Coadministration of the Aromatase Inhibitor Formestane and an Isopropanolic Extract of Black Cohosh in a Rat Model of Chemically Induced Mammary Carcinoma

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Original Paper

Abstract

Non-steroidal as well as steroidal aromatase inhibitors are currently being discussed as alternatives to tamoxifen in the first-line treatment of patients with hormone-dependent breast cancer. Many of these women are in a postmenopausal state and additionally troubled by climacteric complaints. Naturally occurring symptoms like hot flushes and night sweats can be triggered or augmented by anti-hormonal drugs. At the aromatase molecule, steroidal inhibitors like exemestane and formestane compete with the hormonal precursors for the substrate binding site and inactivate the enzyme irreversibly. An isopropanolic extract of the rootstock of black cohosh (iCR), which is a common comedication of aromatase inhibitors in breast cancer patients suffering from climacteric symptoms, contains triterpene glycosides and cinnamic acid esters, both of which possess structural similarities to steroids. We therefore tested a high dose of iCR, guaranteeing an effective uptake of 60 mg herbal substance per kg body weight and shown to influence rat bone and uterus, for putative interactions with two low dosing regimens of 3.5 mg or 5.0 mg formestane per animal and day. We chose a rat model of chemically induced breast cancer and evaluated tumor growth and serum estrogen levels. Compared to a tumor area of 1400 mm^2 after 21 days of unopposed tumor growth, formestane treatment, irrespective of concomitant black cohosh application, significantly reduced neoplastic growth by 50%. Formestane also significantly reduced serum estrogen levels, an effect which was also not abolished by iCR. Therefore, in this experimental setting, when challenging two low doses of formestane with a high dose of iCR, our data do not raise concerns against combining aromatase inhibitors with black cohosh.

Key words
Cimicifuga racemosa · *Actaea racemosa* · Ranunculaceae · aromatase inhibitor · black cohosh · formestane · mammary carcinoma · phytotherapy

Introduction

Breast cancer is the most frequent malignant disease in women. Between 8 and 10% of German women will develop breast cancer during their lifetime. Anti-cancer therapy with cytostatics, analogues of gonadotropin-releasing hormone, aromatase inhibitors (AI) or antiestrogens like tamoxifen frequently induce climacteric-like complaints or aggravate preexisting menopausal symptoms. Within this disease complex, especially hot flushes and other vasomotor symptoms are bothersome for the affected women. In women with a history of hormone receptor-positive breast cancer, estrogen substitution against climacteric-like complaints is not recommended due to the supposable risk of hormone-induced tumor progression [1].

Among the treatment options against breast cancer recurrence, tamoxifen has long been the drug of first choice. AI are, however, gaining importance as first-line treatment for estrogen receptor (ER)-positive breast cancer due to the absence of endometrial side effects and a prolonged period of disease-free survival compared to tamoxifen [2]. Aromatase is responsible for the conversion of androgens, e.g., androstenedione, to estrone and of tes-

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tosterone to estradiol. It also represents the main source of estrogens in postmenopausal women and is thus a target for chemotherapy of estrogen-dependent breast cancer. Formestane is a member of the first generation of steroidal AI.

Patients treated with AI are frequently harassed by climacteric complaints like hot flushes, night sweats and sleep disturbance. The symptoms that are naturally occurring during menopausal transition are often triggered or augmented by the drug and patients urgently seek relief from this additional inconvenience [3]. As synthetic hormones are contraindicated in these cases of hormone-dependent breast cancer, plant-derived alternatives are gaining importance.

