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Effects of bisphenol A, (-)-epigallocatechin-3-gallate, green tea, quercetin and rutin on the male reproductive tract function in rodents.

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Contents

Abstract

Over the last ten years Prostate Specific Antigen (PSA) based screening and heightened awareness has lead to a substantial increase in the number of men diagnosed with early stage, localized prostate cancer. Treatment of localized prostate cancer can be broadly divided into four categories: surgery, hormone therapy, radiation therapy or watchful waiting. With the recent success of the anti-androgen Bicalutamide 150mg (Casodex®), Early Prostate Cancer (EPC) Programme, many physicians are now encouraging their patients to add Casodex® to other standard therapies. These same patients are being exposed to information in the lay literature regarding the benefits and risks of dietary components like phytoestrogens (particularly green tea, soy products and cooked tomato products) as well as herbal extracts such as saw palmetto and PC-SPES (PC for Prostate Cancer; SPES lat. for Hope) and also xenoestrogens like bisphenol A (BPA).

A number of so-called xenobiotics, including pesticides (p,p'-DDT), plasticizers (BPA) and a variety of other industrial chemicals (polychlorinated biphenyls) contain a phenolic ring that mimics the A-ring of estradiol and have been reported to have hormonal or anti-hormonal activity. Although the level of exposure to these xenobiotics may be, if any, very low, they may exert their potential toxicity or endocrine disturbance in human beings and wildlife.

(Anti-)androgen like effects of environmental and nutritional compounds were evaluated using new immunohistochemical and morphometric methods. Therefore, orchiectomized Wistar rats (n=13) were treated s.c. with 1 mg/kg bw/day testosterone propionate (TP) for 7 days and compared to orchiectomized rats without TP substitution (OX) and to an untreated intact control group. Sections obtained from prostates and seminal vesicles were stained with polyclonal and monoclonal antibodies against the androgen receptor (AR) and assessed densitometrically (intensity of the immunoreaction) and morphometrically (epithelial height and luminal area). TP caused an enhancement of staining intensity and an increase in organ weights, epithelial height and luminal area. The use of proliferation markers (PCNA, MIB-5) showed also a highly significant increase of immunoreactive cells in TP-substituted orchiectomized rats compared with the OX group. Based on the present data, the densitometric analysis of AR-immunoreactivity as well as the assessment of proliferation markers, epithelial height and luminal area proved to be sensitive parameters for the evaluation of androgen effects on prostates and seminal vesicles. In further studies these parameters will be used to test several industrial xenoestrogens as well as phytoestrogens on their possible androgenic capacity.

Using this test methods, we evaluated (anti-)androgen like effects of BPA. Animals were treated p.o. either with vehicle or with 3, 50, 200, 500 mg/kg bw/day BPA (n=13) for seven days. One group was treated s.c. with 1 mg/kg bw/day TP. FL (3 mg/kg bw/day, p.o.) was

used to antagonize androgen effects of the suprapharmacological dose (500 mg/kg bw/day) of BPA. Androgen like effects of BPA on prostates and seminal vesicles were assessed by the Hershberger Assay, densitometric analysis of AR immunoreactivity, cell proliferation-index and a morphometric analysis. Absolute weights of prostates and seminal vesicles were not increased by BPA, whereas the relative weights were increased at higher doses of BPA, most likely due to a decrease in body weight indicating toxic effects (diarrhea, acidity) of this compound. Staining intensity for AR immunoreactivity was increased at low but not at higher doses of BPA in comparison to the orchiectomized rats. BPA at all doses tested did not cause an increase of the cell proliferation-index. Epithelial height and glandular luminal area were increased by low doses of BPA, whereas higher doses caused a decrease of these parameters.

While epidemiological, and molecular and cellular studies have shown that some of dietary components are associated with lower incidence of prostate cancer, there have been no studies that have examined the effects of these components on prostate cancer progression. Furthermore, the possible (adverse) effects of these dietary components on standard therapies, particularly hormone therapy, have not been examined. We are using the PC-346C orthotopic model of prostate cancer to examine the interactions between green tea consumption and hormone therapy in regulation of prostate tumor progression and metastasis. These experiments will serve as a paradigm for the study of the interactions of other nutrients, herbal extracts or natural products with standard therapy. These studies have the potential to impact on dietary recommendations for men diagnosed with early stage prostate cancer who contemplate standard therapies. We hypothesize that (-)-epigallocatechin-3-gallate (EGCG), green tea, quercetin and rutin consumption may alter the sensitivity of prostate tumor cells to hormone therapy, resulting in dysregulation of the mitotic and apoptotic pathways in early stage, hormone confined cancers, leading to the induction of hormone resistance and tumor progression. The flavonoids EGCG, quercetin, rutin are known *inter alia* as antioxidants common in green tea and apples. It was recently shown, that they may have significant growth inhibiting effects on human cancer cell lines and in tumor xenografts in different animals like mice and rats.

The primary catechine in green tea is the flavanol (-)-epigallocatechin-3-gallate — a polyphenolic compound with a flavonoid structure. Quercetin is a flavonoid that forms the “backbone” for many other flavonoids, including the citrus flavonoid rutin. Quercetin and rutin are flavonols.

EGCG (50μM) inhibited cell proliferation significantly after 24 hours of all mentioned cell lines. Human androgen-dependent LNCaP and human androgen-independent PC-3 cells were treated with EGCG (10μM, 25μM, 40μM, 50μM, 100μM) for 2h, 4h, 6h, 12h, 24h, 36h. Adherent cells remaining after treatment were detected by Crystal Violet Assay (CV). 10μM

EGCG inhibited cell growth in LNCaP cell line significantly after 2h compared to vehicle control (Ethanol). 50µM of EGCG inhibited cell growth in PC-3 cells after 2h significantly. Androgen-dependent PC-346C cells were treated with EGCG (10µM, 25µM, 40µM, 50µM, 100µM) for 2h, 4h, 6h, 12h, 24h and 36h). AR antagonists like Casodex® and 50µM EGCG inhibited cell growth in human PC-346C cell line significantly after 24 hours. Higher concentrations and longer exposure of EGCG led to a dose-dependent PC cell death in all treated groups in the used cell lines. Cell cycle data showed significant S-phase arrest in all cell lines used. EGCG induced significant apoptosis in human PC-346C and LNCaP cells.

Furthermore, we inoculated nude mice with human PC-346C and PC-3 cells and treated them with green tea for 6 weeks. Preliminary data showed a significant reduction of tumor weights after 6 weeks. A combination treatment with green tea and Casodex also led to a significant reduction of human PC-346C tumors. Taken together, these findings indicate, that EGCG and green tea respectively inhibit PC cell growth by inducing apoptosis in human LNCaP, PC-346C and PC-3 cells *in vitro* as well as *in vivo* as a function of dose and time. Similar data could be observed for quercetin using four human cancer cell lines. In spite of chemical relation to EGCG and quercetin, rutin showed no effects on cancer cell lines.

These findings indicate the potential of dietary compounds or even xenoestrogens as mild agents for PC prevention and/or even treatment.

Zusammenfassung

Aufgrund von PSA (Prostate Specific Antigen) basiertem Screening und gleichzeitig höherem Risikobewusstsein ist die Anzahl der Patienten mit lokalisiertem Prostatakrebs im Frühstadium in den letzten 10 Jahren stark angestiegen. Behandlung von lokalisiertem Prostatakarzinom kann im Wesentlichen in vier Kategorien eingeteilt werden: OP, Hormontherapie, Strahlungstherapie und Beobachtung. Der Behandlungserfolg mit dem Antiandrogen Bicalutamid 150mg (Casodex®), Early Prostate Cancer (EPC) Programm, bei Prostatakrebs im Frühstadium, führte von vielen Ärzten zu vermehrten Empfehlungen von Kombinationsbehandlungen (Casodex®) mit den bekannten Standardtherapien. Diese Patienten werden durch diverse Informationsquellen auf Risiken und mögliche Vorteile von Phytoöstrogenen (Grüner Tee, Sojaprodukte, Tomatenprodukte) als auch Kräuterextrakte wie Palmettoextrakt und PC-SPES (PC für Prostatakrebs; SPES für Hoffnung) und auch Xenoöstrogenen wie Bisphenol A (BPA) hingewiesen.

Eine Reihe sogenannter „Xenobiotika“ wie Pestizide (p,p’-DDT), Weichmacher (BPA) und viele andere Industriechemikalien (polychlorierte Biphenyle) beinhalten einen phenolischen Ring, welcher den bekannten A-Ring vom weiblichen Geschlechtshormon Östradiol zu imitieren vermögen und bekannt sind für ihre Fähigkeit hormonelle und anti-hormonelle Aktivität zu entfalten. Auch wenn der Grad der Exposition solcher „Xenobiotika“ sehr gering erscheinen mag, könnten sie ihre potentielle Toxizität oder ihr endokrines Störpotential im Menschen und in Tieren ausüben.

(Anti-)androgen ähnliche Effekte von Umwelthormonen (Xeno- und Phytoöstrogene) und hormonell aktiven Nährstoffen, sind mit neuen immunhistochemischen und morphologischen Methoden untersucht worden. Orchiektomierte Wistar Ratten (n=13) wurden s.c. mit 1 mg/kg KG/Tag Testosteronpropionat (TP) über 7 Tage behandelt und mit orchiektomierten Ratten ohne TP Behandlung (OX) und einer unbehandelten intakten Gruppe verglichen. Immunhistochemische Schnitte der Prostata und der Samenblase wurden mit polyklonalen und monoklonalen anti-AR (anti-Androgen Rezeptor) Antikörpern gefärbt und densitometrisch (Intensität der Immunreaktion) und morphometrisch (Epithelhöhe und Lumenfläche) vermessen. TP führte zu einer Steigerung der Farbintensität und der Organgewichte, der Epithelhöhe und der Lumenfläche. Die Anwendung von Proliferationsmarkern wie (PCNA, MIB-5) zeigte ebenfalls eine hoch signifikante Steigerung von immunreaktiven Zellen in TP behandelten orchiektomierten Ratten im Vergleich zur unbehandelten OX Gruppe. Basierend auf diesen Daten, erwiesen sich sowohl die densitometrische Analyse der AR-Immunreaktivität als auch Abschätzung der Proliferationsmarker, Epithelhöhe und der Lumenfläche als zuverlässige Parameter bei der Evaluation von androgenen Effekten auf die Prostata und der Samenblase. In weiteren Studien werden diese Parameter genutzt, um

einige industrielle Xenoöstrogene als auch Phytoöstrogene auf ihre mögliche androgene Kapazität hin zu testen.

Zur Untersuchung (anti-)androgen ähnlicher Effekte von BPA wurden, unter Anwendung obiger Testparameter, orchiektomierte Wistar Ratten (n=13 pro Gruppe) mit 3, 50, 200, 500 mg/kg KG/Tag über 7 Tage behandelt. Eine Gruppe wurde mit 1 mg/kg KG/Tag TP behandelt. FL (3 mg/kg KG/Tag) wurde gegeben, um mögliche androgene Effekte der suprapharmakologischen Dosierungen (500 mg/kg KG/Tag) von BPA zu antagonisieren. Neben dem industriell validierten Hershberger Assay wurden densitometrische Auswertung der Immunreaktivität (AR), Proliferationsindex und morphometrische Analyse angewandt. Absolute Gewichte von Prostata und Samenblase wurden durch BPA nicht verändert. Relative Gewichte waren aufgrund der Abnahme des Körpergewichts (toxische Effekte wie Diarrhö und Übersäuerung) leicht erhöht. Farbintensität der AR-Immunreaktivität und Epithelhöhe und Lumenfläche waren bei niedrigen Dosierungen von BPA leicht erhöht. Höhere Dosierungen von BPA führten im Gegensatz zu einer Erniedrigung aller Werte im Vergleich zu den orchiektomierten unbehandelten Tieren. Der Proliferations-Index wurde in allen getesteten Dosierungen von BPA nicht beeinflusst. Epithelhöhe und glanduläre Lumenfläche waren erhöht bei den niedrigen Dosierungen von BPA, wobei die höheren Dosierungen zu einer Abnahme dieser Parameter führten.

Während epidemiologische, molekularbiologische und zelluläre Studien aufzeigen, dass Nahrungsinhaltsstoffe mit einer geringeren Häufigkeit von Prostatakrebs in Verbindung gebracht werden, gibt es keine Daten die die Effekte dieser Substanzen auf das Fortschreiten des Prostatakrebs aufzeigen. Weiterhin sind die möglichen nachteiligen Effekte solcher Substanzen auf Standardtherapien, speziell der Hormontherapie, vollkommen unklar. Zur Untersuchung der Interaktion zwischen grünem Tee Konsum und der Hormontherapie auf den Prostatakrebs (PC) benutzen wir ein PC-346C orthotopisches Tiermodell des PC. Diese Studie soll Vorreiter für weitere Studien zur Untersuchung weiterer Xeno- und Phytoöstrogene und deren Einfluss auf den PC sein. Diese geplanten Studien werden Ernährungsempfehlungen für Patienten mit PC im Frühstadium hervorbringen. Wir nehmen an, dass EGCG, grüner Tee, Quercetin und Rutin die Sensitivität der Prostata Krebszellen auf die Hormontherapie zu verändern vermag, resultierend in einer Dysregulation der mitotischen und apoptotischen Signalwege des sich im Frühstadium befindlichen Hormon abhängigen Krebs', welche zu einer Induzierung der Hormonresistenz und des Tumorwachstums führen. Die Flavonoide EGCG, Quercetin und Rutin sind unter anderem als Antioxidantien bekannt und in grünem Tee und Äpfeln vertreten. Es konnte kürzlich gezeigt werden, dass diese Substanzen einen signifikant wachstumshemmenden Einfluss auf humane Krebszelllinien und humane Tumore in Mäusen und Ratten haben.

Das primäre Katechin in grünem Tee ist das Flavonol (-)-Epigallocatechin-3-Gallat - eine polyphenolische Komponente mit einer Flavonoidstruktur. Quercetin ist ein Flavonoid, welches als Grundgerüst vieler anderer Flavonoide wie dem Citrus-Flavonoid Rutin dient. Quercetin und Rutin sind Flavonole.

