxifen was started after the first month of fenretinide administration, at a daily dose of 20 mg, and continued for 24 months. The toxicity and pharmacodynamics of 4-HPR as single agent as well as after tamoxifen administration was evaluated. No interference of tamoxifen on retinoid plasma concentrations was observed. The incidence of fenretinide-induced nyctalopia was 6% only, and reversed completely after fenretinide discontinuation. Four subjects stopped treatment due to side effects, and hot flushes were reported in 84% of women after the start of tamoxifen. The combination tamoxifen plus fenretinide had acceptable tolerability for this high-risk cohort.

In the prevention trial of Decensi et al. (54), a total of 235 premenopausal women with pT1mic/pT1a breast cancer (n = 21), or intraepithelial neoplasia (n = 160), or 5-year Gail risk  $\geq 1.3\%$  (n = 54) were randomly allocated to tamoxifen 5 mg/day, fenretinide 200 mg/day, their combination, or placebo. The aims of the trial are: data for plasma insulin-like growth factor I (IGF-I), mammographic density, uterine effects, and breast

neoplastic events after 5.5 years of treatment. During the 2-year intervention, tamoxifen significantly lowered IGF-I and mammographic density by 12% and 20%, respectively, fenretinide by 4% and 10% (not significantly), their combination by 20% and 22%, with no evidence for a synergistic interaction. The combination of low-dose tamoxifen plus fenretinide did not reduce breast neoplastic events compared to placebo, whereas both single agents, particularly fenretinide, showed numerical reduction in annual odds of breast neoplasms. Further follow-up is indicated. In another study with 235 women, fenretinide positively balanced the metabolic profile in overweight premenopausal women. After 2 years of fenretinide treatment, 25% of women improving their insulin sensitivity, and this may favorably affect breast cancer risk in these women (55).

### NOVEL RETINOIDS

The activation of both RAR and RXR pathways may potentiate the effect of retinoids on cell growth and differentiation. **Alitretinoin** (9-cis-RA) binds to both receptor and therefore is a pan-agonist. Alitretinoin has been shown to suppress mammary carcinogenesis effectively in female rats with acceptable side effects (56). In this experiment, combination of alitretinoin and fenretinide has shown additive effects, suggesting that they may inhibit mammary cancers by different mechanisms. A phase I trial of alitretinoin (50 to 140 mg/m<sup>2</sup>/day) and tamoxifen (20 mg/day) was conducted in 12 patients with metastatic breast cancer (57). The combination of tamoxifen and alitretinoin was well tolerated and demonstrated antitumour activity in metastatic breast cancer.

The current focus is on the development and validation of RXR-selective ligands (rexinoids), with the aim of maximizing antineoplastic efficacy while minimizing adverse side effects. Apart from modulation of the cell growth and differentiation, rexinoids have been found to down-regulate COX-2 expression in normal mammary epithelial cells (58). Compounds of particular interest include LGD1069 (bexarotene, Targretin) and LG100268.

Rexinoid **bexarotene** was shown to suppress mammary tumorigenesis in transgenic mice and its preventive effect was separated from toxicity (59). It was raised from this experiment that cutaneous toxic effects of retinoids are mediated through activation of RAR receptor, whereas cancer preventive activities of these agents are caused by RXR receptor activation. Bexarotene also effectively suppressed chemically induced rat mammary carcinogenesis (60). The substance suppressed both ER-positive (60) and ER-negative tumours (61) and was well tolerated in animal models. The preliminary results of our group pointed out to apparent antitumour activity of orally administered bexarotene and additive effects of combination bexarotene plus pineal hormone melatonin in NMU-induced mammary carcinogenesis in rats (unpublished results). Results of Yen and Lamph (62) suggested a role for bexarotene in combination with chemotherapeutic agents (paclitaxel, doxorubicin, cisplatin) in prevention and overcoming acquired drug resistance in advanced breast carcinoma in vitro. The activity and safety of oral bexarotene (200 mg/m<sup>2</sup>/day) have been evaluated in 148 patients with metastatic breast cancer (63). Approximately 20% of the patients experienced a clinical benefit. There were no drug-related deaths, and only two subjects had serious adverse events. Based on these data, a chemoprevention trial testing the ability of oral bexarotene (once daily for 28 days) to modulate breast tissue biomarkers has recently been initiated in women at increased risk for breast cancer. In this biomarker study, known carriers of BRCA-1 or BRCA-2 mutations or women with a  $\geq 10\%$  risk for carrying such mutations, were randomized for oral bexarotene or placebo. The different biomarkers — including markers of proliferation, growth factors and COX-2 will be analysed (NCI web site).

Another RXR-selective retinoid, **LG100268** has shown promising antineoplastic activity in experimental models. The rexinoid LG 100268 was an effective chemopreventive agent in preventing the development of both malignant and premalignant mammary

lesions in MMTV-erbB2 mice. The cancer prevention effect was associated with reduced expression of Ki67 and cyclin D1 in mammary glands by >80% (64). In another experiment, LG100268 and arzoxifene have synergized to promote apoptosis in a rat model of ER-positive breast carcinoma and in ER-positive human breast cancer cells in culture (65). This combination induced apoptosis by up-regulation of TGF- $\beta$  and inhibition of the pro-survival nuclear factor  $\kappa$ B and PI3K signalling pathways. Authors have shown that it is not necessary to administer both drugs continuously during tumour progression to prevent cancer in the rat model because dosing of these drugs in combination for relatively short periods, each followed by drug-free rests, is highly effective. This new approach to chemoprevention uses high doses of drugs that are too toxic for long-term administration but, when given for short periods, the agents are nontoxic and still induce apoptosis in breast cancer cells. And finally, LG100268 and arzoxifene were tested in the prevention and treatment of mouse mammary tumour virus-neu model of ER-negative breast cancer (66). The both substances as individual drugs, delayed the development of ER-negative tumours. Moreover, the combination of the drugs was strikingly synergistic, as no tumours developed in any mouse fed by this combination. This combination inhibited proliferation and induced apoptosis in the tumours and also induced significant tumour regression when used therapeutically.

## CONCLUSIONS

Tamoxifen and raloxifene are both indicated for the prevention of breast cancer in postmenopausal women at high risk for development of the disease. Raloxifene is comparable to tamoxifen in its ability to reduce the risk of breast cancer in these women and has fewer side effects as shown in the STAR trial. Combination of fenretinide with other agents, such as SERMs, could manifest additive or synergistic effects. This combination with low-dose tamoxifen has been proved to be safe. Great interest has been recently focused on rexinoids, which appear to have a high antineoplastic potential and due to their selective binding to RXR have a limited toxicity. Rexinoids also exhibits oncostatic effects in ER-negative breast cancer experimental models, what have not been observed in the prevention trials with SERMs. The rexinoid bexarotene is currently tested in women at increased breast cancer risk.

The important questions relating to breast cancer prevention will be the scope of the ongoing trials and include: documenting the extent to which breast cancer prevention translates into improved survival, determining optimal duration of therapy, how age of therapy initiation may interact with optimal duration, identifying women who are likely to develop ER-positive breast tumours and developing therapies directed at decreasing risk of ER-negative breast cancers or therapies applicable to premenopausal women.

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## PROTECTIVE ROLE OF NON-STEROIDAL ANTIINFLAMMATORY DRUGS IN EXPERIMENTAL MAMMARY CARCINOGENESIS

ORENDAS P.<sup>1</sup>, AHLERS I.<sup>1</sup>, KUBATKA P.<sup>2</sup>, BOJKOVA B.<sup>1</sup>, KASSAYOVA M.<sup>1</sup>, AHLERSOVA E.<sup>1</sup>, KAJO K.<sup>3</sup>, PEC, M.<sup>2</sup>

<sup>1</sup>Department of Animal Physiology, Institute of Biology and Ecology, Faculty of Science, P.J.Safarik University, Kosice, Slovak Republic, <sup>2</sup>Department of Medical Biology, Jessenius Faculty of Medicine, Comenius University and <sup>3</sup>Department of Pathological Anatomy, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic and BB Biocyt, Diagnostic Centre, Ltd., Banska Bystrica, Slovak Republic

### Abstract

Prolonged administration of non-steroidal antiinflammatory drugs (NSAIDs) in clinical praxis decreased markedly the risk of various neoplasia incidence, especially that of colorectal and breast cancer. Using preventive-curative protocols with NMU- or DMBA- induced mammary carcinogenesis in female Sprague-Dawley rats we have analyzed possible protective effects of indomethacine, diclofenac, nimesulide, rofe-, etori- and celecoxib. Indeed, all used drugs influenced to various degree the parameters of mammary tumor formation. Respecting the clinical importance of COX-1 inhibitors (eg. ibuprofen and diclofenac) celecoxib as COX-2 inhibitor is supposed to be currently the best choice, for drugs combination too, in experimental and clinical oncology.

**Key words:** mammary carcinogenesis, female rats, non-steroidal antiphlogistics, chemoprevention

### INTRODUCTION

Non-steroidal antiinflammatory drugs (NSAIDs) have been used in pain and inflammation treatment for more than three thousand years. NSAIDs inhibit synthesis of prostaglandins (PGs) and leukotrienes while eicosanoid synthesis (including PGs, thromboxanes and prostacyclines) is mediated by cyclooxygenase activity. There are two cyclooxygenase forms: constitutive (COX-1) and inducible (COX-2) type, the latter is up-regulated in inflammation and carcinogenesis. Mammary neoplasia showed increased prostaglandin (PGE<sub>2</sub> in particular) synthesis and content (1). In tumor tissues the COX upregulation (COX-2 in particular) was proved, 80% of human precancerous lesions showed COX-2 upregulation (2; 3). COX-2 upregulation induced mammary tumorigenesis in transgenic mice (4). However, COX-1 role in tumorigenesis was confirmed by experimental oncology too; COX-1 knockout rodents were less sensitive to skin and intestinal carcinogenesis induction (5). COX activation stimulates carcinogenesis through increased proliferation, invasiveness, angiogenesis, and apoptosis inhibition and immune response modulation (2). COX-2 upregulation is associated with human epidermal growth factor 2 (HER2) overexpression and COX/PGs system stimulates estrogen aromatase (6). Considering target effectivity and specificity, there are NSAIDs with selective COX-1 activity (acetylsalicylic acid -ASA- administered in doses from 30-300 mg), non-selective COX inhibitors (ASA administered in doses from 500 mg, indomethacin, ibuprofen, diclofenac), preferential-selective COX-2 inhibitors (nimesulide, meloxicam, piroxicam), and selective COX-2 inhibitors (coxibs – celecoxib, etoricoxib, valdecoxib, parecoxib, lumiracoxib). Chronic ASA administration was prescribed as antiagregative compound in thromboembolism prevention, other NSAIDs (mainly ibuprofen and diclofenac, nimesulide, meloxicam, celecoxib) were administered for prolonged periods in osteoarthrotic and rheumathoid arthritic patients. While ASA is administered daily over

Address for correspondence:

RNDr. Peter Orendas, PhD. Department of Animal Physiology, Institute of Biology and Ecology, Faculty of Science, P.J.Safarik University, Moyzesova Str. 11, 040 01 Kosice, Slovak Republic  
e-mail: orendas@upjs.sk

several years, other NSAIDs are administered intermittently even in case of several-year administration. ASA dosage is region-dependent, in Europe single daily dose of 100mg prevails, in USA the single daily dose is higher, cca 300-400 mg; other NSAIDs doses are similar. These facts complicate statistical evaluation of NSAIDs chemoprevention effect.