Extracts of the root of black cohosh (*Actaea/Cimicifuga racemosa*), a herb native to North America, are the most frequently used preparations and the unique isopropanolic extract iCR (Remifemin®) is the preparation which has been tested most profoundly in laboratory experiments and animal studies [4], [5], [6], [7], [8], [9] as well as in clinical trials [10], [11]. Its main characteristic compound groups are triterpene glycosides (TTG) and cinnamic acid esters (CAE), both of which possess structural similarities to steroids [12], [13]. The iCR at a daily dose of 60 mg herbal substance per kg body weight has been shown to exert bone protective effects [5] and to influence gene expression levels in rat uterus [9]. It has, however, been shown to be devoid of negative estrogen agonistic effects on hormone-dependent breast cancer *in vitro* [4] and *in vivo* [14] in an estrogen-free environment as well as in the presence of residual estrogenic substances.

This is strongly in contrast to data obtained with phytoestrogenic isoflavones, which exert their antiestrogenic effect only when residual estrogens are displaced from their receptors, but which show intrinsic estrogenic effects in the absence of other estrogens [5]. Consequently, comedication of tamoxifen and black cohosh has been shown to be safe. Even under highly competitive conditions, iCR does not antagonize tamoxifen's actions [6], whereas such an undesired interaction has been observed for phytoestrogenic isoflavones [15], [16].

In the well established rat model of ER-positive mammary cancer, tumors are chemically induced by intragastric administration of 7,12-dimethylbenz[*a*]anthracene (DMBA) [17], [18], [19], [20]. Neoplasias occur with a frequency of > 90%, have repeatedly been shown to be ER-positive and are sensitive to treatment with formestane [21], [22]. We chose two different, comparatively low doses of the AI for facilitating competitory interactions with iCR and in order to demonstrate dose-dependency of formestane effects on tumor kinetics or serum estradiol levels or both.

As AI are starting to replace tamoxifen as first-line prophylaxis against breast cancer recurrence, women who take black cohosh against their drug-induced menopause-like complaints are increasingly concerned about putative interactions between their two medications. Thus, we used the above animal model of chemically-induced mammary cancer to investigate whether interactions occur between two different low doses of a steroidal AI and a dose of iCR known to influence bone and uterus.

Materials and Methods

Animals and treatments

The rat model of DMBA-induced breast cancer has been validated in various settings and is sensitive to treatment with formestane [17], [18], [19], [20], [21], [22]. Fifty days after birth, 54 female Sprague-Dawley rats (Schaper & Bruemmer breeding colony; Salzgitter, Germany) received a single intragastric dose of 20 mg DMBA (Sigma Aldrich Chemie; Taufkirchen, Germany) dissolved in olive oil.

Rats were housed by treatment group, 3 animals per macrolon cage, in a room providing alternating 12-hour periods of light and dark. Diet and water were permitted *ad libitum*. All conditions of husbandry were in accordance with local regulations, and experimental procedures were approved and conducted under the auspices of a local German animal care and oversight committee. Following DMBA treatment, the test animals were monitored weekly for mammary tumor development.

First tumors were diagnosed at 35 days after DMBA application. Then, following tumor occurrence, the 54 female Sprague-Dawley rats were randomly allocated to the following treatment groups.

Group A (n = 14) was treated with 3.5 mg formestane/animal and iCR; group B (n = 13) was treated with 5.0 mg formestane/animal and iCR; group C (n = 13) was treated with 5.0 mg formestane/animal and isopropyl alcohol, and group D (n = 14) remained untreated.

Study drugs and administration

The iCR liquid extract originated from the validated manufacturing process for Remifemin® tablets and was provided by Schaper & Bruemmer GmbH & Co. KG (Salzgitter, Germany).