EGCG (50µM) hemmte in unseren Untersuchungen die Proliferation in allen getesteten PC-Zelllinien nach 24 Stunden signifikant. Humane androgen-abhängige LNCaP und humane androgen-unabhängige PC-3 Zellen wurden mit EGCG (10, 25, 40, 50 und 100µM) über 2, 4, 6, 12, 24 und 36 Stunden behandelt. Adhärente Zellen nach der Behandlung wurden mit dem Kristallviolett Assay (CV) detektiert. 10µM EGCG hemmte die Proliferation der LNCaP Zellen nach 2 Stunden signifikant im Vergleich zur Ethanol Kontrolle. In PC-3 Zellen wurde das Zellwachstum nach 2 Stunden in einer Dosierung von 50µM EGCG signifikant gehemmt. Androgen-abhängige PC-346C Zellen wurden mit EGCG (10, 25, 40, 50 und 100µM) über 2, 4, 6, 12, 24 und 36 Stunden behandelt. AR-Antagonisten wie Casodex® und 50µM EGCG hemmten das Wachstum von humanen PC-346C Zellen signifikant nach 24 Stunden. Höhere Konzentrationen und längere Einwirkdauer von EGCG führte zu einem dosis-abhängigen PC Zelltod in allen behandelten Gruppen der genutzten Zelllinien. Zellzyklusdaten zeigten einen signifikanten S-Phasenarrest in allen genutzten PC-Zelllinien. EGCG verursachte weiterhin eine signifikante Anzahl apoptotischer Zellen in humanen PC-346C und LNCaP Zellen.

Aufgrund dieser Datenlage injizierten wir orthotopisch immundefizienten Nacktmäusen humane PC-346C und PC-3 Zellen und behandelten sie über 6 Wochen mit grünem Tee. Vorläufige Daten zeigten eine signifikante Reduzierung des Tumorgewichts nach 6 Wochen. Eine Kombinationsbehandlung von grünem Tee mit Casodex® führte ebenfalls zu einer deutliche Reduzierung der absoluten PC-346C Tumorgewichte. Zusammengefasst zeigen unsere Daten, dass EGCG beziehungsweise grüner Tee das Wachstum von PC-Zellen signifikant über u.a. Apoptose in LNCaP, PC-346C und PC-3 Zellen *in vitro* als auch *in vivo* dosis- und zeitabhängig hemmt. Für Quercetin konnten weitestgehend ähnliche Daten erhoben werden. Trotz der chemischen Relation von Rutin im Vergleich zu Quercetin und EGCG, zeigte Rutin keinerlei die Proliferation von PC-Zellen hemmende Eigenschaften.

Unsere Untersuchungen könnten das Potential von Nahrungsinhaltsstoffen oder auch Xenoöstrogenen in Bezug auf Prostatakrebs Prävention und/oder gar Behandlung in Zukunft deutlicher aufzeigen.

Introduction

Interaction between Dietary and Natural Products with Conventional Therapy for Prostate Cancer

Prostate cancer now is the second most commonly diagnosed male cancer in many western countries after lung cancer [1]. The major risk factors for prostate cancer include age and race, and the consumption of a high fat diet. Epidemiological studies have identified several additional components of the diet that appear to be protective, including consumption of green tea [2], which appear to exert its effect through its action as an antioxidant.

The main cause of death from prostate cancer is the invasion and metastasis of prostate cancer to the bone, liver and brain. However, for many men the disease will remain localized and slow growing, and extensive PSA screening programs have lead to the increased identification of early stage tumors in younger men. Approximately 70% of these tumors are indolent and will not need treatment during the patients life time [3]. Unfortunately at present there is no way to distinguish between aggressive, clinically significant tumors that need to be treated and indolent tumors. As a result, many patients are treated more aggressively than is necessary. In the case of most patients, anti-androgen therapy will be the first choice.

While the mechanism of anti-androgens such as Flutamide® and Casodex® have been studied extensively, particularly as it relates to the treatment of advanced disease, the ramifications of long term use for organ confined disease or chemoprevention and the effects of diet on the effectiveness of the drug have not been investigated. Anti-androgens induce prostate tumor regression by initiating cell death, or apoptosis [4]. *In vitro*, anti-androgens induce apoptosis in most androgen-dependent epithelial cells, however a small population fail to die and become resistant to anti-androgen therapy. This phenomenon is reflected in the clinic, where resistance to anti-androgens eventually develops in nearly all patients. The mechanisms underlying this progression of prostate cancer, and the influence of diet on this process have not been investigated.

Bisphenol A (BPA)

Is BPA an anti-androgen or androgen?

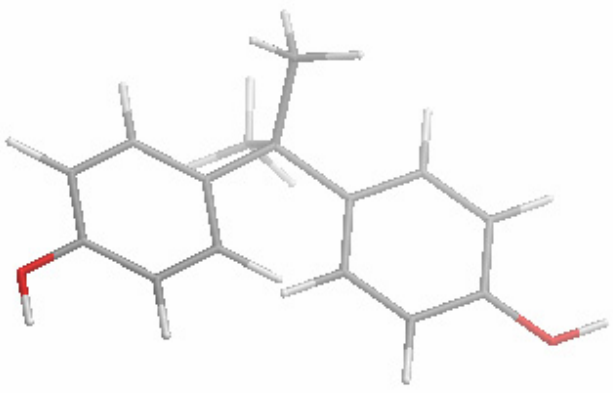
We were interested to ask this question, since the endocrine profile of BPA may lead to the development of a new therapeutic for PC. On the other hand, it is also important to evaluate the hormonal properties of BPA, it may exert its potential toxicity or endocrine disturbance in human beings and wildlife, although the level of exposure to this xenobiotics may be, if any, very low.

Recent *in vitro* studies demonstrate, in fact, that xenobiotics can bind with estrogen receptors and activate them, resulting in gene expression [5-7]. BPA slightly induced MCF-7 cell proliferation at a level of 0.1µM and maximum proliferation at 10µM [7]. Also in *in vivo* studies, BPA showed estrogenic activity. Plasma free testosterone levels were dramatically decreased following eight weeks of BPA treatment [8]. It was claimed by vom Saal that, the exposure of pregnant mice to extremely low concentrations of certain xenobiotics, for instance, results in offspring with lower sperm production, increased prostate size or alters maternal behaviour, postnatal growth rate and reproductive function in female mice [9-12]. Other work groups, in contrast, found an uterotrophic response (increase in uterine wet weight) at doses up to 100 mg/kg BPA for 3 days [13-15].

Also Gupta [16] claimed an enhancement of the anogenital distance and the prostate size of fetuses, when pregnant CD-1 mice were treated with BPA in the microgram range per kg BW/day. BPA induces inappropriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells (LNCaP). Takao reported a significant decrease in plasma free testosterone levels at 50µg BPA / ml in drinking water (14mg/kg BW/day). No significant effect (although a trend in the same direction) was observed neither after 4 weeks of exposure nor after 4 and 8 weeks exposure to 5µg of BPA / ml drinking water (0,14mg/kg BW/day) [8].

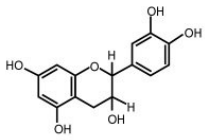
In addition, Kim et al. did not detect any androgenic or anti-androgenic activities of BPA in Hershberger Assay [17]: the BPA doses used were 10 - 1000mg/kg BW/day.

Evaluation of (anti-)androgen like effects of xenoestrogens in regard to usually androgen-dependent early prostate cancer growth and progression using standard Hershberger Assay with new immunohistochemical and morphometric methods. Additional parameters such as morphometric and qualitative data are required to determine the (anti-)androgenicity of a given substance, in particular if the expected effects are of lower degree (xenoestrogens like BPA). The present study was carried out to clarify the (anti-)androgenic potential of BPA in a broad dose range from “ultralow”, “pharmacological” to “suprapharmacological” in rats using the standard Hershberger Assay with additionally androgen sensitive parameters.



Bisphenol A (BPA)

Dietary Modifiers of Prostate Cancer



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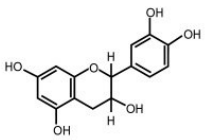

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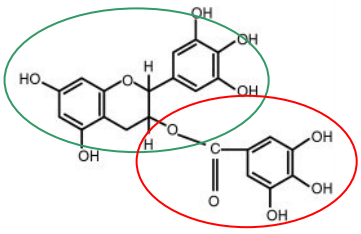

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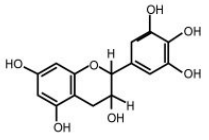

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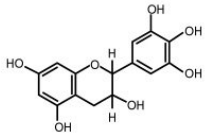

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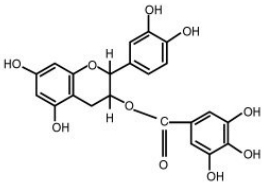

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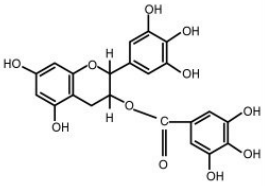


(-) Epigallocatechin gallate

In the last ten years a number of well designed epidemiological studies have shown that consumption of green tea [2], tomato products [18,19] and soy [20] may be associated with a lower risk of prostate cancer. The beneficial effects of these diets have been attributed to the polyphenols, particularly (-)epigallocatechin-3-gallate (EGCG), in green tea [2,21,22], the isoflavones (genistein, daidzein and biochanin A) in soy; and the carotenoids (lycopene) in cooked tomato products [23].

Green tea, the dietary component contains a number of polyphenols which have been tested alone and in combination in a variety of *in vitro* model systems of prostate cancer. The most active ingredient is EGCG, which induces cell cycle arrest and apoptosis *in vitro* in several of the commonly used human prostate cancer cell lines, including PC-346C, LNCaP, PC-3 and DU-145 cells [24,25]. The mechanism of action of EGCG is still under active investigation, and several different mechanisms have been proposed including the inhibition of 5α-reductase [26,27] and down-regulation of the androgen-receptor AR [28], both of which have the potential to limit the androgen mediated stimulation of tumor growth. In addition administration of EGCG also it is clear that as well as the modulation of ornithine decarboxylase [21] and anti-oxidant enzymes such as catalase, manganese superoxide dismutase, glutathione peroxidase and glutathione-S-transferase [29,30]. *In vitro* analysis has shown that in PC-346C, LNCaP, PC-3 and DU-145 cells treatment with purified EGCG or genistein modulates cell cycle arrest through the upregulation of p21/WAF-1 and p27/KIP-1 and their increased binding to CDK4 and CDK2, cyclin D1 and cyclin E a process that induces G_0/G_1 cell cycle arrest and apoptotic cell death [24,31], possibly through the suppression of Erb B1-Shc-ERK1/2 signaling [32]. In addition the expression of a number of other genes including the

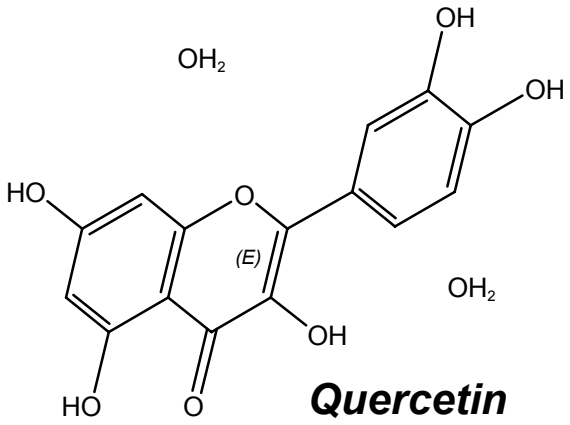
apoptosis inhibitor, surviving, DNA topoisomerase II, CDC6 and mitogen-activated protein kinase is downregulated [30]. Furthermore the ability of EGCG to induce these effects appears to be independent of the androgen receptor or p53 status of the cells [24]. Similar findings have been reported for genistein and other soy isoflavones, suggesting that there may be a common pathway that modulates the effects of diverse anti-oxidants in prostate tumors [33].

While there have been a limited number of animal studies examining the efficacy of green tea as a chemopreventive agent for prostate cancer, it has been demonstrated that oral infusion of green tea polyphenols resulted in a significant delay in primary tumor incidence and tumor burden in TRAMP mice [34], and our own results suggest that consumption of green tea significantly decreases the incidence and progression of urogenital tumors in the LOBUND-Wistar model system. Whether this decrease is mediated by the changes intracellular signaling outlined above, or due to the effects of green teas on peripheral tissues has not been fully evaluated. There is considerable evidence that green tea or its constituents have significant effects on several important peripheral tissues. It has been shown that administration of EGCG induces increases in LH and testosterone levels in rats treated for 8 weeks [35], and alters testosterone, IGF-1 and food intake in nude mice [36]. These changes in the endocrine status induced by parenteral administration of EGCG may have profound impact on the growth inhibition and regression of human prostate. In addition EGCG has been shown to induce several hepatic P450 enzymes including P450 1A1, 1A2 and 2B1 as well as glucuronosyl transferase [37]. These enzymes are involved in the metabolic clearance of steroids such as 3α- and 3β-androstanediol, the major catabolic intermediates of 5α-DHT as well as anti-androgens such as Casodex®. Thus not only does green tea have the potential to decrease serum testosterone levels, but it may lead to more rapid metabolism and conjugation of the anti-androgen, resulting in a substantially lower effective dose of Casodex®.