Sporadic notes from clinical practice on NSAID effect in neoplasias, especially of colorectal origin (including familial adenomatous) were confirmed by papers documented the inhibiting effect of indomethacin in experimental DMBA-induced mammary carcinogenesis in female rats (7) or indomethacin effectiveness on spontaneous intestinal tumor occurrence in rats (8), or piroxicam effects in experimental colorectal carcinogenesis in rats (9).

Numerous reports on NSAIDs chemopreventive effects in various neoplasia have been published. NSAIDs inhibited mammary carcinogenesis (10), particularly the coxibs (11). Some results were surprising: Harris et al. (10) summarised results of 91 epidemiological trials. Long-term (over 5 years) NSAIDs (ASA and ibuprofen) administration decreased breast cancer incidence by 39%, colorectal cancer incidence by 73%, lung cancer incidence by 39, and prostatic cancer incidence by 39%; the effect of ASA was more prominent. NSAIDs decreased oesophageal cancer incidence by 39%, stomach cancer incidence by 62%, and ovarian cancer by 47% too. On the other hand, Zhao et al. (12) excluded 21 reports out of 47 for inconsistency after detailed statistical analysis; in remaining 26 reports even the long-term NSAIDs administration decreased the breast cancer incidence but slightly (with marginal statistical significance), the average relative risk (RR) was 0.91 (ASA) and 0.81 (ibuprofen), respectively. Most clinical trials, however, reported significant breast cancer incidence decrease in long-term NSAIDs administration, approximately 20% (without difference among individual substances, 13, 14, 15).

The aim of our work was to analyze the effect of selected NSAIDs in mammary carcinogenesis in young (virgin) female Sprague-Dawley rats. We have chosen indomethacin and diclofenac as preferential COX-1 inhibitors; nimesulide as non-selective COX-2 inhibitor; and rofecoxib, etoricoxib, and celecoxib as selective COX-2 inhibitors. In some experiments the combination of NSAIDs and melatonin was used.

## MATERIAL AND METHODS

Female rats of Sprague-Dawley strain obtained from Charles River Laboratories (Sulzfeld, Germany; Budapest, Hungary), An-Lab (Prague, Czech Republic) aged 33-37 days were adapted to standard vivarium conditions with the temperature  $23\pm 2^{\circ}\text{C}$ , relative humidity 60-70%, artificial regimen light:dark = 12:12 h, with lights on from 7.00 h (light intensity 150 lux per cage). The animals were fed the MP diet (Top Dovo, Dobrá Voda, Slovakia) resp. Purina Mills Test Diet (Richmond, USA), ST-1 Bergman (Jesenický u Prahy, Czech Republic), Ssniff (Soest, Germany) and drank tap water *ad libitum*. Each group involved about 20 animals; exact numbers see Tab.1. Mammary carcinogenesis was induced by N-methyl-N-nitrosourea (NMU) and dimethylbenz/a/anthracene (DMBA). NMU (Sigma, Deisenhofen, Germany) was administered intraperitoneally in two doses, each per 50mg/kg b.w., in a six-day interval between 41<sup>st</sup>-58<sup>th</sup> postnatal days to all animals. NMU was freshly prepared by dissolving in isotonic saline solution (0.5 ml per animal). DMBA (Sigma, Deisenhofen, Germany) was dissolved in corn oil and applied intragastrically (1 ml per animal) in three doses of 10 mg per animal between postnatal days 45-59.

### **Chemopreventive effect of some NSAIDs compounds was analyzed:**

**Indomethacin** (Léčiva, Prague, Czech Republic) was administered in concentration of 20 µg/ml of tap water 3 days a week daily (Tuesday, Thursday, Saturday); for preparation of 1000 ml of solution 20 mg of indomethacin (INDO) were dissolved in 3.1 ml of 60% ethanol and mixed up with tap water to desired volume. **Diclofenac** (Diclofenac sodium, produced by: Ningbo Smart Chemical & Pharmaceutical Co., LTD, Ningbo, Zhejiang, China, provided by: Zentiva a.s., Hlohovec, Slovakia). Diclofenac (DICLO) was administered in tap water in the concentration of 0.01 mg/ml (7 days before and 22

weeks after the first NMU dose). **Nimesulide** (Helsinn Healthcare SA, Lugano, Switzerland) in a dose of 5 mg/kg b.w. was administered subcutaneously twice a week in the afternoon. PEG 400-5 w/w, ethanol 96% – 15 w/w, 1N NaOH – 3.76 w/w and distilled water H<sub>2</sub>O 76.24 w/w were used to prepare nimesulide (NIM) solution. The solution was applied in a volume of 0.5 ml per animal (either 5 days before and 27 weeks after the first NMU dose or 3 days before and 13 weeks after the first DMBA dose, respectively).

**Rofecoxib** (Vioxx, Merck Research Laboratories, Merck Frosst, Montreal, Canada) was finally prepared with Purina Mills Test Diet (Richmond, USA). Rofecoxib (ROFE) was administered in the chow in two concentrations – 0.01 mg/1 g (ROFE 0.001%), or 0.05 mg/1 g (ROFE 0.005%) 7 days before and 17 weeks after the first NMU dose. **Etoricoxib** (Merck Research Laboratories, Merck Frosst, Montreal, Canada) was applied in a chow ST-1 Bergman (Jesenický u Prahy, Czech Republic). Etoricoxib (ETO) was administered in the chow in two concentrations: 0.01 mg/1 g (ETO 0.001% or ETO10) and 0.025 mg/1 g (ETO 0.0025% or ETO25) 7 days before and 22 weeks after the first NMU dose.

**Celecoxib** (Celebrex, Pfizer Pharmaceuticals Group, New York, USA) was applied in the Ssniff diet (Soest, Germany). Celecoxib (CELE) was administered in a concentration of 1.666 g/kg food (CELE 0.1666%) 7 days before and 20 weeks after the first NMU dose.

The animals were divided into several groups (20 animals per group as minimum): control group without chemoprevention but with carcinogen application, chemoprevention with NSAIDs – INDO, DICLO, NIME, ROFE (ROFE 0.001% a ROFE 0.005%), ETO (ETO 0.001% a ETO 0.0025%), CELE. Chemoprevention with chemopreventive substance began 3-12 days before carcinogen administration and lasted until the end of the experiment (13-27 weeks after the first NMU administration). The animals were weekly weighed and palpated in order to record the incidence, number, location, and size of tumors. The effect of chemopreventives on body mass gain (evaluated from the initiation until the end of the experiment), food and water intake was observed. In the last week of each experiment all animals were sacrificed by quick decapitation and final investigation was performed: inspection of body organs, mammary tumor excision and size measurement, tumor samples were preserved in 10% formalin solution for further investigation. Statistical evaluation of selected parameters of mammary carcinogenesis (incidence, tumor frequency per group and animal, tumor volume and latency), body mass, food and water intake were evaluated by a combination of non-parametric and parametric tests. Tumor incidence was evaluated by Mann-Whitney U-test, other parameters by one-way analysis of variance or Kruskal-Wallis test, respectively. Tumor volume was calculated according to formula:  $V \text{ (mm}^3\text{)} = \pi \times S_1^2 \times S_2 / 12$ ; where ( $S_1 < S_2$ ),  $S_1$  is tumor width and  $S_2$  tumor length in mm,  $S_1$  and  $S_2$  were measured perpendicularly.

The experiments were conducted according to the principles provided in the Law No. 115/1995, 289/2003, 489/2003 and 23/2009 of Slovak Republic for the Care and Use of Laboratory Animals.

## RESULTS

In NMU experiment the indomethacin chemopreventive effects were recorded only as decreased tumor frequency per animal (by 17.7%). In DMBA experiment indomethacin improved all tumor parameters, the incidence decreased by -63%, tumor frequency per animal by 66.5%, tumor frequency per group by 88%, and latency period was lengthened by 63%. Moderate diclofenac effect in NMU experiment was recorded in all tumor growth parameters (except of latency period), mainly in incidence (-34.5%) and tumor frequency per group (-40%). Nimesulide in NMU experiment decreased especially tumor incidence and frequency per group. Rofecoxib showed better efficacy in almost all parameters, mainly in tumor incidence at both concentrations (-40% and -42.5%, respectively), in tumor frequency per group at higher concentration (-40%) and in tumor volume at both concentrations (-35% and -43%, respectively). Etoricoxib was partially effective at higher

concentration – in all parameters except of tumor volume; at lower concentration only latency was lengthened (+7.96%). Celecoxib efficacy in mammary carcinogenesis inhibition was confirmed (except of tumor volume) in all parameters, particularly in incidence (-24%), tumor frequency per group (-54.4%) and animal (-40%).

None gross macroscopic changes were seen in sectioned animals; general mortality was below 10% per group.

Histological analysis: In experimental mammary tumorigenesis ductal adenocarcinomas prevailed. Malignant/benign lesion ratio is approximately 90/10 and is not altered by successful NSAIDs treatment. As for adenocarcinomas, invasive (mainly cribriform) forms prevail, the rest are carcinomas in situ (16).

**Table 1:** Effects of NSAIDs in prevention of mammary carcinogenesis in the experiments of physiological group of the Dept. Animal Physiology, Faculty of Science, P. J. Safarik University Košice

| Experiment                                                           | Group                 | Tumor incidence (%) | Tumor frequency per group | Tumor frequency per animal | Tumor volume (cm <sup>3</sup> ) | Latency (days)           |
|----------------------------------------------------------------------|-----------------------|---------------------|---------------------------|----------------------------|---------------------------------|--------------------------|
| <b>INDO-NMU</b>                                                      | CONT<br>n=20          | 70.00               | 2.10±0.58                 | 3.00±0.70                  | 1.28±0.43                       | 116.79±9.07              |
| (17)<br>(20 ug/ml tap water)<br>12 d + 25 w<br>(September to March)  | INDO<br>n=20          | 85.00<br>(+21.5%)   | 2.10±0.32<br>(0%)         | 2.47±0.29<br>(-17.67%)     | 2.89±0.62<br>(+126%)            | 99.88±6.10<br>(-14.5%)   |
| <b>INDO-DMBA</b>                                                     | CONT<br>n=15          | 100.00              | 3.40±0.63                 | 3.40±0.63                  | 2.69±0.66                       | 93.40±6.37               |
| (17)<br>(20 ug/ml tap water)<br>9 d + 26 w<br>(November to May)      | INDO<br>n=19          | 36.84<br>(-63%)     | 0.40±0.13<br>(-88%)       | 1.14±0.14<br>(-66.47%)     | 2.24±1.45<br>(-16.7%)           | 152.29±10.42<br>(+63%)   |
| <b>DICLO-NMU</b>                                                     | CONT<br>n=20          | 80.00               | 3.65±0.67                 | 4.56±0.66                  | 1.65±0.22                       | 90.60±4.80               |
| (16)<br>(0.01 mg/ml tap water)<br>7 d + 22 w<br>(August to January)  | DICLO<br>n=24         | 70.83<br>(-11.46%)  | 2.21±0.41<br>(-39.45%)    | 3.12±0.40<br>(-31.58%)     | 2.61±0.99<br>(+58.18%)          | 105.06±7.37<br>(+15.96%) |
| <b>NIM-NMU</b>                                                       | CONT<br>n=18          | 85.00               | 1.30±0.19                 | 1.53±0.17                  | 1.39±0.31                       | 116.79±9.07              |
| (18)<br>5 mg/kg b.w.<br>5 d +27 w<br>(October to May)                | NIM<br>n=18           | 55.56<br>(-34.5%)   | 0.78±0.21<br>(-40%)       | 1.40±0.22<br>(-8.50%)      | 1.02±0.34<br>(-26.62%)          | 99.88±6.10<br>(-14.5%)   |
| <b>NIM-DMBA</b>                                                      | CONT<br>n=24          | 95.83               | 4.33±0.47                 | 4.52±0.67                  | 1.38±0.47                       | 64.00±3.03               |
| (18)<br>5 mg/kg b.w.<br>3 d+13 w<br>(April to July)                  | NIM<br>n=25           | 96.00<br>(+0.2%)    | 4.60±0.67<br>(+6%)        | 4.79±0.64<br>(+5.97%)      | 2.28±0.44<br>(+65.22%)          | 64.90±2.82<br>(+1.40%)   |
| <b>ROFE-NMU</b>                                                      | CONT<br>n=23          | 69.60               | 1.74±0.32                 | 2.60±0.32                  | 2.85±1.23                       | 80.06±6.03               |
| (19)<br>(0.001 a 0.005 mg/g chow)<br>7 d + 17 w<br>(June to October) | ROFE<br>0.001<br>n=24 | 41.70<br>(-40%)     | 1.42±0.43<br>(-18.4%)     | 3.40±0.64<br>(+30.8%)      | 1.85± 0.72<br>(-35%)            | 80.50±6.37<br>(+0.5%)    |
|                                                                      | ROFE<br>0.005<br>n=25 | 40.00<br>(-42.5%)   | 1.04±0.32<br>(-40%)       | 2.60±0.50<br>(0.0%)        | 1.63± 0.42<br>(-43%)            | 88.20±6.70<br>(+10%)     |