The concentration of the extract was 77.4 mg/mL in relation to the dry residue. The concentration of the total TTG in iCR, calculated as standard triterpene glycoside 26-deoxyactein ($C_{37}H_{56}O_{10}$; MW: 660.8), amounted to 27 mg/mL. Concentration of CAE was measured according to the calibration of isoferulic acid. In addition to the free acids caffeic, ferulic, and isoferulic acids, fukiic acid esters as well as piscidic acid esters were easily detectable. Of these, fukinolic acid showed the highest concentration (max. 0.3%) in iCR. Cimicifugic acids A (max. 0.15%), B (max. 0.25%), E and F were also frequently found in varying amounts. Based on the above method and composition, the total concentration of CAE was 3.7 mg/mL. The extract was prepared using 40% (v/v) isopropyl alcohol.

iCR was administered via the animals' drinking water in a dose that had previously been established to guarantee effective uptake of 60 mg herbal substance per kg body weight. In previous animal experiments, this dose had been shown to exert bone protective effects [5] and to influence gene expression levels in rat uterus [9]. The mixture was freshly prepared on a daily base. Formestane was obtained from Sigma Aldrich Chemie. The daily doses of 3.5 mg formestane/animal (group A) or 5.0 mg formestane/animal (groups B and C) were suspended in 100 μL olive oil and injected subcutaneously. Animals of group C received iso-

propyl alcohol at a dose corresponding to the alcohol intake of extract-treated animals of group B. Treatments were started on the day of tumor diagnosis.

Serum estradiol levels

14 days after the onset of experimental treatment, 4 randomly chosen animals of each group were anesthetized with enflurane, and 1 mL blood was drawn from the tail vein. Serum was obtained by centrifugation, and levels of 17-beta-estradiol were evaluated by a commercial laboratory according to standard procedures.

Clinical evaluation and necropsy findings

The test animals were monitored daily for general clinical symptoms and examined twice a week for tumor and body weight changes. Tumors were measured by calipers, and tumor area is given in mm^2 as calculated from two perpendicular diameters. Upon completion of 30 days of experimental treatment, the animals were sacrificed by CO_2-asphyxiation and exsanguination; individual animals that had shown signs of distress during the course of the experiment were killed prematurely. Tumor findings of these animals were handled according to the method of "last observation carried forward". Post-mortem examinations were carried out, and all organs were inspected macroscopically for abnormalities. Tumors were excised, weighed and evaluated microscopically.

Statistical methods

All comparisons were tested for statistical significance in Student's two-tailed t-test with a p-value level of significance of 0.05.

Results and Discussion

Mammary tumors occured between 35 and 78 days after DMBA application. This marked the beginning of the study, and the various treatments commenced. No statistically significant differences in the time of first tumor diagnosis were observed between groups, which was 54 ± 14 days after DMBA treatment in group A, 43 ± 12 days after DMBA treatment in group B, 53 ± 16 days after DMBA treatment in group C and 39 ± 12 days after DMBA treatment in group D. Initially, animals had an overall mean tumor area of approx. 200 mm^2 which, when unopposed, increased within 21 days up to 1400 mm^2 in the untreated animals of group D.

Aromatase is responsible for the conversion of androgens, e.g., androstenedione to estrone and testosterone to estradiol. In the DMBA rat model of breast cancer, formestane has a marked inhibitory effect on the number and size of tumors. Consequently, compared to the vehicle control, the three formestane test groups showed significantly retarded tumor growth of up to 700 mm^2 within 30 days (Fig. **1**). No significant differences in the kinetics of mammary tumor growth were observed between the three formestane test groups A, B and C. Irrespective of iCR coadministration, formestane (5.0 mg per day/animal) reduced tumor mass by > 50 %, whereas tumor number was reduced by 32 %.

Palpation results obtained during the phase of tumor growth were further corroborated by necropsy data. Thirty days after initial tumor diagnosis, the animals of group D showed a significantly greater tumor mass per animal and significantly larger individual tumors than animals of groups A, B and C. The number of tumors per animal was likewise reduced in the formestane treated animals of groups A, B and C. However, with p-values of 0.15 (A vs. D), 0.14 (B vs. D) or 0.12 (C vs. D) in Student's two-tailed t-test, it did not reach statistical significance (Table **1**).

No significant differences between group B and group C were observed while analyzing the necropsy parameters indicating that iCR did not antagonize or diminish the antitumoral effects of formestane. Significant differences between groups A and B were also not observed in necropsy data.