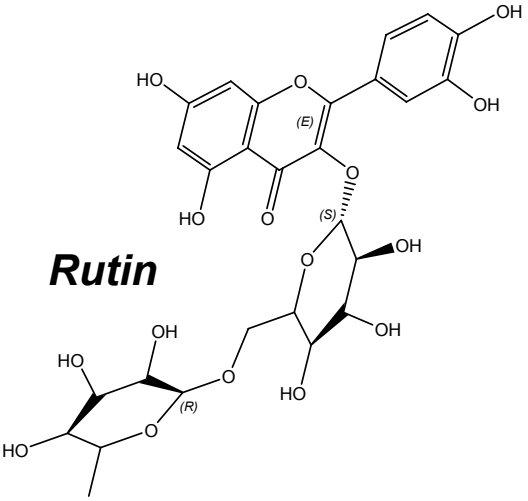
Interactions Between Standard Therapies and Dietary Modifiers

The dramatic increase in the number of men being diagnosed with early stage prostate cancer, and with the increased popularity of dietary supplements it is entirely likely that many men being treated with standard therapies will also be taking unregulated dietary supplements which alter the cell biology of prostate cancer cells and may influence the clinical course of the disease and response to therapy. While the studies outlined above and in the preliminary data suggest that green tea, lycopene and other dietary supplements may delay the initiation of prostate cancer no studies have been performed to study the effects of these compounds on tumor progression or to establish whether they interact with standard interventions for early stage prostate cancer (either radiation or hormone therapy). These questions could have considerable clinical implications since many men are self medicating with over the counter

formulations of green tea and green tea extracts, lycopene (in the form of One-a-Day Vitamins plus lycopene), saw palmetto (for general prostate health), as well as more controversial formulations such as PC-SPES.



Quercetin



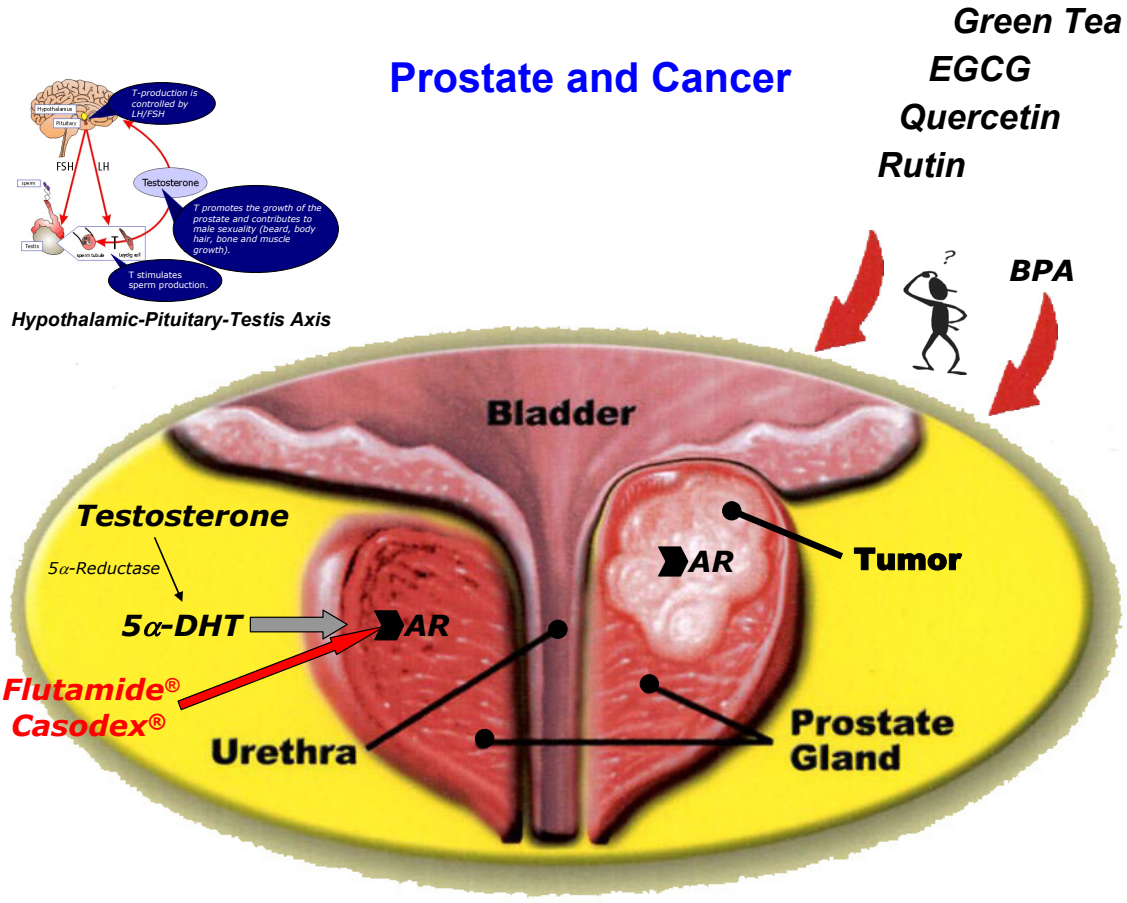
Rutin

Animal Model Systems

The investigation of androgen action *in vivo* has relied on two complimentary approaches: autochthonous model systems and xenografts of human prostate tumors into immunocompromised mice. There are strengths and weaknesses to both systems that are briefly outlined below (Results and Discussion). Autochthonous models offer the major advantage that they can be used to study the initiation of prostatic disease, while xenograft models are most useful for studies related to tumor progression, but have the added advantage that the observations are made in human, rather than rodent tissues thus obviating the problems associated with species specific responses.

Aim of the Study

The aim of this study was to evaluate (anti-)androgen like effects of the xenoestrogen BPA using standard Hershberger Assay with new immunohistochemical and morphometric methods. Additional parameters such as morphometric and qualitative data are required to determine the (anti-)androgenicity of a given substance, in particular if the expected effects are of lower degree (xenoestrogens like bisphenol A) and phytoestrogens and dietary compounds like green tea, EGCG, quercetin and rutin in regard to usually androgen-dependent EPC growth and progression. The major outcomes of these studies will be an estimation of the impact of xeno- and phytoestrogens on the growth and progression of human prostate cancer and prostate tumor sensitivity to the anti-androgens Flutamide® and Casodex®. These studies will determine whether these compounds interfere with the effects of the mentioned anti-androgens in the initiation of apoptosis in androgen sensitive prostate cancer cells, either by altering the redox potential in the cancer cells or by altering the metabolism of Flutamide® and Casodex® in peripheral tissues.



Material and Methods

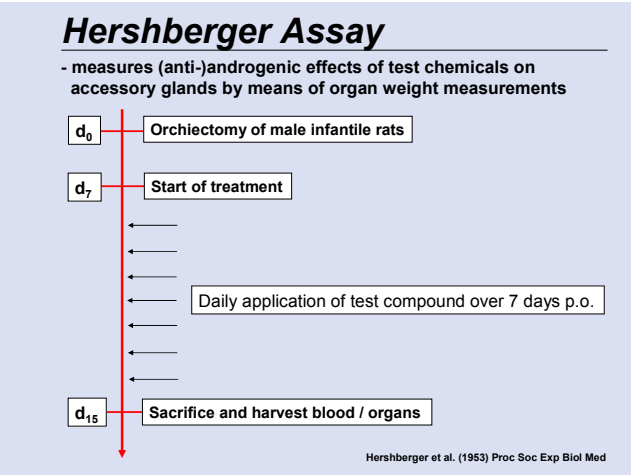
3.1 Animals and housing

Wistar rats, weighing about 150g were separated into different groups by randomized procedure. They received tap water and laboratory standard rat diet *ad libitum*. Groups of two to four animals were kept in Makrolon cages type IV with bedding at 22°C, a relative humidity of 30-70% and artificial 24h light. After acclimatization, animals were orchiectomized [*Nishino et al. Annals of Anatomy 2004*].

3.2 Treatment of animals

Hershberger Assay

The rodent Hershberger Assay using castrated peripubertal male rats measures the androgenic or anti-androgenic effects of test chemicals on several accessory glands/tissues (e.g. ventral and dorso-lateral prostate and seminal vesicles with coagulating glands) by means of organ weight measurements [38]. Generally, accessory sex glands and tissues are dependent upon androgen stimulation to gain and maintain weight during or after puberty. If endogenous testicular sources of androgens are necessary to increase or maintain the weights of these tissues [17,39-40]. All anabolic / (anti-)androgenic compounds (drugs) in clinical use have been screened by means of this in vivo bioassay [41,42]. This assay is one of the assays in the proposed Tier I screening battery by EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee). Tier I sites are those that have greater potential to require long-term or emergency cleanup work under the Federal Superfund program. These are sites which have a release of a hazardous substance, pollutant or contaminant that has caused, or is likely to cause exposure to humans or contamination of a sensitive environment. They typically involve contamination of drinking water, surface water, air or soils which have either caused or are likely to cause exposure to nearby populations, or have contaminated, or are likely to contaminate sensitive environments such as for example wetlands, national parks and habitats of endangered species [43].



However, the measurements of body and organ weight deliver only a limited amount of information, especially if this assay will be used to carry out a more subtle assessment of the androgenic properties of an unknown substance. Additional parameters such as morphometric and qualitative data are therefore required to determine the androgenicity of a given substance, in particular if the effects are of low degree.Seven days after orchiectomy, animals were partitioned into three groups (n=13 in each group). Two groups were orchiectomized. One group was treated subcutaneously with testosterone propionate (TP) in arachis oil for seven days and compared to vehicle treated, orchiectomized rats without TP substitution (OX) and to a vehicle treated intact control group (intact). After the treatment animals were sacrificed by decapitation. Seminal vesicles and prostates were harvested surgically, weighed and immediately fixed in 4% neutral buffered paraformaldehyde [Nishino et al. Annals of Anatomy 2004].

Seven days after orchiectomy animals were partitioned into eight groups (n=13 in each group). They were treated p.o. with 3, 50, 200 and 500mg BPA/kg BW/day dissolved in propylene glycol for 7 days. BPA was purchased from Fa. Bayer (PtNr. 97.001/Prod.Nr. 04111095, CasNr. 80-05, Leverkusen, Germany). Another group of orchiectomized animals

were treated s.c. with TP 1mg/kg BW/day in arachis oil. FL 3mg/kg BW/day p.o. in combination with 500mg BPA was used to antagonize possible androgen like effects of the "suprapharmacological" dose of BPA. These groups were compared to vehicle (propylene glycol) treated, orchiectomized rats without any other substitution (OX) and to vehicle treated intact control group (intact). Propylene glycol was purchased from Merck, Darmstadt, Germany. TP and FL were kindly provided by Schering AG, Berlin, Germany. After the treatment animals were sacrificed by decapitation and seminal vesicles and prostates were harvested surgically, weighed and immediately fixed in 4% neutral buffered paraformaldehyde for 24h [*Nishino et al. Journal of Steroid Biochemistry & Molecular Biology 2006*].

3.3 Immunohistochemistry

After fixation the specimens (prostate, seminal vesicle) were dehydrated in ascending series of alcohol, embedded in paraffin and cut in sections of 5μm thickness (10 sections per specimen). For the immunohistochemical visualization of androgen receptor and proliferation markers the following primary antibodies were applied: anti-androgen-receptor (1:100, sc-815, rabbit polyclonal, Santa Cruz Biotechnology, California, USA), anti-androgen receptor (1:100, 554224, mouse monoclonal, BD PharMingen, Germany), anti-PCNA (1:200, PC-10, mouse monoclonal, Novocastra, New Castle, United Kingdom) and anti-MIB-5 (1:100, M 7248 mouse monoclonal, DakoCytomation, Denmark).

After deparaffinization in xylene and descending series of alcohol the sections were treated in 0.3% hydrogen peroxide in distilled water to quench endogenous peroxidase activity, rinsed 2 times in 0.01M phosphate buffered saline (PBS) containing 0.1% Triton X-100 (PBS-TX), incubated for 10 minutes in 10% normal goat serum (NGS) in PBS-TX to block non-specific antigen-binding sites, incubated with the above mentioned primary antibodies diluted in PBS containing 1% NGS overnight, rinsed in PBS, incubated with biotinylated secondary antibodies (1:200, Dako, Denmark) for 1h, rinsed in PBS and incubated with StreptAvidin-Biotin (SAB)-Complex (Biocare, Medical, USA) conjugated to horseradish peroxidase for 1h. Peroxidase activity was revealed with the chromogen 3-amino-9-ethyl-carbazole (AEC, Zymed, USA) for 2 minutes. Sections stained with proliferation markers were counterstained with Mayer's hematoxylin for 10 minutes. Omission of primary antibodies resulted in the absence of a specific labeling.

3.4 Morphometry and Densitometry

Intensity of immunohistochemical staining was determined densitometrically, while epithelial height and luminal area of the glandular ducts were measured morphometrically. Microscopy was performed with an Axiophot light microscope equipped with a high resolution scanner camera.

All images have a uniform size of 1300x1030 pixel. Since the images were generated by using a 20x objective and 1.0 Optovar the final resolution of the edge lengths of one pixel in the resulting image is 0.32μm. This resolution was adequate for deciding which profile of glandular ductus in the field of vision is suitable for densitometric measurements. By using densitometry, gray values were transformed pixel by pixel into optical densities [44], followed by calculation of the sum, mean and standard deviation. Densitometry was performed in gray level images with a dynamic range of 256 intensities. Each pixel had a value between 0 and 255, where 0 is darkest (black) and 255 is lightest (white) [*Nishino et al. Annals of Anatomy 2004*].

The glandular duct to be measured was centered into the optical field at the resolution mentioned above. After focusing and adaption of light intensity (3200K) the section was removed and a white image (w) was generated iteratively. The adaption of light intensity was carried out by the following procedure. Firstly the illumination was adjusted, so that the objects of interest could be clearly identified. Thereafter, an image without any histologic object was grabbed and the mean pixel intensity was calculated. If the mean pixel intensity differed much from the pixel intensity obtained from preceding measurements, the light intensity was adapted until the mean pixel intensity adapted similar values to those in the previous measurements. The mean gray value of the white image was determined for each section in order to obtain the same illumination condition within each specimen examined. After white image calibration the glandular duct of interest was grabbed and its immunoreactive epithelium was surrounded interactively. Within this defined epithelial area the mean optical density (OD) and the variance of OD were determined. Five measurements were performed within each section. Five sections were examined per animal resulting in 25 measurements for each animal. The mean values were calculated and compared between the different groups.