| Experiment                                                          | Group          | Tumor incidence (%)     | Tumor frequency per group | Tumor frequency per animal | Tumor volume (cm <sup>3</sup> ) | Latency (days)          |
|---------------------------------------------------------------------|----------------|-------------------------|---------------------------|----------------------------|---------------------------------|-------------------------|
| <b>ETO-NMU</b>                                                      | CONT<br>n=20   | 55.00±11.41             | 0.90±0.28                 | 1.64±0.39                  | 0.91±0.30                       | 103.09±7.32             |
| (20)<br>(0.01a 0.025 mg/g chow)<br>7 d + 22 w<br>(October to March) | ETO 10<br>n=18 | 55.56±12.05<br>(+1.02%) | 1.39±0.41<br>(+54.44%)    | 2.50±0.50<br>(+52.44%)     | 1.63±0.80<br>(+79.12%)          | 111.30±7.70<br>(+7.96%) |
|                                                                     | ETO 25<br>n=19 | 52.63±11.77<br>(-4.31%) | 0.84±0.26<br>(-6.67%)     | 1.60±0.34<br>(-2.44%)      | 1.69±1.03<br>(+85.71%)          | 110.60±9.78<br>(+7.28%) |
| <b>CELE-NMU</b>                                                     | CONT<br>n=17   | 94.12                   | 6.12 ±1.01                | 6.50 ±1.00                 | 1.48 ±0.26                      | 77.63 ±3.36             |
| (21)<br>(1666 mg/g chow)<br>7 d + 20 w<br>(December to May)         | CELE<br>n=14   | 71.43<br>(-24%)         | 2.79 ±0.64<br>(-54.4%)    | 3.90 ±0.59<br>(-40%)       | 2.26 ±0.61<br>(+53%)            | 79.90 ±5.57<br>(+3%)    |

In the column "Experiment" the abbreviations of chemopreventive agent and of carcinogen used in presented experiment, are shown. A line below denominates the authors and year of article's release, followed by the chemopreventive agent dose and duration of experiment, where "d" means the start - days before the first carcinogen dose and "w" means duration of agent administration after the first dose of carcinogen. Finally, the season of the experiment realization is shown.

**Abbreviations for chemopreventives:** **INDO**-indomethacin, **DICLO**-diclofenac, **NIM**-nimesulide, **ROFE**-rofecoxib, **ETO**-etoricoxib, **CELE**-celecoxib

**Abbreviations for carcinogens:** NMU - N-methyl-N-nitrosourea, DMBA -7,12- dimethylbenz/a/anthracene, CONT- control group with only carcinogen administered (NMU or DMBA)

## DISCUSSION

Beneficial NSAIDs effects in familial adenomatous polyposis, colorectal cancer, and other neoplasia revealed their protective (as well as therapeutic) effects in carcinogenesis; this became clear especially after chronic NSAIDs administration in arthrotic and arthritic patients. The chemopreventive effect, however, had to be verified in animal models using only a single substance (as arthritic patients were being treated with several NSAIDs simultaneously) and mechanism of protective/therapeutic efficacy had to be clarified. As for genetic background, neoplasia induction and tumor properties had to be as close to those in humans as possible, the animal model choice was crucial. Laboratory rodents, rats in particular, represent the most suitable model (22). Apart from genome similarity, the animal species/strain sensitivity to carcinogenesis induction is critical too; as Wistar-Han strain is insensitive to common carcinogens we had chosen susceptible Sprague-Dawley strain as experimental mammary carcinogenesis model (23). Young virgin (sensu strictiori premenopausal) female rats were used, carcinogenesis induction was performed in the most sensitive period, during mammary gland maturation. The experiments were of preventive/curative type, chemoprevention lasted approximately for 7 days prior to and 18 weeks after the first carcinogen dose. Short-term chemopreventive administration (only 2 weeks after the second carcinogen dose) in the period of presumptive tumor promotion was not effective (diclofenac experiment, not shown). We had not considered strictly preventive scheme (when chemopreventives are administered only before carcinogen application), however, this strategy brought intriguing results in Lenoir et al. (24) report using melatonin in experimental mammary carcinogenesis. Our group administered NSAIDs up to 25 weeks after the first NMU dose, longer administration may be useful in approaching the clinical situation e.g. NSAIDs administration in postmenopausal women.

The tumor growth parameters improvement by indomethacin in our DMBA experiments is of particular interest. Similarly Carter et al. (7) and McCormick et al. (25) reported the same effect, especially when using high-fat diet. However, indomethacin is not used in

current medical practice. ASA in low doses regarded as the model of “selective” COX-1 inhibitor has no chemopreventive properties. Suzui et al. (26) used aspirin (400 ppm in the diet) as second chemopreventive agent in female rats, mammary carcinogenesis was induced by 2/Amino-1-methyl-6-phenylimidazol /4, 5-b/ pyridine. Aspirin diminished mammary tumor frequency (-44%). In NMU experiments diclofenac slightly decreased incidence and tumor frequency, and we found no other relevant reports as well. The preventive effect of nimesulide was surprisingly confirmed only in NMU experiment; the same effect (but using high-fat diet) was reported by Nakatsugi et al. (27). Considerable effort had been made to coxib efficacy evaluation, results on rofecoxib and celecoxib are prominent. We believe the lack of ETO efficacy is due to low daily dose, rofecoxib (especially in higher dose) showed better chemopreventive effect. After publication of these results, serious side effects of some coxibs were revealed: long-term treatment increased fatal thromboembolism rate in patients (28), therefore we turned down valdecoxib analysis. As meta-analysis in patients treated with chronic low CELE doses showed less cardiovascular complications (29) and this coxib was re-introduced to clinical practice, we launched celecoxib efficacy evaluation in NMU-induced mammary carcinogenesis using standard protocol instead. We found the CELE effect in experimental mammary carcinogenesis is consistent and potentiated in combination with other substance (melatonin in our case). Similar mammary carcinogenesis suppression was reported by Harris et al. (30) using several CELE doses in DMBA protocol in female Sprague-Dawley rats. Nowadays, CELE in combination with other compounds is breaking through to cancer treatment, especially in colorectal (CRC) and breast cancer (BC) treatment (31). CELE combined with capecitabine was effective in metastatic BC pretreatment, and CELE not only improved capecitabine effectiveness but reduced its toxicity too (32). After rofecoxib and valdecoxib withdrawal from the market, it is necessary to reevaluate early results of Harris group (33) reporting 71% BC incidence reduction in patients with long-term coxib treatment gained during 2003-2004. We have cited above cautious evaluation of Zhao et al (12) on protective NSAIDs effect in BC.

## CONCLUSIONS

Elementary knowledge on COX role in carcinogenesis should favor COX-2 inhibitors role in carcinogenesis prevention. However, we should acknowledge the COX-1 superiority in long-term treatment (ASA, ibuprofen, diclofenac). Apart from coxibs, non-specific COX-2 inhibitors nimesulide and meloxicam (neglected in experimental oncology) are often used in clinical practice. Reported results (33) favour celecoxib for combination therapy. We find NSAIDs with NO-releasing activity (which should improve efficacy and reduce toxicity) to be promising. Apart from NSAIDs with NO-releasing activity CELE analogues with tetrahydropyridin dependent nitric oxide donor moiety seem to be promising too (34): we anticipate their antiinflammatory/antineoplastic effect.

The only way how to reduce toxicity and side gastrointestinal and cardiovascular effects is to use the lowest possible dose in shorter intervals. The epidemiological trials favoring continual prolonged treatment exclude other NSAIDs but ASA, all other NSAIDs treatment is under clinical practice restrictions. Combination of selected NSAIDs (e.g. celecoxib) with approved chemotherapeutics or radiotherapy, respectively, in chosen neoplasia treatment is a promising way.

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## STATINS: THEIR POTENTIAL USE FOR CANCER PREVENTION AND TREATMENT

ZIHLAVNIKOVA K.<sup>1</sup>, KUBATKA P.<sup>1</sup>, PEC M.<sup>1</sup>, KASSAYOVA M.<sup>2</sup>, BOJKOVA B.<sup>2</sup>, ORENDAS P.<sup>2</sup>,  
AHLERS I.<sup>2</sup>, KAJO K.<sup>3</sup>, STOLLAROVA N.<sup>4</sup>

<sup>1</sup>Department of Medical Biology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic.

<sup>2</sup>Institute of Biological and Ecological Sciences, Science Faculty, P. J. Safarik University, Kosice, Slovak

Republic, <sup>3</sup>Department of Pathological Anatomy, Jessenius Faculty of Medicine, Comenius University, Martin,

and BB Biocyt, Diagnostic Centre, Ltd., Banska Bystrica, Slovak Republic, <sup>4</sup>Department of Biology and

Ecology, Faculty of Education, Catholic University, Ružomberok, Slovak Republic

### Abstract

Statins, 3- hydroxy- 3 methylglutaryl coenzyme A reductase inhibitors are commonly used drugs in the treatment of lipid disorders. Recently, many epidemiological and preclinical studies pointed to antineoplastic properties of statins and thus exhort to their use in cancer therapy and prevention. Probable mechanisms by which statins influence the carcinogenesis are induction of apoptosis, the inhibition of proliferation and angiogenesis, consequently metastasis growth. Results of our group suggested chemopreventive potential of some statins in the chemoprevention of mammary carcinogenesis in female rats. Mechanism of action, preclinical studies and clinical efficacy of statins are reviewed in this paper.