Formestane is well known to influence plasma levels of various sex hormones and their precursors (e.g., progesterone, testosterone, androstenedione, dehydro-epiandrosterone). We chose 17-beta-estradiol as marker substance for aromatase activity. As expected and as previously described [23], serum levels of 17-beta-estradiol were lower (32 – 36 %) in the groups treated daily with 5.0 mg formestane than in animals treated with the lower dose of 3.5 mg formestane or than in untreated controls. Whereas in

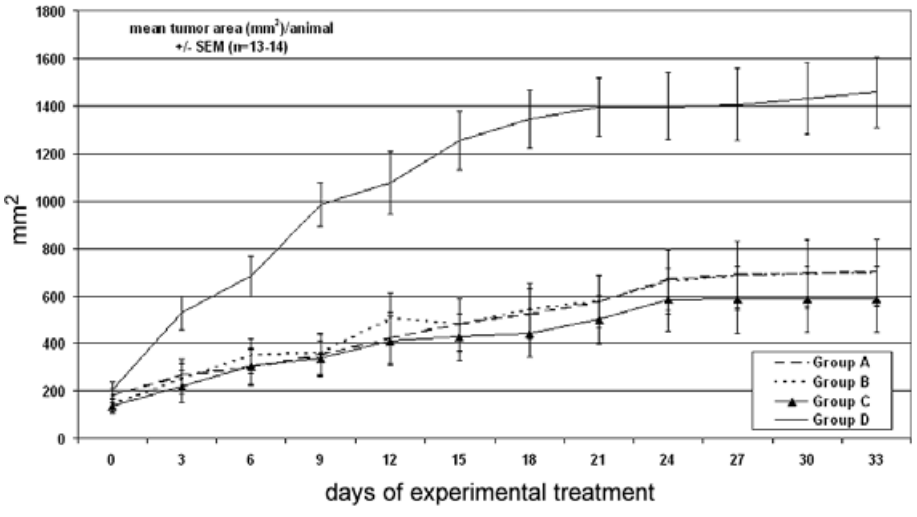

Fig. **1** Development of mammary tumors. Tumors were measured by calipers, and tumor area is given in mm^2. Treatment with formestane (groups A, B, C) reduced significantly (Student's two-tailed t-test) tumor growth as measured by tumor area compared to control treatment (group D). Treatment with iCR (groups A, B) had no impact on formestane-reduced tumor growth compared to formestane-only treated animals (group C).

Table **1** Mammary tumors at necropsy

Mean ± SEM	*Group A (3.5 mg FOR + iCR)*	*Group B (5.0 mg FOR + iCR)*	*Group C (5.0 mg FOR)*	*Group D (control)*
Tumor mass per animal (g)	2.0 ± 1.4	1.9 ± 1.2	1.9 ± 1.8	4.4 ± 2.8
Maximal tumor mass per animal (g)	1.1 ± 0.9	1.0 ± 0.6	1.1 ± 0.9	2.5 ± 2.0
Tumor number per animal	4.7 ± 3.7	4.9 ± 2.7	4.8 ± 2.8	6.9 ± 3.9

Compared to controls (group D), treatment with formestane (groups A, B, C) significantly reduced tumor growth. Animals of group D showed a significantly greater tumor mass ($p < 0.01$) per animal and significantly larger individual tumors ($p < 0.01$) than animals of groups A, B and C. The number of tumors per animal was likewise reduced in the formestane treated animals of groups A, B and C. With p-values of 0.15 (A vs. D), 0.14 (B vs. D) or 0.12 (C vs. D) in the Student's two-tailed t-test, however, it was not statistically significant. Treatment with iCR (groups A, B) did not interfere negatively with the formestane effect (group C).