The epithelial height was determined by using a 40x objective and 1.0 Optovar. For the determination of the luminal area a 10x objective and 1.0 Optovar were used. The quantitative assessment of proliferating cells was performed using a 40x objective and 1.0 Optovar. We have counted 1000 of those cells per section exhibiting nuclei. A two-sided t-test at a significance level of $p<0.05$ was applied for statistical comparison. Data were depicted as means ± standard deviation (SD).

Standard Operating Procedures for Handling Nude Mice

Purpose

Nude and other immunocompromised mice are at greater risk than conventional mice for the development of infectious disease. Frequently, infectious pathogens are transferred to nude mice by contaminated equipment or exposure to contaminated environments. The procedures described here are implemented to minimize the risk of infectious disease to nude mice:

General Procedure

All equipment and caging is autoclaved prior to using with nude mice. The night prior to manipulating the mice under the laminar flow hood, the hood is sprayed with 70% ethanol with the blower on overnight. After each use, the hood is disinfected with Sani-plex 128 spray and again sprayed with the 70% ethanol. The hood blower is left on at all times when mice are present. The light is turned off when the hood is not in use. All individuals handling the nude mice must wear a steam sterilized gown, hair bonnet, and sterile gloves. Two individuals are present during the handling of the mice, one that is designated as the animal handler and maintains sterility, while the other handles the non-sterile items and opens the sterile materials for the handler. All individuals present when the mice are handled must minimally wear a bonnet and mask. Any persons reaching into the hood must wear a clean gown or smock and gloves to prevent contamination of the sterile interior. Aseptic technique should be used at all times when handling the nude mice. Any objects or surfaces that will come in contact with the mice should have been sterilized by steam, UV radiation, or contact with 70% ethanol for at least 30 minutes.

Unpacking Newly Arrived Mice

Shipping containers are sprayed with a choline dioxide solution before they are placed in the hood. The designated animal handler opens the sterile food and water under the hood. The non-sterile person opens the cage and shipping box under the hood. Care must be taken by the non-sterile person to avoid contact with the inner surfaces of the hood and the inside of the shipping box. The animal handler then adds food and water to the cage top and removes the mice from the shipping box and places them in the cage. Unused water bottles are resealed in the container and will be stored in the room for future use. Mice will undergo a two week acclimation period prior to experimental use.

Cage changing

The designated animal handler should re-glove after every 6 – 10 cages or if gloves are torn or contaminated or if a cage has ill animals. Any cages with animals appearing sick will be placed at the end of the cage changing order to avoid possible exposure of healthy animals to

pathogens or contaminants. The mouse cages are changed at least once weekly or more often, if deemed necessary. If food and or water needs to be added between the scheduled cage change, it must be done aseptically under the prepared hood.

Routine Inspection of the Mice

All animals will be checked daily, including on the weekends and holidays. Examine the mice by viewing them through the cage. The micro-isolator top should not be opened. NEVER open the top outside the sterile hood.

3.5 Experimental Design: Green Tea and Prostate Cancer

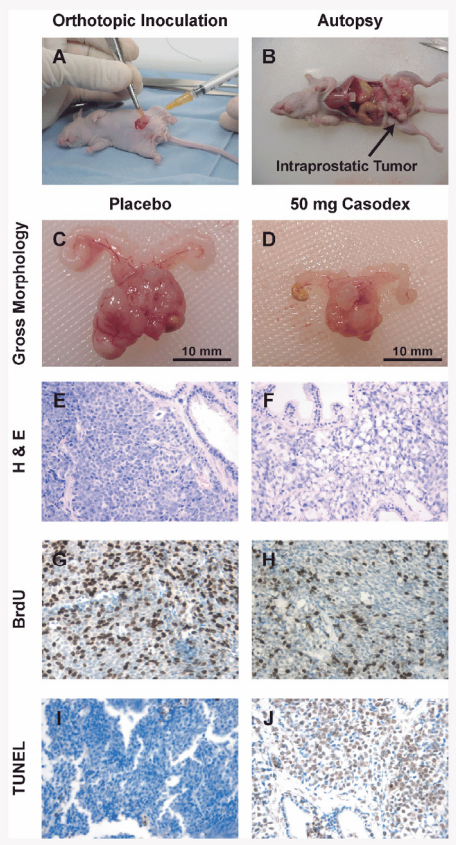
[Published by oral and poster presentations]

The overall goal of this specific aim is to examine the effects of green tea on the growth of human PC-346C and PC-3 orthotopic tumors in nude mice and to determine whether green tea influences the effects of Casodex® administration on the tumor growth, progression and metastasis in this model system.

After acclimatization 100 three week old male weanling nude mice will be randomly assigned to four arms. Three arms will be placed on PC-346C, PC-346C/Casodex® and PC-3 tumors. The fourth arm provide positive control group for each tumor type in which tumors are known to grow rapidly. The animals will be maintained on their respective diet for the duration of the experiment, and will have access to water in the control groups *ad libitum*.

These animals will be implanted with 12.5mg/90days sustained release testosterone pellets (custom made by Innovative Research, Sarasota) designed to maintain a constant level of

testosterone (4-6ng/mL) in the blood of the nude mice. This stabilization of serum testosterone is necessary due to the substantial variation of endogenous testosterone levels in nu/nu mice [45]. One week later, 2 x 10^5 PC-346C and PC-3 cells suspended in growth factor replete Matrigel™, will be injected orthotopically into the prostate. Three weeks later all animals will be implanted with Casodex® (50mg/90day) pellets. Tumor growth will be monitored weekly by palpation.

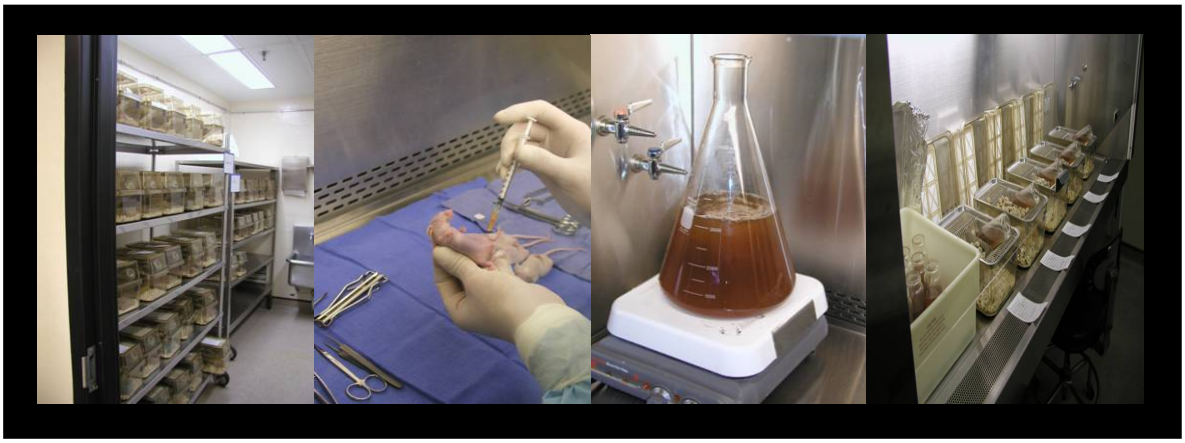


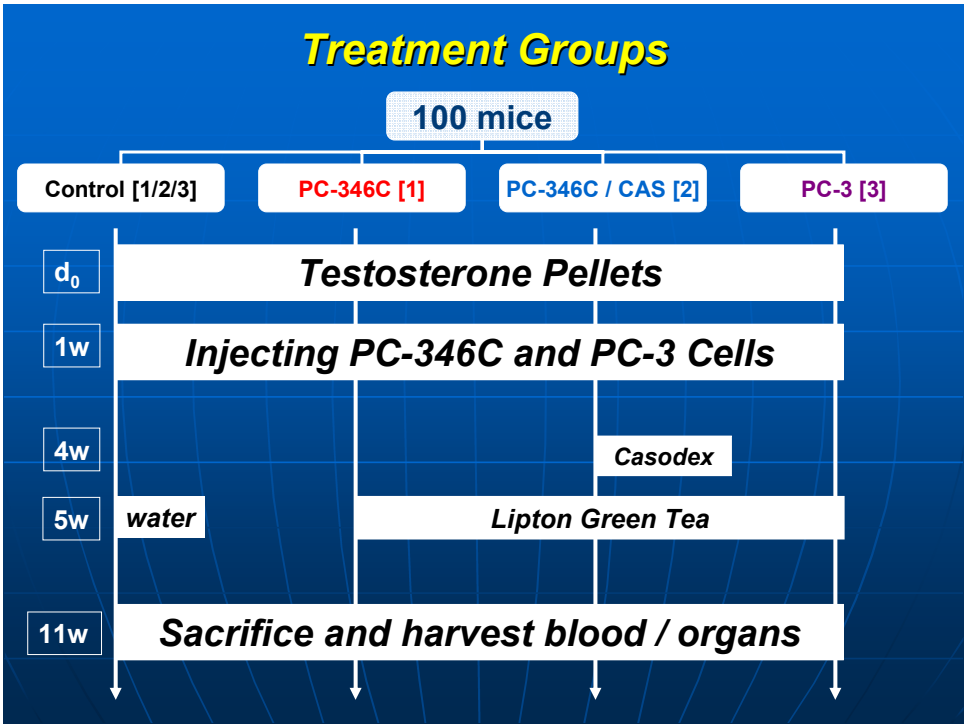
When mixed with growth factor replete Matrigel™ and injected into the prostates of testosterone-implanted male NCR nu/nu, immuno-compromised mice (panels A and B) PC-346C cells form large, well vascularized, orthotopic tumors (panel C), which reach between 1.2 and 1.5 grams after 5 weeks. This growth reflects a combination of a high rate of proliferation (as measured by bromodeoxyuridine (BrdU) incorporation-panel G) and a low rate of apoptosis (as measured by TUNEL staining-panel I). These orthotopic tumors are sensitive to Casodex® administered by implantation of 50mg, 90day slow release pellets (custom-made by Innovative Research, Sarasota), starting two weeks after injection of the cells. This treatment results in substantial shrinkage of the primary tumor (panel D), and considerable loss of tumor vasculature (panel F). In addition, Casodex® induced a considerable decrease in the rate of proliferation (panel H) and a large increase in the rate of apoptosis (panel J). Data demonstrate that the 50mg, 90-day slow release pellets induce approximately 50% reduction of tumor volume during the short three-week course of treatment. These implants produces a serum level of Casodex® of 8.9±3.5μg/mL, a level that is very similar to that achieved in men receiving 150mg Casodex® per day as a first line treatment for localized prostate cancer. It is important to note that although the tumor weight does decrease there is also a clear decrease in the cellularity of the tumor after treatment with Casodex® which indicates that dead cells are not being cleared rapidly by macrophages in nude mice. This would suggest that the decrease in tumor weight may provide an under estimate of the effectiveness of Casodex®.

We will use this orthotopic xenograft model to investigate the effect of green tea consumption on the growth and progression of early stage prostate cancer and to examine the interactions between these nutrients and anti-androgen therapy.

One week later green tea treatment was started. The animals in the treatment groups had

access to green tea *ad libitum*. The green tea was prepared fresh every other day to ensure that there was no degradation of the polyphenols with time [46], by adding 0.6% (w/v) dehydrated green tea powder to deionized water and heating to 55°C, a procedure that does not degrade the polyphenols present in the tea [47]. The green tea was provided by Thomas J. Lipton Inc. (Englewood Cliffs NJ) and contained 4.5% EC, 7.2% EGC, 5.3% ECG, and 10.6% EGCG (% dry weight) as the major polyphenols and had a caffeine content of 0.2%. After six weeks all animals were sacrificed and the urogenital complex including tumors was removed in toto. Tumors were weighed separately.

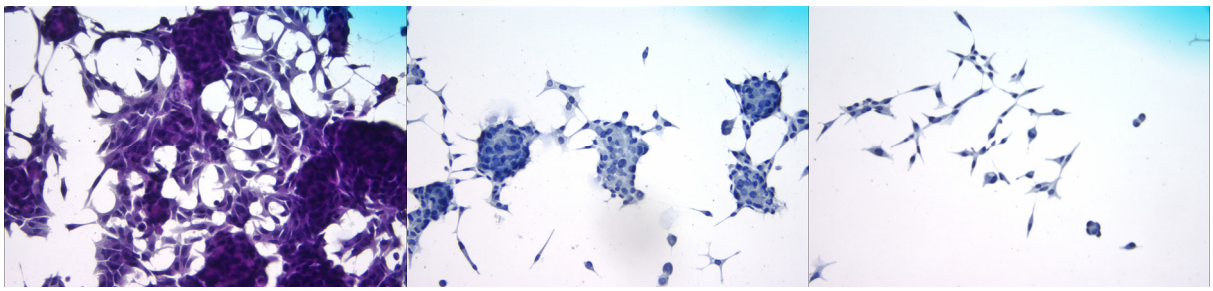




3.6 Cell Culture Methods and Human Cancer Cell Lines

[Published by oral and poster presentations]

LNCaP and PC-3 human prostate cancer cells, obtained from American Type Culture Collection (Rockville, MD), were cultured in RPMI-1640 (Life Technologies, Gaithersburg, MD) and Ham's F12K (Sigma, St. Louis, MO) respectively, with 10% Fetal Bovine Serum (Atlas, Fort Collins, CO). DU-145 prostate cancer cells were cultured in Minimum essential medium (Eagle) with 2mM L-glutamine and Earle's BSS adjusted to contain 1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids, and 1.0mM sodium pyruvate, 90%; fetal bovine serum, 10%. Another human prostate cancer cell line PC-346C used for *in vitro* and *in vivo* experiments were cultured in DMEM/F12 media containing 2% charcoal stripped serum (Atlas Biologicals, Fort Collins, CO), 0.1mg/mL BSA, 20ng/mL EGF, 1% ITS-G, 10nM testosterone, 1.4μM hydrocortisone, 1nM T3, 0.1mM phosphoethanolamine, 50ng/mL choleratoxin, and 20μg/mL fetuin.



All cell lines were routinely passaged every 3-4 days.