**Key words:** statins, antineoplastic properties, carcinogenesis, chemoprevention, cancer therapy

### INTRODUCTION

Statins are most-widely used drugs for the treatment of hypercholesterolemia. They had become a first choice in current prescribing practice and are pivotal in the primary and secondary prevention of cardiovascular diseases [1, 2]. The discovery of statins significantly changed the approach in dislipidemic therapy and tremendously decreased morbidity and mortality from cardiovascular events by 50% [3]. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase. This enzyme is a rate limiting step in cholesterol biosynthesis in the liver. Current preclinical studies have proved pleiotropic properties of statins that can be useful for cancer therapy and prevention.

First member of statin group drugs – mevastatin (compactin) was derived from *Penicillium citrinum* in 1976 [4]. Similarly as mevastatin, next statin – lovastatin is natural, simvastatin and pravastatin were created as analogs of the first two drugs. All other statins are synthetic. Two statins (pravastatin, rosuvastatin) are hydrophilic, others are lipophilic (mevastatin, lovastatin, simvastatin, atorvastatin, fluvastatin, cerivastatin, cirlevastatin, pitavastatin) [5].

Statins are generally well tolerated drugs and this is one of the reasons why they replaced previous drugs (nicotinic acid, cholesteramine, estrogens, dextrotyroxene) used to reduce cardiovascular events. However, there are few side effects of statins that are monitored and reported. Most statins are metabolized in part by one or more hepatic cytochrome P450 (CYP 450) enzymes, leading to an increased potential for drug interactions and problems with certain foods (such as grapefruit juice) [6]. Statins can increase activity in hepatic transaminases up to 5% of patients. This effect is dose-related. Myotoxicity, including myopathy and rhabdomyolysis led to acute renal insufficiency are rare (0.1 %) and associated with treatment of statins with various drugs [6, 7] such as bile acid sequestrants, fibrates, niacin, gemfibrozil, and ezetimibe [8]. Statin's impact on the nerv-

Address for correspondence:

Mgr. Katarína Žihlavníková Department of Medical Biology Jessenius Faculty of Medicine  
Comenius University Malá Hora 4 036 01 Martin Slovak Republic

Tel.: +4210434131425, Fax: +4210434134807, e-mail: zihlavnikova@jfm.uniba.sk

ous system is debatable. Several studies had dealt with memory, dementia or Alzheimer disease and the results are contradictory [9]. Few cases reported link of pravastatin and simvastatin to lichenoid drug eruptions [10, 11], lovastatin and simvastatin have been reported to cause lupus-like syndromes [12, 13]. Generally, incidence of adverse effects caused by statin treatment is very low and benefits definitely outbalance the risks [14].

As an essential step in biosynthesis of mevalonate pathway, statins affect levels of cholesterol and others downstream products (isoprenoids) important in many physiologic processes such as cell signaling, apoptosis, protein synthesis, cell growth, posttranslational modifications and differentiation. Changes of these processes in neoplastic cells may therefore result in control of tumor initiation, growth, and metastasis [7]. The anti-neoplastic properties and potential tumor-suppressive effect of statins, and their use in cancer therapy or prevention is the main subject of this review.

## ANTINEOPLASTIC PROPERTIES OF STATINS

### Induction of apoptosis

Several experimental studies of carcinogenesis have shown pro-apoptotic effect of statins. Mechanism of impact is mostly indirect. Direct impacts of statins to apoptosis are not examined as well as subsidiary. Some published studies are describing interactions between molecules of statins and proteasome. A small amount of unconverted lovastatin remains in the body and stops the activation of the proteasome due to inhibition of p21 and p27 degradation in the cell cycle. These cyclin-dependent kinase inhibitors stay in the cell and stop the unregulated cell growth that characterizes cancer cells. Therefore are known to inhibit cancer cell growth [15]. Higher pro-apoptotic activity showing lipophilic statins compare to hydrophilic [16].

Statins inhibit processes of geranylgeranylation and farnesylation (posttranslational modifications), which are essential for the activity of many proteins (e.g. small GTP-binding proteins Ras and Rho) responsible for activation and function cell signaling, important for cell growth of normal and transformed cells [17]. Apoptosis induced by statins is caused by regulation changes of pro-apoptotic and anti-apoptotic proteins expressions. For example, lovastatin (5  $\mu\text{mol/l}$ ) increased the regulation of Bim protein and induced apoptosis in human glioblastoma cells [18]. Pretreatment with lovastatin (10-30  $\mu\text{mol/l}$ ) significantly increased apoptosis in four tested colon cancer cell lines with different sensitivity. In this case, decreased expression of the antiapoptotic protein Bcl-2 and increased the expression of the proapoptotic protein Bax was observed [19]. Treatment with as little as 1  $\mu\text{mol/l}$  lovastatin over a period of 72 hours led to DNA degradation into nucleosome-sized fragments characteristic of apoptosis [20]. Dimitroulakos et al. [21] have manifested lovastatin-induced apoptosis of the acute myeloid leukemic cell lines and the neuroblastoma cell.

Actual results of our group demonstrated an apparent antineoplastic effect of dietary administered atorvastatin in the chemoprevention of rat mammary carcinogenesis in the specimens of mammary tumors, atorvastatin at a concentration of 100 mg/kg, significantly decreased mRNA expression of *Bcl-2* gene but did not influence *Bax* mRNA expression compared to control group [unpublished results]. In this regard, pro-apoptotic shift of ratio in *Bax/Bcl-2* mRNA expression caused by atorvastatin in our experiment was confirmed.

Statins seem to be suitable for activation of caspases, which are substantial part process of apoptosis. Caffario et al. [22] found out, that cerivastatin caused death of human myeloma cells by activating caspase-3, caspase-8, and caspase-9. Lovastatin induced apoptosis in prostate cancer cell line through activation of caspase-7 [23] and in leukemic cells by activation of caspase-3 [24]. Similarly, Shellman et al. [25] have pointed to fact that lovastatin 50-times enhances activity of caspase-3, while they worked with mice and human model of melanoma. Simvastatin has been shown to activate mitochondrial caspase 9 and to induce apoptosis in chronic lymphocytic leukemia cells [26].

Finally, atorvastatin induced apoptosis in activated hepatic stellate cells and highly increased protease activity of caspase-9 and -3. Gene expression of the proteins of Bcl-system revealed that Bid is involved in apoptosis induced by atorvastatin [27].

### **Inhibition of proliferation**

Almost 20 years ago, it was already suggested that statins could inhibit tumor cell growth and possibly prevent carcinogenesis [28]. Statins inhibit proliferation of cancer cells *in vitro*, either *in vivo* by inhibition of HMG-CoA reductase and subsequently through decrease of isoprenoids levels. Cell proliferation inhibition depends on the sensitivity of the particular statin and cell type of which it interact [5]. For example, fluvastatin inhibited the proliferation and viability in murine renal cancer cells [29]. On the other hand, lovastatin have been caused inhibition of farnesyl biosynthesis in human astrocytomas. This led to a block in Ras mediated signaling, indicated by lower MAPK activity and consequently, tumor cell proliferation was reduced up to 80% [30].

Several studies demonstrated positive results in breast cancer models. Treatment with lovastatin at a concentration of 50 mg/kg did inhibit the formation of pre-neoplastic mammary intraepithelial neoplasias *in vivo* [31], cerivastatin induced dose-dependent decrease in cell proliferation of breast cancer cell line [32]. Statins have shown to be able to inhibit the cell proliferation of receptor-positive and -negative human breast cancer cells but failed to completely abrogate the  $E_2$ -induced proliferation of receptor-positive breast cancer cells [33]. Atorvastatin, fluvastatin, lovastatin, simvastatin investigated on the proliferation of the human breast cancer cell line MCF-7 were able to inhibit cell proliferation up to 90 % at a concentration of 50  $\mu\text{mol/l}$  [34]. Clinical studies have recorded similar positive results, fluvastatin decreased tumor proliferation and increased apoptosis in high-grade, stage 0/1 breast cancer. Proliferation (Ki-67) of high grade tumors decreased by a median of 7.2% ( $P = 0.008$ ), which was statistically greater than the 0.3% decrease for low grade tumors. Study suggests that statins have biologic effects on the high grade subset of early stage breast cancers [35]. Moreover, embryonic stem cells treated with statins exhibited morphological change and significant reduction in cell proliferation [36].

Generally, statins can be categorized into three groups with regard to their antiproliferative properties. The inhibitory potency of simvastatin, lovastatin, fluvastatin and atorvastatin are in the same order of magnitude, whereas pravastatin is significantly less potent, and cerivastatin is more potent [37]. Inhibition of cell proliferation caused by HMG-CoA reductase inhibitors has been suggested to be due to prevention of transition from G1 to S phase in the cycle, down-regulation of cyclin- dependent kinases that assist the progress of cell cycle or up-regulation of cell cycle inhibitors [38]. This inhibition can be reversed by the availability of free mevalonate, FPP (farnesylpyrophosphate), or GGPP (geranylgeranylpyrophosphate), indicating that the observed effects of statins are indeed mediated by HMG-Co A reductase inhibition [5].

### **Impact on angiogenesis and metastasis**

Angiogenesis play an important role in tumor growth and metastases. Statins can by direct or indirect mechanisms inhibit or stimulate angiogenesis depending on type of tumor cells and concentration of drug [1].

Statins by unknown mechanism, but demonstrably decrease the levels of one of the most important molecule of angiogenesis, vascular endothelial growth factor (VEGF), and thereby inhibit capillary formation. In contrast, statins have been shown to stimulate protein kinase B in endothelial cells, which activates eNOS (endothelial nitric oxide synthase). Increased synthesis of nitric oxide (NO) improves blood circulation and pro-angiogenic activity [1, 17, 39] as was proved in certain studies [40, 41]. Weis et al. [42] demonstrated biphasic effect of statins, when low concentration of cerivastatin and atorvastatin (0.5 mg/kg/per day) increased proliferation of endothelial cells, while high doses (2.5 mg/kg/per day) significantly inhibit tumor vascularization and reduced tumor growth [42].

Several lines of evidence suggest that statins impair the metastatic potential of tumor cells by inhibiting cell migration, attachment to the extracellular matrix, and invasion of the basement membrane [1]. Lovastatin reduced endothelial leukocyte adhesion molecule (e.g. E-selectin) expression. Inhibition of E-selectin expression gives rise to a significant reduction in TNF- $\alpha$  stimulated adhesion of colon carcinoma cells to human endothelial cells. Moreover, low concentration of lovastatin ( $\leq 1 \mu\text{mol/l}$ ) attenuated TNF- $\alpha$  induced tumor cell invasion *in vitro* [43]. Wang et al. [44] demonstrated a reduction in gene expression of matrix metalloproteinase (MMP) – 9 and decreasing membrane localization of Ras proteins after treatment with lovastatin *in vitro*. Statins have been shown to inhibit epithelial growth factor (EGF) – induced tumor cell invasion. Treatment of pancreatic cancer cells with fluvastatin markedly attenuated EGF-induced translocation of RhoA from the cytosol to the membrane fraction and actin stress fiber assembly, whereas it did not inhibit the tyrosine phosphorylation of EGF receptor and c-erbB-2. HMG-CoA reductase inhibitors affect RhoA activation by preventing geranylgeranylation, which results in inhibition of EGF-induced invasiveness of human pancreatic cancer cells [45].