untreated control animals, the mean value (± SEM) for estradiol was 170.8 (± 19.7) pmol/L, it was reduced to 108.7 (± 22.7) pmol/L in formestane treated (5.0 mg per day) animals of group C. In animals of group B, which had received formestane and iCR, mean estradiol levels of 116.7 (± 9.5) pmol/L were found (Fig. **2**). With p-values (Student's two-tailed t-test) of 0.08 (C vs. D) or 0.06 (B vs. D) respectively, none of these differences was, however, statistically significant. It is likely that the lower dose of formestane could not reduce 17-beta-estradiol levels compared to control animals, whereas the higher dose of formestane was closer to a threshold level of effectivity. On the other hand, serum estradiol levels have to be regarded as a rather variable parameter, as even a very high dose of twice daily 7.5 mg formestane per animal could not reduce serum estradiol levels in another experimental setting [21]. In a further experiment, serum levels and especially tumor tissue levels of, e. g., testosterone or dehydro-epiandrosterone should be investigated.

Steroidal AI are currently again in the focus of clinical research as a third-line therapy in metastatic breast cancer cases where non-steroidal AI have failed [24]. As they compete with androgens for the enzyme's substrate binding site, the potential of frequent co-medications to interfere with this binding should be investigated. We therefore tested two AI dosing regimens able to partially hinder tumor growth in the DMBA model. In parallel, we administered high doses of a black cohosh extract frequently used for alleviating menopausal symptoms and evaluated tumor growth and serum estrogen levels as parameters of unopposed formestane action.

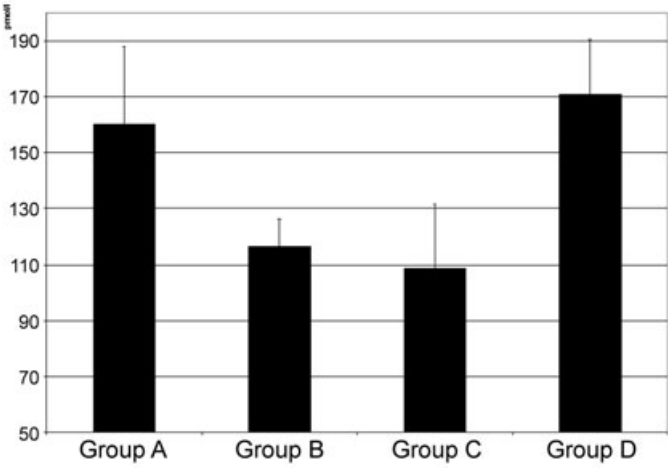

Fig. **2** Serum levels of 17-beta-estradiol. Whereas treatment with low dosed-formestane (group A) could not reduce 17-beta-estradiol levels compared to control animals (group D), a higher dose of formestane (groups B, C) markedly reduced estradiol levels. Concomitant treatment with iCR (group B) had no influence on the lower hormone levels compared to formestane-only treated animals (group C).

Unlike as for ER, where black cohosh competes with steroidal natural ligands [4], the interaction between aromatase enzyme and its steroidal inhibitor is neither hindered nor competed for by any of the compounds that are present in the black cohosh extract. Common structures of several extract compone classes resemble a steroidal conformation. Triterpenes, which in their glycosidic form are the substances most characteristic for *Actaea*, and cinnamic acid esters, the second most prevalent compound class, both possess structural similarities to steroids. As inhibition of proliferation and induction of apoptosis in the ER-positive cell line MCF-7 have been demonstrated, both compound classes have been made responsible for the interactions between black cohosh extracts and ER [4], [8].

We here show for the first time that in a relevant animal model of hormone-dependent cancer, the black cohosh extract iCR does not interfere with the beneficial effects of a steroidal AI. A high dose of iCR, which has been shown to influence bone quality [5] and uterine gene expression levels in rats [9], does not impede the AI, even if dosed sub-therapeutically. In conclusion, our results are in favor of comedication of AI with a long used herbal remedy, iCR, for the treatment of climacteric hot flushes and night sweats associated with AI treatment.

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