Crystal Violet Growth Assay

For growth assays, cancer cells were plated at a density of 2 x 10^4 cells/well in 24 well plates. Treatments with EGCG, quercetin and rutin (Sigma, St. Louis, MO), or DMSO/Ethanol vehicle (as a time matched negative control) were initiated 24h after plating. After 2-96h of treatment, Crystal Violet growth assays were performed according to manufacturer's directions (Fisher Scientific, Pittsburgh, PA).

3.7 FACS Analysis

Cells were seeded in T-150 flasks at a density of 5 x 10^6 cells/flask, and 24h after plating were treated for a period of another 24h. Cells were stained with 5μg/ml propidium iodide (PI), (Sigma) containing RNAse (Roche Diagnostics) for 30 minutes. At least 10.000 cells per treatment were analyzed on a Coulter Epics XL cytometer (Coulter, Miami, FL) and cell cycle modeling was performed using Multiplus AV software (Phoenix Flow Systems).

Apoptosis was measured by flow cytometry by terminal-transferase mediated labeling of DNA strand breaks. Cells were fixed in 2% formaldehyde, washed twice in PBS/0.2% BSA, and permeabilized with 70% Ethanol at -20°C for at least 1h. DNA strand breaks were labeled with bromodeoxyuridine by terminal transferase (Roche Diagnostics) and detected using the APO-BrdU detection kit according to manufacturer's directions (Phoenix Flow Systems, San Diego, CA). Cells were counterstained with PI, and at least 10.000 cells were analyzed and modeled utilizing Multiplus AV software (Phoenix Flow Systems).

Statistical Analysis

Data are expressed as the Mean ± standard error (SE). One way analysis of variance (ANOVA) was used to assess statistical significance between means. Difference between means were considered significant when $p<0.001$ using the Bonferroni post-test. All statistical analyses were performed with the GraphPad Instat software (Intuitive Software for Science, San Diego, CA).

Results and Discussion

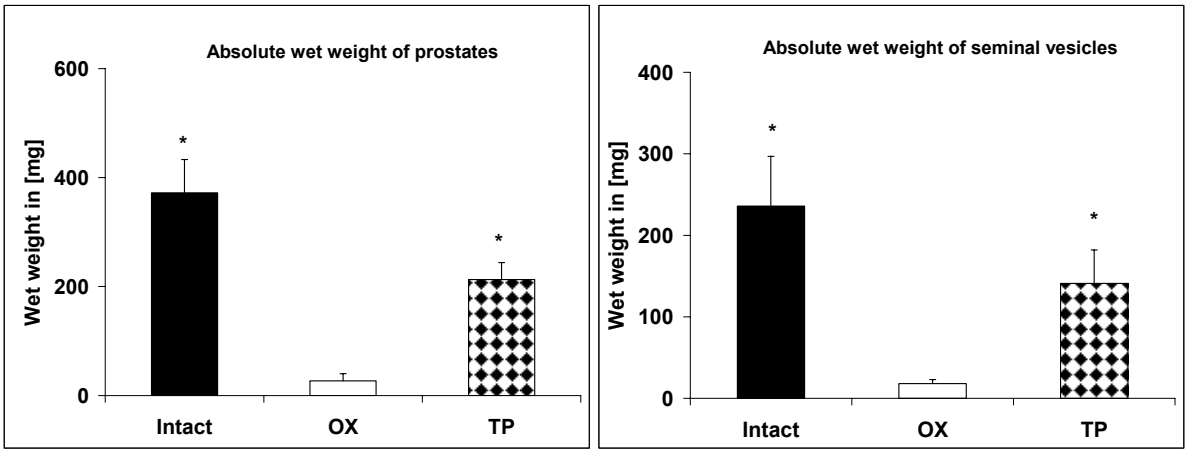
4.1 Modified Hershberger Assay

[Nishino et al. Annals of Anatomy 2004]

[Nishino et al. Journal of Steroid Biochemistry & Molecular Biology 2006]

Although the Hershberger Assay is a valid quantitative method for evaluating androgenic or anti-androgenic properties of substances by measuring the organ weight of prostates and seminal vesicles, the findings obtained by this assay provide only limited information on the specifity of the observed effects when only the reactions of the organ weights are judged: For example, the growth of seminal vesicles can be stimulated not only by androgens but also by estrogenic substances, well known as a paradoxical effect of estrogens [48-50]. Morphologic and functional analysis of cellular parameters in male accessory organs may allow a more subtle and reliable assessment of the (anti-)androgenicity of substances; in previous studies we analyzed the regulation of tenascin expression [51]. Since the amount of nuclear AR present in the rat prostate has been demonstrated to be influenced by androgens [52,53], a densitometric analysis of AR-imunoreactive cells in prostate and seminal vesicles was performed by using immunohistochemical methods [54,55].

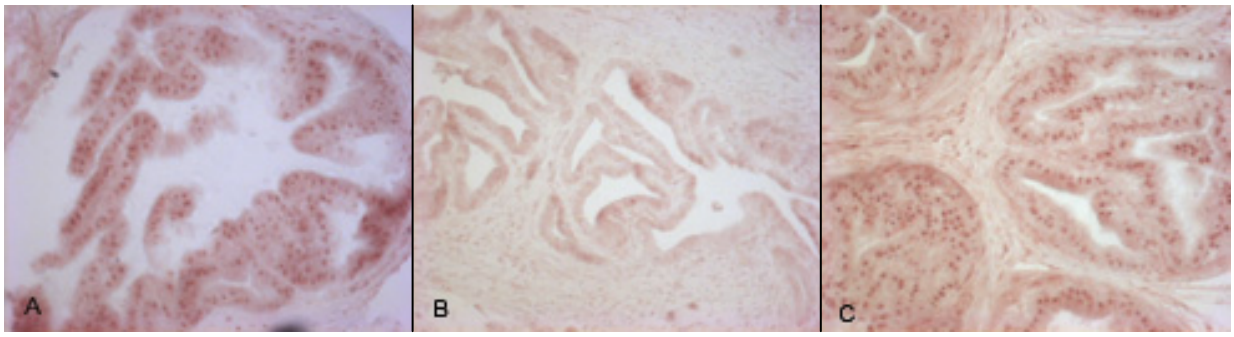
Measurement of the body weight yielded no significant differences between the intact group, OX group and TP group. However, examination of wet weights (absolute and relative) of prostates and seminal vesicles showed that orchiectomy in the OX group induced a significant decrease in organ wet weights in comparison to the intact group, which was nearly reversed by an administration of a pharmacological dose of TP in the TP group.



4.1.1 Densitometric Measurements

It has been previously described that the concentrations of biochemically active substances can be estimated from the optical density of the immunoreactive signal [56,57]. The advantages of a computer-assisted densitometry are a faster scoring procedure of sections from large series and a higher reliability. However, the disadvantage of a semiquantitative approach is the possibility that relevant signals can easily be missed, so that comparative studies should be based on rather robust signals [58]. Using the monoclonal antibody (androgen receptor, AR) the staining intensity of AR in the prostates revealed the following optical density values: 104±20 (intact), 56±17 (OX), and 77±22 (TP). Statistical analysis confirmed that the intensity of staining was significantly higher in both the intact and TP group compared to the OX group.

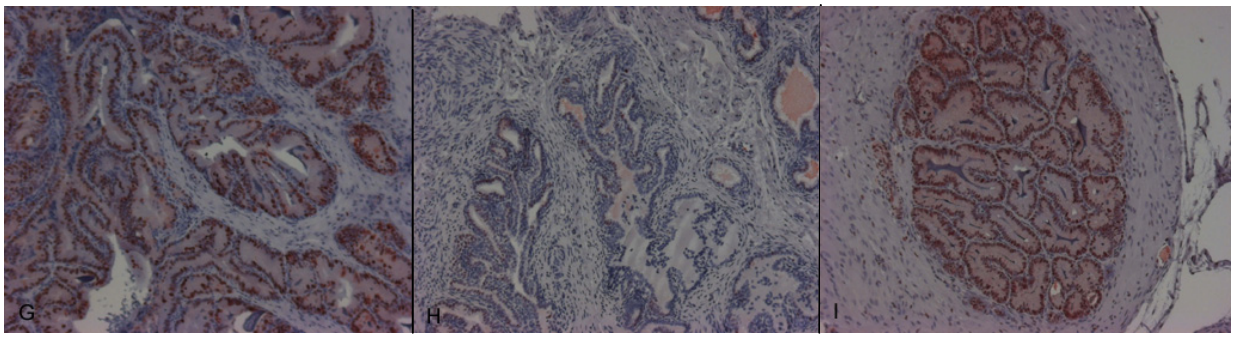
The data provide evidence that orchiectomy results in a reduced staining intensity of AR, whereas substitution with TP enhances the immunoreactive signal of AR.



4.1.2 Cell Proliferation

In order to further characterize androgenic effects two proliferation markers were used in this study. The proliferation associated antigen Ki-67 (MIB-5) and proliferating cell nuclear antigen (PCNA) have been used as proliferation and prognostic markers in a large number of studies, in particular in malignancies [59,60]. PCNA is a highly conserved 36kDa acidic nuclear protein that is expressed during cell replication and DNA repair [61,62]. PCNA interacts with DNA-polymerase delta and with RF-C protein to bind at DNA primer-template junctions. Immunostaining of S-phase nuclei will detect PCNA in sites of DNA synthesis. Ki-67 antigen is expressed during the G_1, S, G_2 and M-phases of the cell cycle, but is not expressed during the G_0 (resting)-phase. Because Ki-67 antigen has a short half-life, it can be used as a marker of actively proliferating cells [63,64]. Endothelial cell proliferation in male reproductive organs of adult rat is high and regulated by testicular factors. In the epididymis, the ventral and dorso-lateral prostate lobes, and the seminal vesicles, endothelial cell proliferation decreased after testosterone withdrawal and increased following testosterone treatment [65].

The assessment of cell proliferation markers yielded the following data in the rat prostate: The percentage of immunoreactive epithelial cells for MIB-5 was 85±9% in the intact group, 9±1% in the OX group and 90±2% in the TP group. The percentage of immunoreactive epithelial cells for MIB-5 in the OX-group was significantly reduced compared to the intact and TP group. Similar results were obtained for the relative amount of cells immunoreactive for PCNA (not shown). Thus, whereas orchiectomy caused a considerable decrease in cell proliferation, administration of TP could reverse this effect and induced a cell proliferation index similar to the intact group.

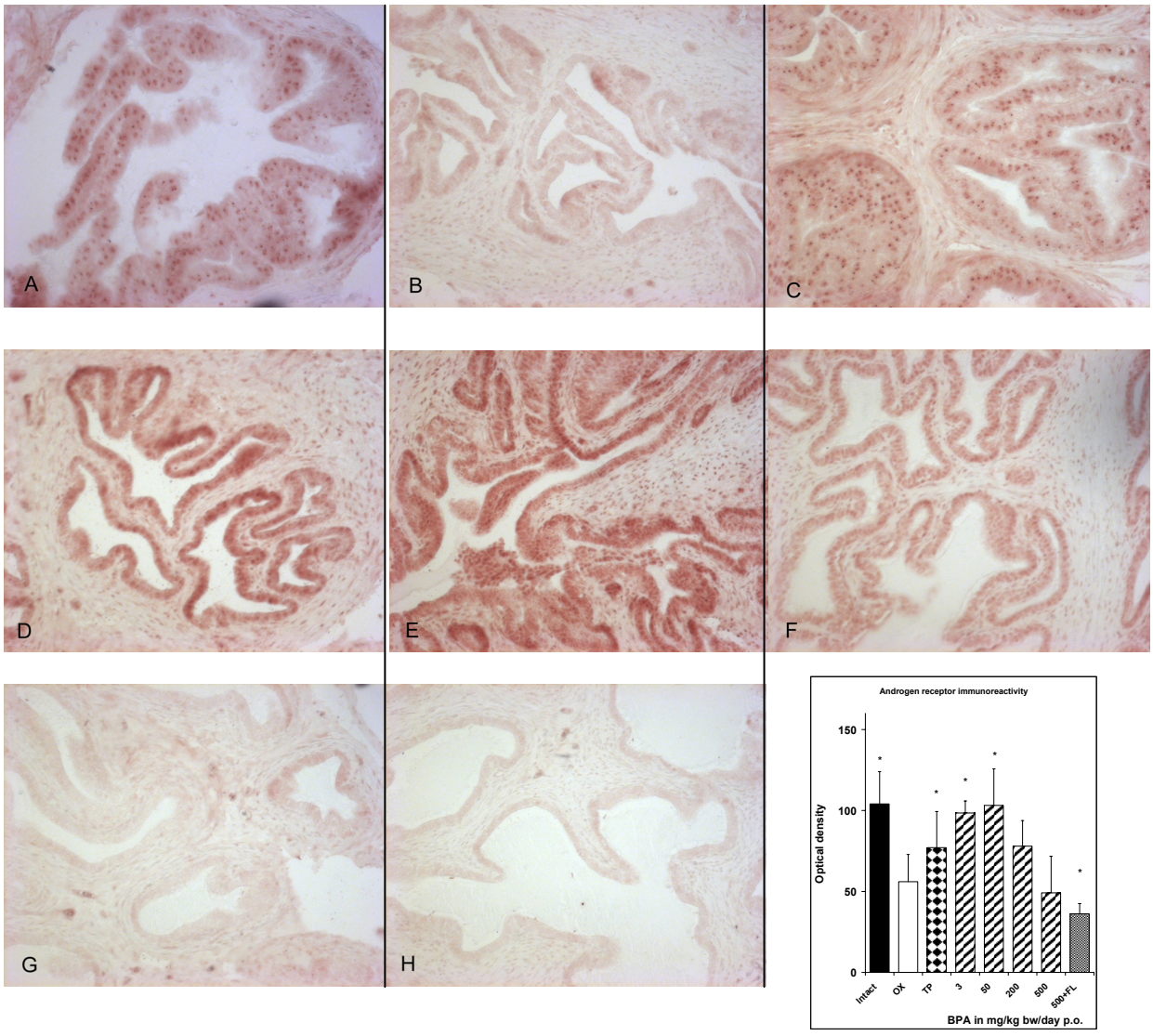


4.2 Androgenic Potential of Bisphenol A

In contrast to TP, BPA induced no effects on absolute weights of prostate and seminal vesicle. BPA at high doses of 200 and 500mg/kg BW/day caused a decrease in body weights and a significant increase in relative weights of prostate and seminal vesicle. A simultaneous administration of flutamide FL had no further effect in BPA treated animals. Animals treated with 200, 500 and 500+FL mg/kg BW/day BPA showed severe signs of gastro-intestinal toxicity.