Although the exact mechanisms by which statins reduce risk of various type of cancer have not been fully elucidated and hypotheses are still subject of many studies, there seems to be considerable overlap of mechanisms by which statins exert their beneficial effects. In addition to the above- mentioned mechanisms, the anti-inflammatory, immunomodulatory, and radiosensitizing properties of statins may further contribute to the inhibition of cancer [5].

### Preclinical studies

Inhibitors of HMG-CoA reductase have demonstrated antineoplastic effects in a variety of cell lines, e.g. neuroblastoma [46], leukemia [47] breast cancer [48], melanoma [25] and glioma cells [49].

Even some researchers have shown that lipid-lowering drugs including statins increase the occurrence of several types of cancer in rodents [50], there is evidence that oral dosing of statins significantly inhibited the growth of a mouse ErbB2 mammary carcinoma model [51]. Another study was comparing effects of all commercially available statins in an *in vitro* and *in vivo* model of human experimental pancreatic adenocarcinoma, whereas simvastatin exerted the highest tumor-suppressive effect *in vitro*, rosuvastatin, cerivastatin and fluvastatin were the most potent compounds in an *in vivo* model [52]. Pravastatin suppressed hepatocarcinogenesis in male Sprague-Dawley rats. These results suggest that this effect might be related to pravastatin's inhibition of p21 (ras) isoprenylation and thus inhibition of cell proliferation and induction of apoptosis in neoplastic lesions [53]. In addition, combinatorial treatment with lovastatin and enzastaurin (Protein kinase C inhibitor) was found to enhance anti-tumor efficacy in human hepatocellular carcinoma *in vivo* and *in vitro* [54]. Furthermore, animal model with female mice proved that pravastatin and simvastatin may prevent colon tumorigenesis [55]. Recently, tumor growth was significantly reduced in the mice model treated with lovastatin. However, the tumor grew much faster in the mice treated with 1 mg/kg/day of lovastatin than in the positive control group. Authors suspect this result might be related to vascular endothelial growth factor. In this model, investigators found that lovastatin inhibits tumor growth at a high dosage (5 or 10 mg/kg/day). However, it also promotes tumor growth at a low dosage (1 mg/kg/day) and that this duality effect should be further studied for patients treated with various dosages of statins [56].

Finally, in our experiment antineoplastic effects of atorvastatin in the chemoprevention of N-methyl-N-nitrosourea – induced mammary carcinogenesis in female Sprague-Dawley rats were evaluated. In the group with higher concentration (100 mg/kg), atorvastatin suppressed tumor frequency by 80.5 % and tumor incidence by 49.5%, as well as prolonged the latency period by 14 days compared to control animals. Atorvastatin,

administered at a lower dose in the group (10 mg/kg), did not demonstrate a significant antineoplastic effect in mammary gland carcinogenesis [unpublished results]. In the next experiment performed by our group tumor-suppressive effect of simvastatin in the same rat model of mammary carcinogenesis were investigated. Higher concentration (180 mg/kg) significantly decreased tumor incidence by 58.9 % and suppressed frequency by 80.5 % compared to control group of animals. Similarly, the latency period lengthened by 14.5 days compared to not-treated animals. Lower concentration (18 mg/kg) did not significantly change parameters of mammary carcinogenesis (**Table 1**). Compared to control group, in the groups SIMVA 18 and SIMVA 180 a significant increase in food intake of animals was recorded. The significant changes in final body weight gain among groups with administered simvastatin and control group were not found [unpublished results].

**Table 1.** Effects of simvastatin in NMU-induced mammary carcinogenesis in female Sprague-Dawley rats at the end of experiment.

| Group                                        | CONT       | SIMVA 18             | SIMVA 180                  |
|----------------------------------------------|------------|----------------------|----------------------------|
| all animals/tumor bearing animals            | 19/12      | 19/15                | 19/5                       |
| tumor incidence (%)                          | 63.16      | 78.95 (+25 %)        | 26.32 a,b (-58.5 %)        |
| tumor frequency per group*                   | 1.89±0.57  | 1.84±0.30 (-2.5 %)   | 0.37±0.16 a,b (-80.5 %)    |
| tumor latency* (days)                        | 97.42±5.94 | 95.20±3.73 (-2 days) | 112.00±5.42 c (+14.5 days) |
| average tumor volume* (cm <sup>3</sup> )     | 0.93±0.28  | 0.74±0.29 (-20.5 %)  | 0.71±0.43 (-23.5 %)        |
| cumulative tumor volume** (cm <sup>3</sup> ) | 33.48      | 25.79 (-23 %)        | 4.97 (-85 %)               |

CONT – control group, SIMVA 20 – group with administered simvastatin at a concentration of 20 mg/kg in food, SIMVA 200 – group with administered simvastatin at a concentration of 200 mg/kg in food.

\* Data are expressed as means±SEM.

\*\* Data are expressed as a sum of volumes per group. Values in brackets are calculated as %-ual deviation from the 100% of non-influenced control group (with exception of latency). Significantly different, <sup>a</sup>P<0.05 vs CONT, <sup>b</sup>P<0.01 vs SIMVA 18, <sup>c</sup>P<0.05 vs SIMVA 18.

## Clinical Studies

The results of large clinical trials led to FDA (Food and Drug Administration) approval of first statins in 1987 [57]. Later results of a clinical trials showed that statins may not only decrease LDL cholesterol but moreover statins unveil antineoplastic effects. Scandinavian Simvastatin Survival Study (4S) and its follow up study have yielded not significant but promising result, when cancer risk decreased by 12 % in the group treated with simvastatin compare to placebo group [58].

Nowadays, epidemiologic studies indicated that statins may prevent certain malignancies, including melanoma, breast, colon, and prostate cancer [5948, 6049, 6150, 6251, 6352]. Poynter et al. [61] found out that use of statins were associated with a 47 % relative reduction in the risk of colorectal cancer after adjustment for other known risk factors. The Air Force/Texas Coronary Atherosclerosis Prevention Study showed a 50% lower incidence of new cases of melanoma in lovastatin-treated patients [63]. The benefit of using statins was shown also in randomised clinical study “The risk of cancer in users of statins”. Patients included in this study were after infarct myocardium and were using one of the statins or combination of these drugs longer than 4 years. This had resulted in 20% decrease risk of carcinomas [64]. Similar results were observed in control-case study from Quebec, confirming that users of statins were found to be 28% less likely than users of bile acid-binding resins to be diagnosed as having any cancer [65]. Another large study was provided by Danish investigators. Trial named „Cancer risk among statin users: a population-based cohort study”, studied relative risk for any of the monitored specific cancers (liver, colorectum, lung, breast, prostate, female genital organs and lymphatic and haematopoietic tissue). In summary, individuals prescribed

statins experienced a slightly reduced cancer incidence compared to population controls of nonusers and users of other lipid-lowering drugs [66]. Several studies were focused on mammary gland carcinoma. American multicentric prospective study of old women treated with hypolipidemic therapy exhorts to use statins especially in this group of critical patients, according to decreasing carcinoma occurrence by 68 % [62]. Boudreau et al. [67] carried out study about long term use (5 years ) of statins in postmenopausal women, where statins slightly decreased occurrence of breast cancer (OR = 0.7). However, in 2007, a group of researchers led by the same author did not find association between statins use and breast carcinoma in a large population-based setting [68].

Some statins were used for the treatment of cancer. Pravastatin administered at a daily dose of 40 mg prolonged the survival of patients with advanced hepatocellular carcinoma [69]. Minden et al. [70] described a case when lovastatin had apparent control of the leukemic blast cells in acute myeloid leukaemia patients. This effect was observed at a dose double the usual recommended dose for hypercholesterolemia. Moreover, Katz et al. [71] have proved that statins enhance the efficacy of neoadjuvant chemoradiation in rectal cancer, although with the unusually high incidence of pathologic complete response. In another study, fluvastatin reduced proliferation and increased apoptosis in women with high grade breast cancer [35].

However, not all clinical studies have yielded promising results. Cholesterol and Recurrent Events (CARE), a randomized clinical study, has shown pro-carcinogenic impact in women treated with pravastatin. The incidence of breast cancer in a prospective trial was 5.5% among pravastatin treated patients versus 0.3% in the placebo group [72]. Similarly, other study showed increased breast cancer risk among patients older than 55 years. Statins were not associated with breast cancer risk in women  $\leq 55$  years. [73]. Different study investigated combination of simvastatin (40 mg/per day) and ezetimibe (10 mg/per day) in patients with aortic stenosis (SEAS) caused debate and controversial views. The rate of new cancers was 50% higher in the active treatment arm -106 in the ezetimibe/simvastatin group versus 67 in the controls [74]. On the other hand, procarcinogenic effect of statins used in clinical trials is really sporadic.

Based on favourable results from oncological research, statins may represent a novel clinical approach for cancer risk reduction or maybe treatment. Several questions are unanswered about the role of statins in cancer patients. It is unknown, which types of tumors are responsive to statin therapy. Actual experimental data suggested that statins may be potentially effective in the treatment of melanoma, leukaemia, brain cancer, hepatocellular cancer and squamous cell cancer of the head and neck [75]. Further, it is not known, which statins are most effective in carcinogenesis – hydrophilic statins (pravastatin, rosuvastatin) or lipophilic statins (atorvastatin, simvastatin, fluvastatin, lovastatin). Finally, the optimal statin regimens were not defined yet. Statins administered in combination with other oncostatic substances may enhance tumor suppressive effects. In order to reduce statin adverse effects (myopathy, hepatotoxicity, rhabdomyolysis), it is favoured continuous low-dose drug clinical regimens.

## SUMMARY

Pleiotropic properties of statins with proven anticarcinogenic effects in human cells can open a new era in clinical medicine. New information about antitumor potential of statins had led to the development of clinical therapeutic and preventive trials, aimed to precisely define the role of these drugs in clinical oncology. Many of these studies have given promising initial results, but future large confirmatory trials should definitively answer the question about statins' role in carcinogenesis. The results of our group clearly pointed to atorvastatin and simvastatin favourable effects in experimental rat mammary carcinogenesis and gave the drugs a chance to become classified among substances with chemopreventive efficacy in various neoplasias including breast cancer.

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## MELATONIN IN BREAST CANCER CHEMOPREVENTION AND TREATMENT

KASSAYOVA M.<sup>1</sup>, ORENDAS P.<sup>1</sup>, AHLERS I.<sup>1</sup>, BOJKOVA B.<sup>1</sup>, KUBATKA P.<sup>2</sup>, KISKOVA T.<sup>1</sup>,  
AHLERSOVA E.<sup>1</sup>, PEC M.<sup>2</sup>, KAJO K.<sup>3</sup>

<sup>1</sup>Department of Animal Physiology, Institute of Biology and Ecology, Faculty of Science, P.J. Safarik University, Kosice, Slovak Republic, <sup>2</sup>Department of Medical Biology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic, <sup>3</sup>Department of Pathological Anatomy, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic and BB Biocyt, Diagnostic Centre, Ltd., Banska Bystrica, Slovak Republic

### Abstract

Melatonin is an ubiquitously present natural indole with pleiotropic action. There is much evidence to indicate its regulatory function in circadian and circaannual rhythmicity, immunity, antioxidative defence and inflammation. Melatonin exhibits oncostatic properties in various types of cancer, particularly in hormone-dependent, i.e. breast cancer. In addition to *in vitro* studies, chemically-induced (7,12-dimethylbenz[a]anthracene, DMBA; N-methyl-N-nitrosourea, NMU) mammary carcinogenesis in female rats is commonly used for evaluation of chemopreventive/tumor-suppressive effect of melatonin. Under experimental conditions, melatonin efficacy seems to depend on various factors, e.g. the dose, the way of administration and the time of day, respectively. The beginning of melatonin application (before or after carcinogen administration) as well as the duration of treatment are of importance. During the past ten years, effects of melatonin administered alone or in combination with other chemopreventive substances (retinoids, non-steroidal anti-inflammatory drugs, antiestrogens) in DMBA or NMU-induced rat mammary carcinogenesis were investigated in our laboratory. Basic tumor growth parameters – tumor incidence, frequency per group and per animal, tumor volume and latency were evaluated. The results of our studies suggest inconsistent antitumorigenic effect of melatonin: the administration of melatonin alone is less effective, the combination with other chemopreventive substances displays an enhanced antitumor activity. With respect to the substantial benefits occurring in the cancer patients treated with the combination of chemotherapeutic drugs and melatonin and very low toxicity reported, this substance shows a great potential for further clinical evaluation.

**Key words:** chemoprevention, breast cancer, melatonin

### INTRODUCTION

Melatonin is very old, evolutionary conserved molecule, present in numerous taxa of evolutionary distant organisms like bacteria, unicellular eukaryotes, algae, fungi, plants, invertebrates and vertebrates. In vertebrates, melatonin is a neurohormone produced primarily by the pineal gland. High concentrations were detected also in retina, gastrointestinal tract, bone marrow, skin and other tissues (review: 1,2). Pineal gland-derived melatonin serves particularly as a chemical expression of the darkness, i.e. it is involved in the regulation of circadian and circaannual rhythms of various physiological processes. On the other hand, it is very likely that extrapineal melatonin is used locally in the tissues where it is produced, with autocrine and paracrine effects (3,4). In general, MEL is highly pleiotropic. In addition to its chronobiological function, it exhibits immunomodulating, antioxidative, antiinflammatory and apoptotic properties.

The wide spectrum of melatonin regulatory effects is mediated through its binding to several specific receptors and intracellular targets. In mammals, two high-affinity cytoplasmic membrane receptors have been identified, MT1 and MT2. These receptor subtypes are involved in chronobiotic effects of melatonin, i.e. in all actions directly in-

Address for correspondence:

Assoc. Prof. Monika Kassayová, RNDr. PhD., Department of Animal Physiology, Institute of Biology and Ecology, Faculty of Science, P.J. Šafárik University, Moyzešova 11, 041 67 Košice, Slovakia,  
Phone: 055/234 1214, Fax: 055/622 21 24, e-mail: monika.kassayova@upjs.sk

fluencing a circadian master clock in suprachiasmatic nuclei (5). MT1 and MT2 belong to the family of G-protein-coupled receptors, their activation suppresses adenylyl cyclase activity leading to a reduction in the intracellular level of cAMP and subsequently to the changes in target protein phosphorylation. Modulation of cGMP formation has also been reported (6). The third putative MT3 receptor was identified in various Syrian hamster organs. Recently, it has been purified and designated as quinone reductase 2, a detoxification enzyme (7,8). As melatonin molecule has the lipophilic properties, it is capable passing of plasma membrane and binding directly to the nuclear DNA receptors – ROR and RZR isoforms from the retinoic acid receptor superfamily (9). Their exact role has not been elucidated yet, they seem to be involved in immune modulation, anti-oxidative processes and apoptosis. In addition to membrane and nuclear receptors, the cytosolic melatonin binding sites were identified, considerably significant is calmodulin. Melatonin-calmodulin interactions are relevant to cytoskeleton rearrangements (10). Mitochondrial melatonin binding sites seem to be related to antioxidative cell protection and prevention of  $\text{Ca}^{2+}$ - and NO-dependent cellular stress (5).

Anticarcinogenic, chemopreventive and oncostatic melatonin potential has been reported in *in vitro* and/or *in vivo* experimental studies against a variety of cancer types such as hepatocellular, colorectal, non-small lung, renal, bladder and ovarian carcinoma, neuroblastoma, melanoma, leukemia (11,12). These effects are most pronounced in hormone-dependent cancers, particularly mammary and prostate tumors. The data suggest that melatonin is involved in preventing tumor initiation, promotion, and progression (13).

### **Plausible mechanisms of anticancer melatonin action**

MT1 receptor is a major transducer of melatonin's actions in the breast, suppressing mammary gland development and mediating the anticancer actions of melatonin through multiple pathways (14). Melatonin inhibits the growth of breast cancer acting as an antiestrogen through different mechanisms. The indirect neuroendocrine pathway includes the inhibition of hypothalamo-pituitary-gonadal axis and the consequent reduction of estrogen circulating levels. Melatonin acts directly at tumor cell level by the activation of the estrogen receptor, thus behaving as a selective estrogen receptor modulator (SERM). This indole regulates the enzymes involved in the biosynthesis of estrogens in peripheral tissues (aromatase, sulfatase, sulfotransferase and others), thus having the properties of a selective estrogen enzyme modulator (SEEM) (15). Melatonin inhibits the growth of breast cancer via the decrease in linoleic acid cellular uptake and metabolism, thereby preventing the formation of its mitogenic metabolite, 13-hydroxy-octadecadienoic acid (16). It has been shown that melatonin has strong antioxidative properties. It is a potent scavenger of both reactive oxygen and nitrogen species as well as a strong up-regulator of antioxidative enzymes (glutathione-peroxidase, superoxid-dismutase, glutathione-reductase, catalase) and down-regulator of prooxidant enzymes (lipooxygenases, nitric oxid synthases). Melatonin and its metabolites are potent anti-inflammatory agents due to their ability to downregulate gene expression of the pro-inflammatory enzymes, e.g. cyclooxygenase-2 (17). Melatonin has also been shown to modulate immune function. Various cells of immune system are able to synthesize melatonin, e.g. NK cells, eosinophilic leukocytes, lymphocytes, monocytes, platelets, mast cells. Melatonin has been demonstrated to stimulate cytokine production (IL-2, IL-6, IL-12), enhanced phagocytosis, increased NK cell activity, decreased  $\text{TNF}\alpha$  activity. It is believed that induction of Th2 lymphocytes as well as inhibition of Th1 function contribute to anti-inflammatory action of melatonin (review: 18,19).

It has been suggested that the altered circadian rhythm regulation plays significant role in carcinogenesis (20,21). Nowadays there is a rising evidence that melatonin is able to induce circadian synchronization on the cellular level in cancer cells. Melatonin is involved in the regulation of clock genes in breast cancer cells via  $\text{ROR}\alpha$  transcriptional

activity. Blockade of BMAL1 expression by melatonin is associated with the decreased expression of SIRT1, a member of the silent information regulator family, the histone deacetylase, which is involved in epigenetical control of apoptosis. SIRT1 inhibits the expression of DNA repair enzyme and the expression of apoptosis-associated genes. (14,22). Melatonin participation in apoptosis seems to be different in normal and cancer cells. Melatonin exerts anti-apoptotic effects in immune cells and prevents apoptosis of the cells in central nervous system. On the other hand, in cancer cells, melatonin is able to increase apoptotic cell death (6). Additionally, inhibition of telomerase activity has been shown, both *in vitro* and *in vivo* (23) as well as stimulation of expression of cell surface adhesive molecules (E-cadherin,  $\beta$ 1-integrin) in cancer cells (24).

### **Breast cancer – preclinical studies**

The results of *in vitro* studies have shown that only those breast cancer cell lines are susceptible to antimitogenic effect of melatonin which express estrogen receptors (MCF-7, T47D, ZR75-1). MCF-7 cell line is the most commonly used for study of melatonin effects in mammary carcinogenesis. This line is derived from pleural effusion of woman with metastatic breast carcinoma and expresses both estrogen and progesterone receptors. Melatonin in culture medium inhibits cell proliferation and invasiveness, suppress the mitotic activity of estradiol and EGF, therefore blocks proliferation at stage G<sub>0</sub>/G<sub>1</sub> phase of mitotic cycle (25). Physiological melatonin concentrations (close to 1 nM) and pulsatile exposure that mimics circadian pattern *in vivo* (melatonin concentration was changed every 12 h between 10 pM and 1 nM in culture medium) were the most effective (26). Melatonin inhibits also the proliferative effect of prolactin, growth hormone (27) and some growth factors (IGF-I, EGF) in MCF-7 cells (28,29).

First experimental *in vivo* evidences on oncostatic effect of pineal gland come from the half of last century (review: 30). In 1978, Cohen and collaborators introduced the hypothesis that diminished function of the pineal gland may promote the breast cancer development in humans by inducing a state of hyperoestrogenism (31). Subsequent years have brought great progress in investigation of melatonin role in mammary carcinogenesis, particularly on the animal models. Mammary tumorigenesis in female rats induced by chemical carcinogenes – DMBA (7,12-dimethyl-benz[*a*]anthracene) and NMU (N-nitrosomethylurea) is most commonly used. Mice with the high incidence of spontaneous mammary tumors as well as transgenic mice bearing oncogenes related to mammary carcinogenesis (N-ras, HER-2/neu and others) are used less frequently (review: 32). Depletion of pineal function resulted in the stimulation of experimental mammary tumorigenesis. Aubert *et al.* (33) and Tamarkin *et al.* (34) reported the increased incidence of DMBA-induced mammary tumors in surgically pinealectomized rats; similar results were found in NMU-induced rat neoplasia (35). Long-term exposure to constant light (24 h/day), so-called functional or physiological pinealectomy, resulted in the higher incidence and shorter latency period of tumor appearance in DMBA-treated Holtzman rats as compared to control animals (36), melatonin was able to reverse these effects. On the other hand, experimental manipulations leading to the pineal activation seem to have an opposite effect, for example blinding, anosmia or underfeeding (review: 25). Many other experimental and clinical studies have revealed that melatonin is a crucial substance in antineoplastic effect of pineal gland, even though efficiency of pineal peptides should be taken into consideration too (review: 32).

Anti-tumorigenic effect of melatonin administration in chemically-induced female rat mammary carcinogenesis depends on various experimental factors. Older reports have shown that morning administration of melatonin was followed by the increase in the incidence of mammary tumors induced by DMBA (37). Subsequent investigations have demonstrated a tumor-inhibitory effect of melatonin application during the late light part of day and during the night. Administration of melatonin prior to the nocturnal increase in endogenous melatonin may be most effective because tissues are most sensi-

tive to the hormone at this time (25). As for the way of administering melatonin, i.p., s.c. or oral solution in drinking water is the most common. The most frequently daily doses used are in the ranges 100-500 µg/rat. Dose-dependence of melatonin oncostatic effect was analyzed in the study of Beau Yon De Jonage-Canonico *et al.* (38). Three different melatonin doses were tested – 1, 3 and 10 mg/kg/day, administered daily intragastrically for 6 month, followed by the observation for other 4 months. Melatonin treatment started the day after DMBA administration. At the end of treatment period, maximal incidence decrease was observed in the 3 mg/kg/day group (35 % v.s. 90 % in control animals). The number of tumors (i.e. frequency) per rat was reduced with a maximal effect in the 10 mg/kg/day group. Four months after the treatment cessation, maximal incidence decline was recorded in the 3 and 10 mg/kg/day groups (60% vs 95% in controls), tumor frequency per rat was maximally reduced in the 10 mg/kg/day group.