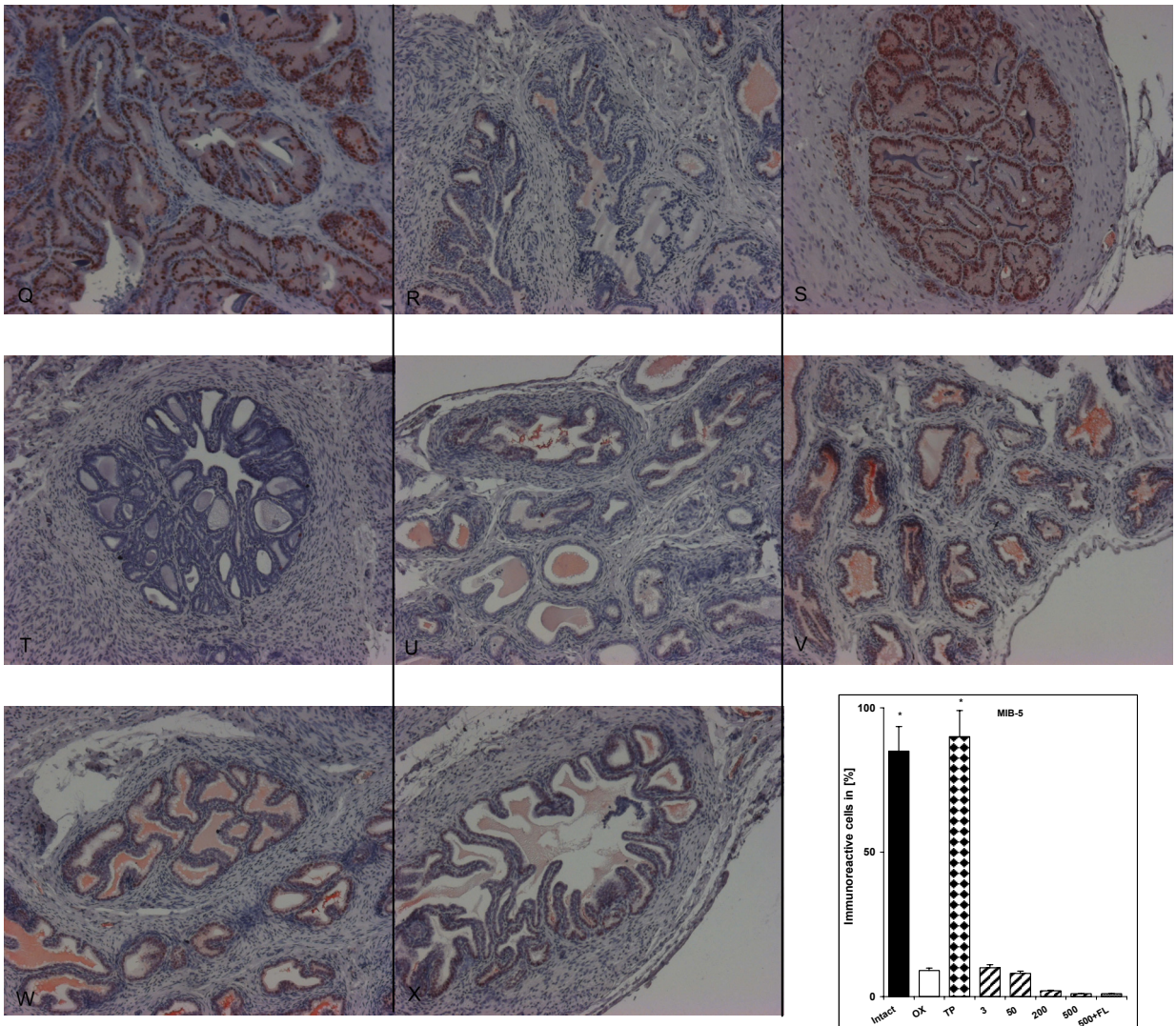
4.2.1 Densitometric Analysis

Using the anti-androgen receptor monoclonal antibody (1:100, 554224, mouse monoclonal, Novocastra, New Castle, United Kingdom) the staining intensity of AR in the prostates revealed the following optical density values: 104±20 (Intact), 56±17 (OX), 77±22 (TP), 99±7 (BPA 3), 103±23 (BPA 50), 78±16 (BPA 200), 49±23 (BPA 500) and 36±6 (BPA 500+FL). Statistical analysis confirmed that the intensity of staining was significantly higher in both the intact and TP group compared to the OX group. Thus, the data obtained provide evidence that orchiectomy results in a reduced staining intensity of AR, whereas substitution with TP enhances the immunoreactive signal of AR. The intensity of staining was significantly increased in prostate after treatment with lower doses of BPA (3 and 50mg/kg BW/day). At 500mg/kg BW/day staining intensity was similar to the castrated control, but the combination of BPA (500mg/kg BW/day) with FL significantly reduced the staining intensity.



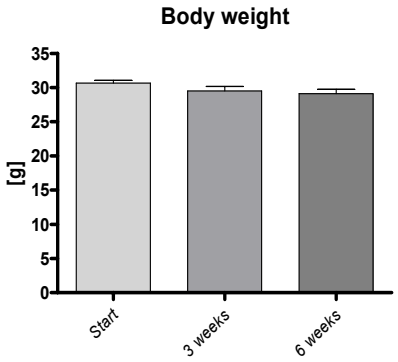
4.2.2 Cell Proliferation

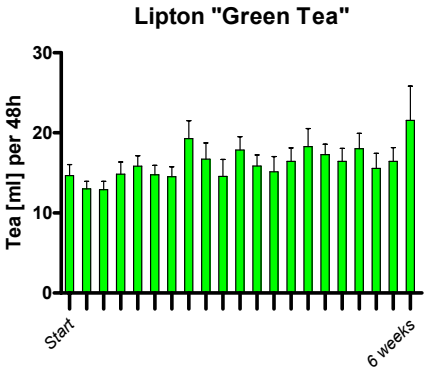
The assessment of cell proliferation markers yielded the following data in rat prostates: The percentage of immunoreactive epithelial cells for MIB-5 was 85±9% in the intact group, 9±1% in the OX group and 90± 2% in the TP group. BPA treated groups displayed: 10±1% (BPA 3), 8±1% (BPA 50), 2% (BPA 200), 1% (BPA 500) and 1% (BPA 500+FL). The percentage of immunoreactive epithelial cells for MIB-5 in the OX group was significantly reduced compared to the intact and TP group. Similar results were obtained for the relative amount of cells immunoreactive for PCNA (not shown). Whereas orchiectomy caused a considerable decrease of cell proliferation, administration of TP could reverse this effect and induced a cell proliferation index similar to the intact group. The assessment of both proliferation markers revealed that BPA showed at all doses tested no stimulation of proliferating activity in prostate.

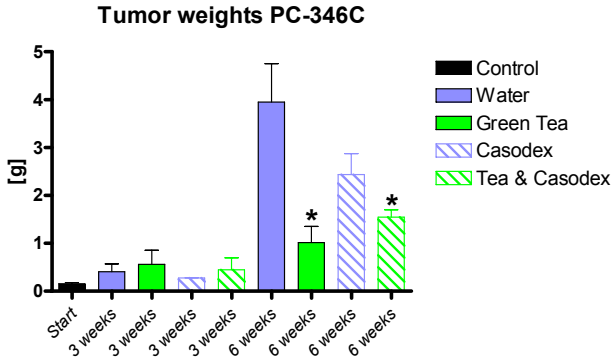


4.3 Green Tea and Prostate Cancer

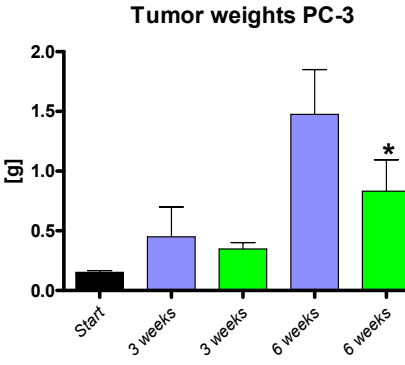
[Published by oral and poster presentations]

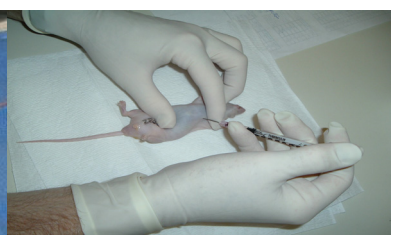






There were no significant changes in body weight over six weeks. Overall average green tea consumption was around 8mL/animal/day. Preliminary data showed a significant reduction of PC-346C and PC-3 tumor weights after 6 weeks. A combination treatment with green tea and Casodex® in PC-346C tumors also led to a significant reduction of androgen-dependent PC-346C tumors. There is a 50% reduction in the incidence of primary tumors in mice consuming green tea when compared to an age-matched cohort receiving tap water. These data demonstrate that long term consumption of green tea in this model system has a substantial effect on the incidence of autochthonous tumor formation. There were almost no metastatic sites in livers and lungs visible in green tea treated animals, whereas positive control groups with metastatic sites were the rule after six weeks. Green tea decreases the incidence of tumors without substantially altering the site of tumor formation.

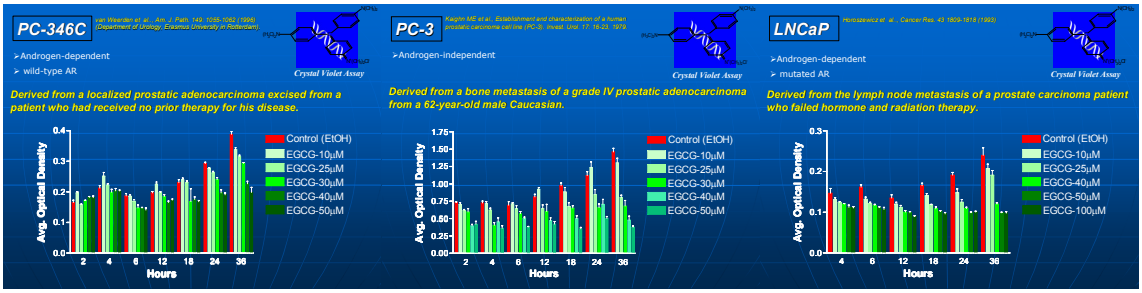




4.4 Effects of Dietary and Natural Products on human Prostate Cancer Cell Lines

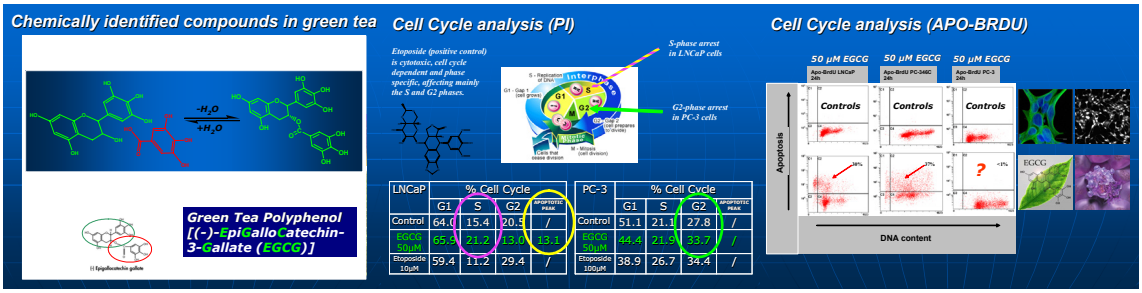
[Published by oral and poster presentations]

EGCG (50µg) inhibited cell proliferation (CV Assay) significantly after 24h of LNCaP, PC-346C, PC-3, DU-145 (not shown) cancer cell lines. Similar data could be observed for quercetin (not shown) using above mentioned cell lines. In spite of chemical relation to EGCG and quercetin, rutin (not shown) showed no effects on cancer cell lines.



Cell cycle analysis with 50µM EGCG for 24 hours showed a significant S-phase arrest in LNCaP cells and a significant G_2-phase arrest in PC-3 cells. Etoposide was used as a positive control.

APO-BRDU with 50µM EGCG for 24 hours showed 30% apoptotic and necrotic cells in LNCaP cells, 37% apoptotic and necrotic cells in PC-346C cells and surprisingly nearly no apoptotic and necrotic cells in androgen-independent PC-3 cancer cells.