The beginning of melatonin treatment is of importance – before, after or simultaneously with the administration of carcinogenic agent (DMBA or NMU). Lenoir *et al.* (39) published the study comparing the effect of preventive and curative treatment with melatonin (10 mg/kg /day, intraperitoneally) in female rats. In preventive group, MEL was administered for only 15 days before DMBA administration; in curative group – long-term (6-month) treatment was used, starting the day after DMBA administration. The rats were followed up for a year after the carcinogen administration. The incidence was decreased in both curative and preventive group, the decline persisted longer (up to 10th month of experiment) in curative group in comparison to preventive group (up to 7th month). Average number of mammary adenocarcinomas per rat was decreased in both groups as compared to the controls (DMBA group) for all the time. The authors concluded almost identical preventive and curative effects of melatonin on the growth of DMBA-induced mammary tumors. In Saez's study (40), using combined preventive-curative approach, the rats were subjected to melatonin treatment (5 mg/rat/day) from one month prior to DMBA administration to the death of the animals. At the end of experiment, latency of mammary tumors was increased as well as overall surviving, tumor growth rate decreased and melatonin returned the prolactin levels to those of the healthy animals. In the following curative study, melatonin administration started after the first tumor appearance and lasted to the death of animals. Increased survival time of tumor-bearing rats was registered, without any changes in tumor incidence or tumor growth. Melatonin improved the prolactin and catecholamine levels in tumor-bearing rats (41). The results of these studies suggest that combined preventive-curative approach is the most effective, with long-lasting melatonin treatment. This idea is supported by the results of our study evaluating the effect of melatonin on NMU-induced mammary carcinogenesis in female rats exposed to repeated stress (immobilization in boxes). Melatonin was administered in drinking water at a concentration of 4 µg/ml daily from 15.00 h to 8.00 h of the next day. Melatonin application started 5 days prior to the first NMU dose and lasted 15 days, i.e. during the promotion phase of tumor development, or long-term until the end of the experiment (week 20). Although the results were non-significant, long-term melatonin administration reduced the number and size of mammary tumors more markedly than its short-term administration: tumor frequency per animal was reduced by 28 % and tumor volume was depressed by more than 40% (42).

During the past 10 years, effects of melatonin administered alone or in combination with other chemopreventive substances (retinoids, non-steroidal anti-inflammatory drugs, antiestrogens) in DMBA or NMU-induced mammary carcinogenesis in female rats were investigated in our laboratory. In our studies, the model of melatonin administration in drinking water at concentrations of 4 µg, 20 µg and 100 µg/ml was used. The continuous (24 hours/day) melatonin administration at concentration of 100 µg/ml (i.e. 3 mg/rat/day) in DMBA-induced tumorigenesis decreased the tumor frequency per animal; melatonin was administered from the first day after DMBA throughout the experiment (25 weeks). Combination of melatonin with retinyl acetate had even more

marked tumor-suppressive effects, depressed the incidence, considerably decreased the frequency per animal and extended the latency period (43). To increase efficiency, we decided to use the melatonin solution treatment from 3.00 p.m. to 8.00 a.m. of the next day (i.e. in the period of maximal tissue sensitivity to melatonin signal) in all other experiments. From 8.00 a.m. to 3.00 p.m. the rats were drinking tap water. We chose the preventive-curative model, when MEL was administered 12-14 days before carcinogen application during the whole experiment (22-25 weeks). MEL administration at the concentration of 20 µg/ml (i.e. 0.5 mg/rat/day) in NMU-induced carcinogenesis extended the latency period; the combined treatment of MEL with retinyl acetate was more effective again: more markedly prolonged latency, declined incidence and decreased frequency per group were observed (44). Similar results were recorded in DMBA-induced tumorigenesis: MEL (20 µg/ml) increased the incidence and frequency per group, while the combined chemoprevention with indomethacine decreased the incidence, frequency per group and per animal as well as prolonged latency (45). In some experiments, the tumor-suppressive effect of neither MEL alone (20 µg/ml) nor its combination with other chemopreventive agents was observed (NMU+MEL+tamoxifen, 46; NMU+MEL+indomethacine, 45). In other experiments the only slight effect of MEL alone was noted, when the decreased tumor volume (MEL+DMBA+tamoxifen, 46) or the decreased incidence (MEL+DMBA+nimesulide, 47) was marked. In one study, even tumor-stimulating effect of melatonin was registered: an increased tumor volume after isolated MEL administration as well as increased frequency per animal in combination was seen (MEL+ NMU+nimesulide, 47). No marked changes in tumor parameters were observed after shortening the MEL treatment period (3 days before NMU application, 20 weeks overall): effect of MEL alone was not expressed, the combination with chemopreventive agent (celecoxib) led to decrease of tumor frequency per group and per animal (48). We also tested the efficiency of lower MEL dose (4 µg/ml, i.e. 0.1 mg/rat/day). The isolated administration of this dose was not effective (the only frequency per group decrease in NMU-induced tumorigenesis was recorded); the combination with raloxifene was more efficient, we observed marked decrease of incidence and frequency per group after NMU as well as DMBA administration (49,50).

### **Clinical studies**

Several clinical trials have suggested the potency of melatonin in the management of breast cancer and other cancer types as well. Much of the evidence came from small trials done by Paolo Lissoni and co-workers. They have demonstrated a wide array of beneficial effects of high dose melatonin – 10-50 mg/day, particularly in patients with advanced cancer. Melatonin acts synergistically with the wide range of chemotherapeutic agents to enhance their antitumor effect, i.e. cisplatin, etoposide, oxaliplatin, 5-fluorouracil, gemcitabine, doxorubicin, paclitaxel, tamoxifen (51,52,11). The presence of melatonin seems to prolong both disease progression-free and overall 1-year survival (53). Concomitant administration of melatonin enhanced the overall tumor regression rate and 5-year survival in patients with metastatic non-small cell lung cancer treated with cisplatin and etoposide (54) as well as the efficacy of irinotecan in metastatic colorectal cancer patients (55). Additionally, melatonin alleviates the side effects of chemotherapy – thrombocytopenia, neurotoxicity, cardiotoxicity, cachexia, chemotherapy-induced asthenia (56). Melatonin may induce the control of neoplastic growth in patients with metastatic solid tumors, for whom no other conventional anticancer therapy is available (57). Patients receiving melatonin have a better performance status and less anxiety than those treated without melatonin. Melatonin improves the quality of life in cancer patients also by consolidating and enhancing nighttime sleep due to its chronobiological effects (11). Large, well-designed, multicentric, randomized, double-blind clinical trials are needed for evaluation of melatonin using in clinical oncology.

## CONCLUSION

The results of several studies suggest that chemopreventive and tumor-suppressive effect of melatonin in experimental chemically-induced rat mammary carcinogenesis is not consistent. The administration of melatonin alone is less effective. The inhibition of tumor growth is more apparent in the combined chemoprevention, where potentiating/enhancing effects can be expected. In addition to the dose, the way and schedule of melatonin administration, it is necessary to take into consideration other factors, e.g. the influence of season. These findings correspond to the results of clinical studies, in which melatonin is applied preferably in supportive and/or adjuvant therapy, considerably in patients suffering from cancer in the developed stage, with poor clinical prognosis. However, oncostatic effect of melatonin was tested on the relatively small numbers of cancer patients so far. With respect to the wide range of beneficial effects of melatonin in organism as well as its low or no toxicity even in high doses, this indole is a candidate for independent clinical evaluation in patients with breast cancer and also with other types of cancer.

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## THE PERSPECTIVES OF PERORAL ANTIDIABETICS IN MAMMARY CARCINOGENESIS PREVENTION

BOJKOVA B.<sup>1</sup>, GARAJOVA M.<sup>1</sup>, ORENDAS P.<sup>1</sup>, KASSAYOVA M.<sup>1</sup>, KISKOVA T.<sup>1</sup>, KUBATKA P.<sup>2</sup>, PEC M.<sup>2</sup>, KAJO K.<sup>3</sup>, AHLERSOVA E.<sup>1</sup>, AHLERS I.<sup>1</sup>

<sup>1</sup>Department of Animal Physiology, Faculty of Science, Institute of Biology and Ecology, P.J. Safarik University, Kosice, Slovak Republic, <sup>2</sup>Department of Medical Biology Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic and <sup>3</sup>Department of Pathology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic

### Abstract

Peroral antidiabetics, biguanides and thiazolidinediones were reported to suppress carcinogenesis both in vitro and in vivo. As the incidence of many cancers including breast cancer is higher in diabetic patients, the choice of suitable therapy may lower the cancer risk. The experimental data are promising, however, the link between cancer and long-term administration of peroral antidiabetics, particularly thiazolidinediones, should be further investigated.

**Keywords:** biguanides, thiazolidinediones, breast cancer, chemoprevention

### INTRODUCTION

Cancer chemoprevention remains a great challenge for oncology. Metabolic pathways and cell growth regulation are interconnected and metabolic disorders, such as diabetes mellitus type 2, are associated with higher cancer risk. The cancer risk for diabetic patients, mainly those with type 2 diabetes, is higher particularly for primary liver and pancreatic cancer, but is elevated for non-Hodgkin's lymphoma, colorectal, endometrial, breast, renal, and bladder cancers too (1-6). Increased cancer incidence in diabetic patients is related to hyperinsulinaemia, impaired insulin receptor and insulin-like growth factor signalling, and subsequent cell proliferation enhancement. On the other hand, cancer patients often develop glucose intolerance (7,8). From this point of view peroral antidiabetics with pleiotropic properties that showed antitumor activity draw more and more attention. So far, the chemopreventive effects were reported in two groups of peroral antidiabetics: biguanides and thiazolidinediones which improve insuline sensitivity. The aim of this paper is to summarise the effects of peroral antidiabetics in carcinogenesis, particularly mammary cancer.

### BIGUANIDES

Biguanides inhibit fatty acid oxidation and liver gluconeogenesis, increase insulin receptor availability and suppress monoamine oxidase activity (9). First reports on oncostatic properties of biguanides were published by Dilman group. Phenformin suppressed dimethylbenz(a)anthracene (DMBA) and N-nitrosomethylurea (NMU)-induced mammary tumorigenesis in female rats (10,11), and 1,2-dimethylhydrazine-induced colon carcinogenesis in rats (12). Phenformin prolonged survival and inhibited spontaneous tumorigenesis in female C3H/Sn mice (13), the same was reported for buformin in female rats (14). However, buformin and phenformin have been withdrawn in most countries because of lactic acidosis risk and nowadays only metformin is used in type 2 diabetes treatment. Metformin exerts its effects through AMP-activated protein kinase (AMPK) activation and subsequent mammalian target of rapamycin (mTOR) pathway inhibition which leads to

Address for correspondence:

RNDr. Bianka Bojková, PhD., Department of Animal Physiology, Institute of Biology and Ecology, Faculty of Science P.J.Šafárik University, Moyzesova 11, 041 67 Košice, Slovak Republic  
e-mail: bianka.bojkova@upjs.sk, Phone: 421 55 234 1209, Fax: 421 55 622 2124

decrease of protein synthesis and cell proliferation. AMPK activation by AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) inhibited breast, glioma, prostate (15), and melanoma cell proliferation (16). Many *in vitro* studies reported tumor suppressive effects of metformin. Metformin suppressed proliferation and induced apoptosis in glioma cells (17), decreased proliferation after metformin treatment was found in prostate (18), ovarian (19), melanoma (16), and breast cancer cell lines (both estrogen receptor  $\alpha$  negative and positive) too (20). Metformin induced translation inhibition and subsequent global protein synthesis decrease in MCF-7 cells (21). The antiproliferative effect of metformin may not entirely depend on AMPK activation. AMPK inhibition by compound C decreased antiproliferative properties of metformin on ovarian cancer cells (19) and glioma cells (17) but AMPK inhibition using siRNA against the two catalytic subunits of AMPK did not prevent the antiproliferative effect of metformin in prostate cancer cell lines (18). Therefore there may be another underlying mechanism of metformin activity to be revealed.