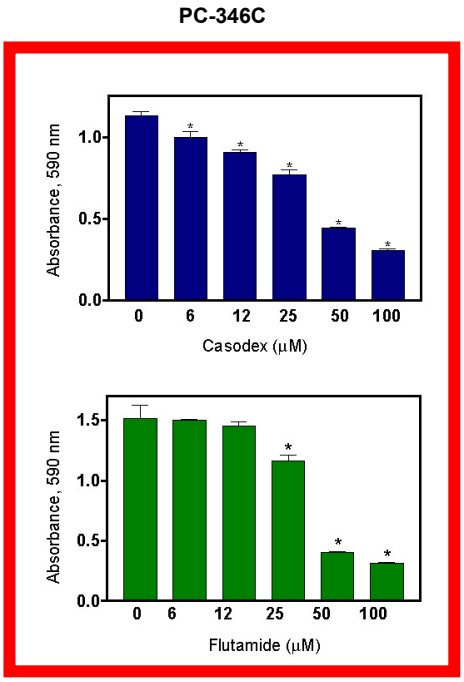


4.5 Animal Model Systems

One of the earliest autochthonous model systems, the LOBUND-Wistar rat was developed at University of Notre Dame by Professor Morris Pollard who observed spontaneous tumor formation in the urogenital system of germ-free rats [66]. Approximately 30% of male LOBUND-Wistar rats spontaneously develop tumors of the urogenital system within 2 years, while treatment with nitrosomethylurea (NMU) increases the tumor incidence to approximately 90% and reduces the time to tumor development to approximately 10 months [67]. Our recent analysis of the effects of green tea on tumor development in this autochthonous model demonstrated that the tumor incidence is reduced by 50% in the animals consuming green tea compared to tap water, demonstrating the utility of the model system for the analysis of diet on tumor formation. However histological analysis demonstrated that 90-95% of the tumors originate in the seminal vesicles of the rats, whether the animals were given water or green tea. Since the seminal vesicles are derived from a different embryological origin than the prostate, this significantly reduces the relevance of this model system [68]. Several transgenic strains have been developed that produce autochthonous prostate cancers. The most widely used transgenic model system for prostate cancer is the TRAMP mouse, which expresses the SV40 large T and middle t antigens under the control of the minimal probasin promoter, targeting the expression of the transgene to the dorso-lateral lobes of the gland [69]. These tumors develop between 8 and 10 weeks, and the majority of the animals die within 28 weeks as a result of metastatic burden [70]. While these tumors are androgen-dependent, the extremely aggressive behaviour of the tumors, the rapid metastatic progression and the neuro-endocrine characteristics of the tumors make them a poor model for the chronic effects of dietary modulation on localized early stage disease. These considerations have lead to the development of a second androgen-dependent transgenic model system the LBP-Tag mouse, which is usually referred to as the LADY model [71]. In this case, the long probasin promoter drives the expression of just the SV40 large T (and thus eliminating effects of SV40 middle t), targeting the transgene to the dorso-lateral and ventral prostate. Like TRAMP mouse, androgen-dependent tumors develop in this model with 100% penetrance, but with a latency of approximately 2-5 months, at which time most mice show evidence of low grade prostatic intraepithelial neoplasia (LGPIN) in the dorso-lateral and ventral lobes of the prostate. Tumor progression to high grade PIN (HGPIN) and local microinvasive and invasive tumors occurs over the next 4 months, leading to the development of androgen receptor negative (AR-)adenocarcinomas and neuro-endocrine carcinomas by 6 months of age. This occurs without significant proliferation of the stroma, reflecting the pathology seen in human disease. Mice eventually develop androgen-independent metastatic disease, primarily in the lymph, liver and lungs between 6 and 9 months, which can be monitored using Tag immunohistochemistry. The long latency and the pathology that accurately reflects the human

disease makes this model system much more representative of the initiation of localized prostate cancer in humans and much more amenable to long term dietary intervention studies.

Prostate cancer research has been hampered by the dearth of good androgen-dependent cell lines of human origin. For many years the standard androgen-dependent cell line has been the LNCaP system. This aneuploid cell line was initially developed from the lymph node metastasis of an individual who failed both radiation and hormone therapy [72]. It is therefore quite surprising that this cell line is androgen-dependent and non-metastatic in the laboratory setting. In addition the cell line contains a mutation in the ligand binding domain of androgen receptor (Thr877Ala), which renders the receptor quite promiscuous, to the extent that the receptor can be activated by a number of endogenous steroids including the active androgen 5α-dihydrotestosterone (5α-DHT) and the adrenal steroid, Δ^4-androstenedione, making it very difficult to control the activation of the receptor *in vivo* [73,74]. This cell line can be grown as xenografts in nude mice as either subcutaneous tumors or as orthotopic tumors, but due to the overriding influence of the adrenal steroids, these tumors fail to respond appropriately to androgen deprivation or anti-androgen therapy (either Flutamide® or Casodex®) [75]. The CWR22 transplantable prostate tumor model system was also developed from a patient who had failed hormone therapy, and while it remains many of the molecular characteristics of early stage prostate cancer, it also contains a mutation in the ligand binding domain of the androgen receptor (His874Tyr) which produces similar ligand promiscuity to the LNCaP cell lines [76], making it less than ideal for *in vivo* evaluation of diet or other interventions designed to regulate progression.



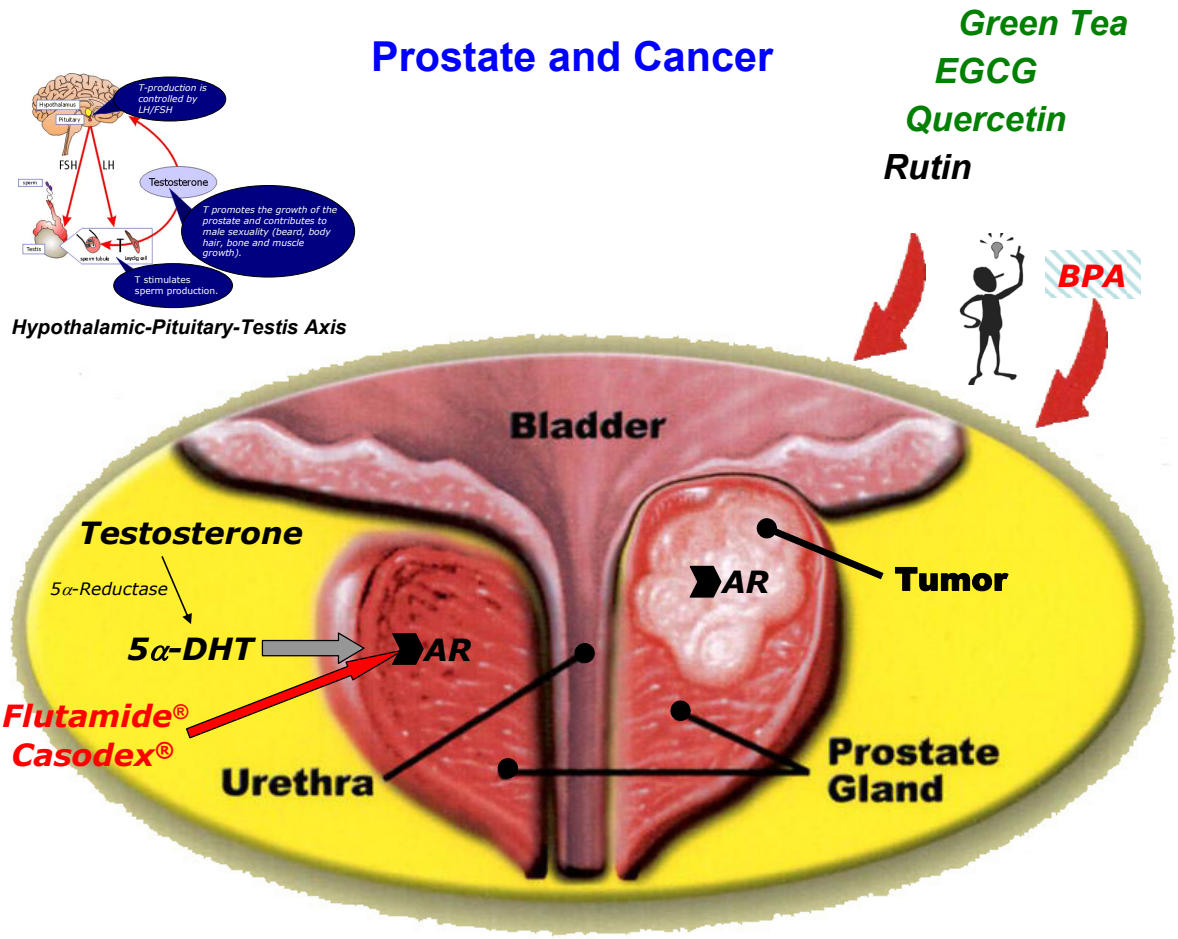
Due to the inherent difficulties of culturing primary prostate epithelial cells a number of groups have developed cell lines immortalized using a variety of viral constructs that interfere with cell cycle or apoptotic mechanisms [77-79]. While these cell lines are very useful for many applications, the disruption of cell cycle and apoptosis mechanisms makes them inherently unsuitable for our studies. A number of androgen-dependent cell lines have also been developed by direct implantation of human tumors into nude mice as subcutaneous xenografts [80]. One of these cell lines, PC-346C, was developed from a prostatectomy of an individual who had received no therapy prior to surgery, and contains a wild type androgen receptor, and wild-type p53. This diploid cell line is androgen-dependent in cell culture, responds appropriately to both androgens and anti-androgens *in vitro* and *in vivo* and has not been immortalized using

viral constructs that disrupt normal cell cycle or apoptotic control pathways, we have chosen to utilize this cell line exclusively for our xenograft.

Conclusion

Based on the present data, the densitometric analysis of AR-immunoreactivity and the assessment of both cell morphology and cell proliferation proved to be independent and sensitive parameters for the evaluation of androgen effects on prostates and seminal vesicles. Using these additional detection tools we found a weak androgenicity of BPA. The combined application of these parameters may provide an additional tool to test the broad spectrum of endocrine active substances, such as endocrine disruptors and phytoestrogens which are currently being discussed as to their potential risk and benefits to the environment and humans.

The xenograft accomplished, represents a comprehensive evaluation of the effects of green tea consumption on prostate tumor progression and metastases, and will determine whether self medication with green tea interferes with standard hormone therapy using Casodex®. The overall outcome of the planned and further studies will have significant impact on the current use of Casodex® for the treatment of early stage localized prostate cancer, particularly in terms of the interactions between diet (in the case of green tea consumption) and standard treatments for the disease. These studies may provide evidence of other important regulatory mechanisms beyond androgens that can be integrated into our understanding of prostate disease and provide new avenues for treatment.



Test Compounds	*Effects of test compounds on prostate and prostate cancer*
Green Tea	Inhibition of human prostate cancer cells *in vitro* / *in vivo*
EGCG	Inhibition of human prostate cancer cells *in vitro*
Quercetin	Inhibition of human prostate cancer cells *in vitro*
Rutin	Inactive *in vitro*
BPA	Up-regulation of AR in prostate

References

1. American Cancer Society Facts and Figures
2. S. Gupta and H. Mukhtar, Green Tea and prostate cancer, Urol. Clin. North Am. 29 (2002) 49-57.
3. R. Choo, L. Klotz, C. Danjoux, G.C. Morton, G. DeBoer, E. Szumacher, N. Fleshner, P. Bunting, G. Hruby, Feasibility study: watchful waiting for localized low to intermediate grade prostate carcinoma with selective delayed intervention based on prostate specific antigen, histological and/or clinical progression, J. Urol. 167 (2002) 1664-1669.
4. M. Tenniswood, Apoptosis, Tumor Invasion and Prostate Cancer, Br. J. Urol. 79 (1997) 27-34.
5. F. Paris, P. Balaguer, B. Terouanne, N. Servant, C. Lacoste, J.P. Cravedi, J.C. Nicolas, C. Sultan, Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines, Mol. Cell Endocrinol. 193 (2002) 43-9.
6. S.C. Nagel, F.S. vom Saal, K.A. Thayer, M.G. Dhar, M. Boechler, W.V. Welshons, Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol, Environ. Health Perspect. 105 (1997) 10-6.
7. H.S. Kim, S.Y. Han, S.D. Yoo, B.M. Lee, K.L. Park, Potential estrogenic effects of bisphenol-A estimated by *in vitro* and *in vivo* combination assays, J. Toxicol. Sci. 26 (2001) 111-8.
8. T. Takao, W. Nanamiya, I. Nagano, K. Asaba, K. Kawabata, K. Hashimoto, Exposure with the environmental estrogen bisphenol A disrupts the male reproductive tract in young mice, Life Sciences 65 (1999) 2351-2357.
9. W.V. Welshons, S.C. Nagel, K.A. Thayer, B.M. Judy, F.S. vom Saal, Low–dose bioactivity of xenoestrogens in animals: fetal exposure to low doses of methoxychlor and other xenoestrogens increases adult prostate size in mice, Toxicol. Ind. Health 15 (1999) 12-25.
10. F.S. vom Saal, P.S. Cooke, D.L. Buchanan, P. Palanza, K.A. Thayer, S.C. Nagel, S. Parmigiani, W.V. Welshons, A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production and behaviour, Toxicol. Ind. Health 14 (1998) 239-60.
11. P.L. Palanza, K.L. Howdeshell, S. Parmigiani, F.S. vom Saal, Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice, Environ.

Health perspect. 110 (2002) 415-422.

12. K.L. Howdeshell, A.K. Hotchkiss, K.A. Thayer, J.G. Vandenbergh, F.S. vom Saal, Environmental toxins: Exposure to bisphenol A advances puberty, Nature 401 (1999) 763-764.
13. P. Diel, T. Schulz, K. Smolnikar, E. Strunck, G. Vollmer, H. Michna, Ability of Xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotrophic activity, J. Steroid Biochem. Mol. Biol. 73 (2000) 1-10.
14. C.M. Markey, C.L. Michaelson, E.C. Veson, C. Sonnenschein, A.M. Soto, The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A, Environ. Health Perspect. 109 (2001) 55-60.
15. H. Tinwell, R. Joiner, I. Pate, A. Soames, J. Foster, J. Ashby, Uterotrophic activity of bisphenol A in the immature mouse, Regul. Toxicol. Pharmacol. 32 (2000) 118-126.
16. C. Gupta, Reproductive Malformation of the Male Offspring Following Maternal Exposure to Estrogenic Chemicals (44516), Proc. Soc. Exp. Biol. Med. 224 (2000) 61-68.
17. H. Kim, S. Han, T. Kim, S. Kwack, R. Lee, I. Kim, J. Seok, B. Lee, S. Yoo, K. Park, No androgenic/anti-androgenic effects of bisphenol-A in Hershberger assay using immature castrated rats, Toxicol. Lett. 5; 135 (1-2) (2002) 111.
18. E. Giovannucci, E.B. Rimm, Y. Liu, M.J. Stampfer, W.C. Willett, A prospective study of tomato products, lycopene and prostate cancer risk, J. Natl. Cancer Inst. 94 (2002) 391-398.
19. E.C. Miller, E. Giovannucci, J.W. Erdman Jr., R. Bahnson, S.J. Schwartz, S.K. Clinton, Tomato products, lycopene and prostate cancer risk, Urol. Clin. North Am. 29 (2002) 83-93.
20. E.P. Castle, J.B. Thrasher, The role of soy phytoestrogens in prostate cancer, Urol. Clin. North Am. 29 (2002) 71-81.
21. S. Gupta, N. Ahmad, R.R. Mohan, M.M. Husain, H. Mukhtar, Prostate cancer chemoprevention by green tea: *in vitro* and *in vivo* inhibition of testosterone-mediated induction of ornithine decaarboxylase, Cancer Res. 59 (1999) 2115-2120.
22. S. Liao, The medicinal action of androgens and green tea epigallocatechin gallate, Hong Kong Med. J. 7 (2001) 369-374.
23. E.J. Johnson, The role carotenoids in human health, Nutr. Clin. Care 5 (2002) 56-65.
24. S. Gupta, N. Ahmad, A.L. Niemenen, H. Mukhtar, Growth inhibition, cell-cycle dysregulation and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-

gallate in androgen sensitive and insensitive human prostate carcinoma cells, Toxicol. Appl. Pharmacol. 164 (2000) 82-90.