*In vivo* reports on metformin oncostatic activity are scarce. Metformin treatment reduced tumor growth in mice bearing LNCaP xenografts (18) and p53 deficient colon cancer HCT116 xenografts, however, the growth of HCT116 p53+/+ cells was not affected (22). Metformin inhibited pancreatic tumorigenesis in hamsters (23) and tobacco carcinogen-induced lung tumorigenesis in A/J mice (24). Metformin administration increased mammary tumor latency and overall surviving in HER-2/neu transgenic mice (25,26) and decreased tumor outgrowth and incidence in nude mice bearing MDA-MB-231 mammary tumor xenografts (27). Our group evaluated chemopreventive effect of metformin (administered in drinking water at a concentration of 50 and 500  $\mu\text{g}/\text{ml}$ , respectively) in NMU-induced mammary carcinogenesis in female Sprague-Dawley rats. Metformin did not significantly alter the tumor growth although a delay in tumor onset was recorded after higher metformin dose during the experiment (28). We suppose the lack of chemopreventive efficacy was due to low doses (corresponding to 5 and 50  $\text{mg}/\text{kg}/\text{rat}/\text{day}$ , respectively), whereas doses used by other authors were several-fold higher (22,23,25).

Human studies showed lower cancer incidence in diabetic patients treated with metformin in comparison with those treated with other hypoglycaemic drugs (29,30). Cancer-related mortality in diabetic patients using metformin in comparison to those using sulfonylureas or insulin was lower too (31). The cancer incidence decrease recorded in diabetic patients may be attributed to so-called metabolic rehabilitation too – biguanides treatment in breast and colon cancer patients improved the survival and slightly decreased contralateral breast tumor incidence (32-35). A short-term clinical trial in nondiabetic patients with rectal aberrant crypt foci (ACF) showed suppression of colonic epithelial proliferation and ACF formation after one-month metformin treatment in comparison with untreated group (36). On the other hand, adverse effects of metformin could not be excluded. A study in athymic nude mice suggested metformin may stimulate angiogenesis and consecutively tumor promotion (20), therefore, its effect *in vivo* should be further analysed.

### THIAZOLIDINEDIONES (GLITAZONES)

The other group of peroral antidiabetics, thiazolidinediones, have been used in diabetes type 2 treatment only from the late 1990s. This group is presented by pioglitazone (Actos TM, Eli Lilly), rosiglitazone (Avandia TM, GlaxoSmithKline), and troglitazone (Rezulin). However, troglitazone was removed from the market in 2000 due to hepatotoxicity risk and rosiglitazone is to be suspended because of cardiovascular risk. A new analogue rivoglitazone is under research for use. Thiazolidinediones are synthetic ligands of peroxisome proliferator-activated receptors  $\gamma$  (PPAR $\gamma$ ). PPAR $\gamma$  and other two isotypes ( $\alpha$ ,  $\beta/\delta$ ) are members of nuclear receptor superfamily comprising steroid, retinoid, and thyroid hormone receptors. PPAR $\gamma$  act as transcription factors and are involved in cell proliferation, lipid transport and accumulation, and immune system modulation. In diabetic patients PPAR $\gamma$  agonists improve insulin sensitivity. Numerous (mostly *in vitro*) reports suggest PPAR $\gamma$  ligands may inhibit carcino-

genesis through cell cycle arrest and apoptosis induction, angiogenesis suppression, and anti-inflammatory actions (37). Grommes et al. (38) reported glioma growth inhibition by pioglitazone (PIO) both in vitro and in vivo, a combination therapy of PIO and 6-OH-11-O-hydroxyfenantrene (IIF, a retinoid X receptor ligand) enhanced reduction of glioma cell proliferation (39). Rosiglitazone (ROSI) decreased neuroblastoma cell proliferation in vitro but showed only minor antitumor effect in a metastatic xenograft mouse model (40). PIO inhibited human gastric cancer cell growth in vitro (41) and pancreatic cancer cell growth both in vitro (42) and in vivo (42,43). Early carcinogenic transformation in chemically-induced liver carcinogenesis in rats was suppressed by PIO (44,45) and ROSI (44). Both ligands inhibited chemically-induced colon carcinogenesis in mice (46) and pheochromocytoma PC12 cell growth (47). PIO and another PPAR $\gamma$  ligand 15-deoxy- $\delta$ 12,14-prostaglandin J2 induced apoptosis and suppressed the production of angiogenic factors in human renal cell carcinoma in vitro (48), the latter substance induced apoptosis in human hepatoma cells (49) and human oral squamous cell carcinomas too (50). PIO suppressed colon cancer liver metastasis in immunodeficient mice (51) and ovarian tumor xenograft growth in nude mice (52). A new PPAR $\gamma$  agonist ciglitazone induced apoptosis in melanoma cells both in vitro and in vivo (53). The effect of PPAR $\gamma$  ligands on breast cancer cell lines is probably modulated by estrogen receptor status. Estrogen receptor  $\alpha$  was found to associate with PPAR $\gamma$  and interfere with its signaling (54). Growth inhibition of ER $\alpha$ -negative MDA-MB-231 cells by troglitazone was more prominent than that of ER $\alpha$ -positive MCF-7 cells, which supports this hypothesis (55). Apoptosis induction by PPAR ligands was not confirmed univocally. Troglitazone (56) and ROSI (57) triggered apoptosis in MCF-7 cells which was enhanced in combination with retinoid X receptor ligand 9-cis-retinoic acid (58), on the other hand, autophagy instead of apoptosis was reported in MDA-MB-231 breast cancer cells treated with ROSI and troglitazone (59). Apoptotic induction may be improved by combination therapy – troglitazone combined with aspirin caused cell cycle arrest and apoptosis in human lung cancer cells (60). ROSI inhibited mammary tumor cell line metastasis (61), impaired adrenocortical carcinoma proliferation in a xenograft mouse model (62), and prevented the progression of preinvasive lung cancer in the A/J mouse model (63). PIO, ROSI, 15-deoxy- $\delta$ 12,14-prostaglandin J2, and another PPAR $\gamma$  ligand, GW7845, suppressed MDA-MB-231 human breast cancer cell invasion (64). Troglitazone inhibited MCF-7 xenograft growth in BNX mice (56). PPAR $\gamma$  ligand, GW7845 (a tyrosine analog optimized for potency on PPAR $\gamma$  inhibited NMU-induced mammary carcinogenesis in rats (65), another PPAR $\gamma$  ligand, N-(9-fluorenyl-methyloxycarbonyl)-L-leucine inhibited mammary tumor growth in the same experimental model as was proved by prominent apoptosis induction; this effect was enhanced in combination with cyclooxygenase-2 inhibitor celecoxib (66). Similarly, ROSI suppressed DMBA-induced mammary tumor growth in rats (67). Our group found prominent PIO effect in NMU-induced mammary carcinogenesis in rats using the same experimental protocol as in the report on metformin mentioned above. PIO was administered in a diet (at a concentration of 10 and 100 ppm). PIO at a higher dose decreased final incidence by 38%, frequency per group by 63%, and extended latency period by 32% when compared with control group. Tumor histopathology showed the most homogenic group was the group administered with higher PIO dose, control group (without chemoprevention) and the group with lower PIO dose were heterogeneous considering tumor type and number of mixed-tumor cases. Mixed-tumor types with high invasive properties were found particularly in control group (68). An important fact to emphasize is the length of in vivo studies. Suh et al. (65) and Badawi et al. (66) initiated chemoprevention one week after the carcinogen application and continued it for 8 weeks, we initiated chemoprevention 12 days prior to tumor induction in order to cover the initiation stage of carcinogenesis and continued it for additional 17 weeks until the experiment termination (68). Similarly Kocdor et al. (67) administered ROSI (200  $\mu$ g/kg b.w.) 2 weeks prior to carcinogen application but the chemoprevention continued only for additional 7 days (the experiment lasted 120 days following DMBA administration). All three authors mentioned above confirmed tumor incidence and frequency decrease after PPAR $\gamma$  ligand treatment, moreover Kocdor et

al. (67) reported no malignant tumor occurrence after ROSI treatment. However, the different chemopreventive protocol used by above mentioned authors and by our group complicates the comparison of chemoprevention efficacy. We have chosen the preventive/curative approach to cover both initiation and promotion stages of carcinogenesis (68) as was suggested by Mehta (69). On the other hand, Kocdor et al. (67) applied chemoprevention during initiation period while Suh et al. (65) and Badawi et al. (66) applied chemoprevention during promotion period, moreover, the length of chemoprevention differed. Therefore, we believe the experimental protocol of in vivo studies (carcinogenesis induction, the onset and length of chemoprevention administration, and termination of experiment in particular) should be standardised to enable proper evaluation of chemoprevention efficacy in individual malignant transformation stages.

Data on long-term thiazolidinedione administration in cancer patients are missing. Clinical phase II trial of troglitazone (stage IV, 8-week median treatment) and ROSI (stages 0-II, 2-6 week treatment) in breast cancer patients did not prove their anticancer effect. PIO in combination with cyclooxygenase-2 inhibitors showed some response in patients with metastatic melanoma, soft tissue sarcoma, and glioma (37). Thiazolidinedione administration in diabetic patients did not reduce proportion of cancer cases in comparison with other treatment; however, these studies either lack duration response analysis (70) or the duration did not exceed 52 weeks (71). Govindarajan et al. (72) showed a 33% lower incidence of lung cancer in thiazolidinedione users in comparison with non-users but the duration of thiazolidinedione use was not considered and the study was not able to account for smoking history which complicate data interpretation. Safety issues, however, should be carefully considered when choosing a long-term therapy. A new meta-analysis confirmed previously suspected increased risk for myocardial infarction although not for cardiovascular mortality or all-cause mortality in patients treated with ROSI (73). As a result, European Medicines Agency recommended in September 2010 to suspend rosiglitazone from the European market. New thiazolidinedione analogues such as rivoglitazone may show better efficacy and safety.

## CONCLUSIONS

Assuming the reports on biguanide and thiazolidinedione anticancer efficacy and our results, we believe they may have big potential as chemopreventives when administered in early stages of malignant transformation or, preferably, prior to initiation. This hypothesis should be further investigated in in vivo studies using standardised experimental protocol. Clinical trials in cancer patients failed to prove an oncostatic efficacy of PPAR $\gamma$  ligands probably because of their inability to reverse advanced stages of cancer. Therefore, preventive-therapeutic strategy rather than just therapeutic one should be chosen in order to inhibit or reverse carcinogenesis, the combination with other drugs (e.g. NSAIDs, selective estrogen receptor modulators or retinoids/rexinoids) should be preferred.

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