25. L.Y. Chung, T.C. Cheung, S.K. Kong, K.P. Fung, Y.M. Choy, T.T. Kwok, Induction of apoptosis by green tea catechins in human prostate cancer DU-145 cells, Life Sci. 68 (2001) 1207-1214.

26. S. Liao, R.A. Hiipakka, Selective inhibition of steroid 5alpha reductase isozymes by tea epicatechin-3-gallate and epigallocatechin-3-gallate, Biochem. Biophys. Res. Commun. 214 (1995) 833-838.

27. R.A. Hiipakka, H.Z. Zhou, W. Dai, Q.Dai, S. Liao, Structure-function relationships for inhibition of human 5alpha reductase by polyphenols, Biochem. Pharmacol. 15 (2002) 1165-1176.

28. F. Ren, S. Zhang, S.H. Mitchell, R. Butler, C.Y. Young, Tea polyphenols down regulate the expression of the androgen receptor in LNCaP cells, Oncogene 19, (2000) 1924-1932.

29. Y. Suzuki, Y. Kondo, S. Himeno, K. Nemoto, M. Akimoto, N. Imura, Role of antioxidant systems in human androgen-independent prostate cancer cells, Prostate 43 (2000) 144-149.

30. K. Suzuki, H. Koike, H. Matsui, Y. Ono, M. Hasumi, H. Nakazato, H. Okugi, Y. Sekine, K. Oki, K. Ito, T. Yamamoto, Y. Fukabori, K. Kurokawa, H. Yamanaka, Genistein, a soy isoflavone, induces glutathione peroxidase in human cancer cell lines LNCaP and PC-3, Int. J. Cancer 99 (2002) 846-852.

31. R. Agarwal, Cell signaling and regulators of cell cycle as molecular targets for prostate cancer, Biochem. Pharmacol. 60 (2000) 1051-1059.

32. N. Bhatia, R. Agarwal, Detrimental effect of cancer preventive phytochemicals silymarin, genistein and epigallocatechin-3-gallate on epigenetic events in human prostate carcinoma DU145 cells, Prostate 46 (2001) 98-107.

33. J. Kumi-Diaka, Chemosensitivity of human prostate cancer cells PC-3 and LNCaP to genistein isoflavone and beta lapachone, Biol. Cell 94 (2002) 37-44.

34. S. Gupta, K. Hastak, N. Ahmad, J.S. Lewin, H. Mukhtar, Inhibition of prostate carcinogenesis in TRAMP mice by oral infusion of green tea polyphenols, Proc. Natl. Acad. Sci. USA 98 (2001) 10350-10355.

35. K. Satoh, Y. Sakamoto, A. Ogata, F. Nagai, H. Mikuriya, M. Numazawa, K. Yamada, N. Aoki, Inhibition of aromatase activity by green tea extract catechins and their

endocrinological effects of oral administration in rats, Food Chem. Toxicol. 40 (2002) 925-933.

36. Y.H. Kao, R.H. Hiipakka, S. Liao, Modulation of endocrine systems and food intake by green tea epigallocatechin gallate, Endocrinol. 141 (2000) 41-48.

37. J.H. Weisbuger, F.L. Chung, Mechanisms of chronic disease causation by nutritional factors and tobacco products and their prevention by tea polyphenols, Food Chem. Toxicol. 40 (2002) 1145-1154.

38. L. Hershberger, E. Shipley, R. Meyer, Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method, Proc. Soc. Exp. Biol. Med. 83 (1953) 175-180.

39. J. Ashby, P.A. Lefevre, The peripubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of anti-androgens, oestrogens and metabolic modulators, J. Appl. Toxicol. 20 (2000) 35-47.

40. M. Breckwoldt, F. Neumann, H. Bräuer, Exempla endokrinologica: Bildatlas zur Physiologie und Morphologie des endokrinen Systems, Bd.1 [Hrsg.: Schering AG, Berlin]. Medical Service München (1991).

41. W. Kühnel, Antiandrogeneffekte an den Geschlechtsorganen männlicher Ratten, Anat. Anz. Suppl. 126 (1970) 149-160.

42. W. Kühnel, Der Einfluss von Antiandrogenen auf die testosteronabhängige Morphologie des männlichen Genitalapparates, Anat. Anz. 136 (1974) 261-265.

43. Endocrine Disruptor Screening and Testing Advisory Committee, Environmental Protection Agency (1998) Final report. Washington D.C.: Environmental Protection Agency.

44. M. Oberholzer, M. Oestreicher, H. Christen, M. Bruehlmann, Methods in quantitative image analysis, Histochem. Cell Biol. 105 (1996) 333-355.

45. T.G. Pretlow, C.M. Delmoro, G.G. Dilley, C.G. Spadafora, T.P. Pretlow, Transplantation of human prostatic carcinoma into nude mice in Matrigel, Cancer Res. 51 (1991) 3814-3817.

46. Z. Chen, Q.Y. Zhu, D. Tsang, Y. Huang, Degradation of green tea catechins in tea drinks, J. Agri. Food Chem. 49 (2001) 477-482.

47. S. Tao Shi, Z. Wang, T.J. Smith, J. Hong, W. Chen, C. Ho, C.S. Yang, Effects of green tea and black tea on 4-(Methylnitrosamino)-1-(3-pyridil)-1-butanone bioactivation, DNA methylation, and lung tumorigenesis in A/J mice, Cancer Res. 54 (1994) 4641-4647.

48. S.E. De Jongh, Paradoxe Wirkungen von Follikelhormon (Menformon) bei männlichen Tieren; ihre Beeinflussbarkeit durch männliches Hormon, Arch. Int. Pharmacodyn. 50 (1935) 348-355.

49. S.E. De Jongh SE, De beinvloeding van het mannelijk genitaal apparaat door hormonen. Hormoon, VII: (1937) 42-51.

50. J. Freud, Conditions of hypertrophy of the seminal vesicles in rats, Biochem. J. 27 (1933) 1438-1445.

51. G. Vollmer, H. Michna, K. Ebert, R. Knuppen, Androgen ablation induces tenascin expression in the rat prostate, Prostate 25 (1994) 81-90.

52. R.J. Moore, J.M. Gazak, J.D. Wilson, Regulation of cytoplasmic dihydrotestosterone binding in dog prostate by 17β-estradiol, J. Clin. Invest. 63 (1979) 351-357.

53. J.P. Blondeau, E.E. Baulieu, P. Robel, Androgen-dependent regulation of androgen nuclear receptor in the rat ventral prostate, Endocrinology 110 (1982) 1926-1932.

54. C. Huggins, C.V. Hodges, Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941, J. Urol. 167 (2002) 948-951.

55. C. Rolf, E. Nieschlag, Potential adverse effects of long-term testosterone therapy, Baillieres Clin. Endocrinol. Metab. 12 (1998) 521-534.

56. L.B. Nabors, E. Songu-Mize, R.R. Mize, Quantitative immunocytochemistry using an image analyzer. II. Concentration standards for transmitter immunocytochemistry, J. Neurosci. Methods. 26(1) (1988) 25-34.

57. R.R. Mize, R.N. Holdefer, L.B. Nabors, Quantitative immunocytochemistry using an image analyzer. I. Hardware evaluation, image processing and data analysis, J. Neurosci. Methods. 26(1) (1988) 1-23.

58. W.I. De Boer, P.S. Hiemstra, J.K. Sont, E. De Heer, K.F. Rabe, J.H.J.M. Van Krieken, P.J. Sterk, Image analysis and quantification in lung tissue, Clin. Exp. Allergy 31 (2001) 504-508.

59. P. Korkolopoulou, J. Oates, J. Crocker, C.Edwards, p53 expression in oat and non-oat small cell lung carcinomas: correlation with proliferating cell nuclear antigen, J. Clin. Pathol. 46 (1993) 1093-1096.

60. T. Kawai, M. Suzuki, S. Kono, N. Shinomiya, M. Rokutanda, K. Takagi, T. Ogata, S. Tamai, Proliferating cell nuclear antigen and Ki-67 in lung carcinoma. Correlation with DNA flow cytometric analysis, Cancer 74 (1994) 2468-2475.

61. M.K.K. Shivji, M.K. Kenny, R.D. Wood, Proliferating cell nuclear antigen is required for DNA excision repair, Cell 69 (1992) 367-374.

62. P.A. Hall, D.A. Levison, A.L. Woods, C.C.W. Yu, D.B. Kellock, J.A. Watkins, D.M. Barnes, C.E. Gillett, R. Camplejohn, R. Dover, N.H. Waseem, D.P. Lane, Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms, J. Pathol. 162 (1990) 285-294.

63. D. McCormick, H. Chong, C. Hobbs, C. Datta, P.A. Hall, Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB-1, Pathol. 22 (1993) 355-360.

64. S.E. Pinder, P. Wencyk, D.M. Sibbering, J.A. Bell, C.W. Elston, R. Nicholson, J. F. R. Robertson, R.W. Blamey, I.O. Ellis, Assessment of the new proliferation marker MIB-1 in breast carcinoma using image analysis: Associations with other prognostic factors and survival, Br. J. Cancer 71 (1995) 146-149.

65. I. Franck Lissbrant, E. Lissbrant, A. Persson, J.E. Damber, A. Bergh, Endothelial cell proliferation in male reproductive organs of adult rat is high and regulated by testicular factors, Biol. Reprod. 68 (2003) 1107-1111.

66. M. Pollard, Spontaneous prostate adenocarcinoma in aged germ-free Wistar-rats, J. Natl. Cancer Inst. 51 (1973) 1235-1241.

67. M. Pollard, The Lobund-Wistar rat model of prostate cancer, J. Cell Biochem. 16H (1992) 84-88.

68. C. Morrissey, A. Buser, J. Scolaro, J. O'Sullivan, A. Moquin, M. Tenniswood, Changes in hormone sensitivity in the ventral prostate of aging rats, J. Androl. 23 (2002) 341-351.

69. N. Greenberg, F. DeMayo, M.J. Finegold, D. Medina, W.D. Tillet, J.O. Aspinall, G.R. Cunha, A.A. Donjacour, R.J. Matusik, J.M. Rosen, Prostate cancer in a transgenic mouse, Proc. Natl. acad. Sci. USA 92 (1995) 3439-3443.

70. J.R. Gingrich, R.J. Barrios, R.A. Morton, B.F. Boyce, F.J. DeMayo, M.J. Finegold, R. Angelopoulou, J.M. Rosen, N.M. Greenberg, Metastatic prostate cancer in a transgenic mouse, Cancer Res. 56 (1996) 4096-4102.

71. N. Masumori, T.Z. Thomas, P. Chaurand, T. Case, M. Paul, S. Kasper, R.M. Caprioli, T. Tsukamoto, S.B. Shappell, R.J. Matusik, A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential, Cancer Res. 61 (2001) 2239-3349.

72. J.S. Horoszewicz, S.S. Leong, E. Kawinski, J.P. Karr, H. Rosenthal, T.M. Chu, E.A. Mirand, G.P. Murphy, LNCaP model of human prostatic carcinoma, Cancer Res. 43 (1983) 1809-1818.

73. X.Y. Zhao, B. Boyle, A.V. Krishnan, N.M. Navone, D.M. Peehl, D. Feldman, Two mutations identified in the androgen receptor of the new human prostate cancer cell line MDA Pca 2a, J. Urol. 162 (1999) 2192-2199.

74. H. Miyamoto, C. Chang, Antiandrogens fail to block androstenedione-mutated androgen receptor transactivation in human prostate cancer cells, Int. J. Urol. 7 (2000) 32.

75. M. Gleave, J.T. Hsieh, C.A. Gao, A.C. Eschenbach, L.W. Chung, Acceleration of human prostate cancer growth *in vivo* by factors produced by prostate and bone fibroblasts, Cancer Res. 51 (1991) 3753-3761.

76. S. MacDonald, L. Brive, D.B. Agus, H.I. Sher, K.R. Ely, Ligand responsiveness in human prostate cancer: structural analysis of mutant androgen receptors from LNCaP and CWR22 tumors, Cancer Res. 60 (2000) 2317-2322.

77. P.C. Weijerman, J.J. Konig, S.T. Wong, H.G. Niesters, D.M. Peehl, Lipofection-mediated immortalization of human prostatic epithelial cells of normal and malignant origin using human papillomavirus type 18 DNA, Cancer Res. 54 (1994) 5579-5583.

78. S.R. Plymate, M. Tennant, R.S. Brinbaum, J.B. Thrasher, G. Chatta, J.L. Ware, The effect on the insulin-like growth factor system in human prostate epithelial cells of immortalization and transformation by simian virus -40 T antigen, J. Clin. Endocrinol. Metab. 81 (1996) 3709-3716.

79. V.L. Bae, C.K. Jackson-Cook, S.J. Maygarden, S.R. Plymate, J. Chen, J.L. Ware, Metastatic sublines of an SV40 large T antigen immortalized prostate epithelial cell line, Prostate 34 (1998) 275-282.

80. W.M. Van Weerden, C.M. de Ridder, C.L. Vandaasdonk, J.C. Romijn, T.H. van der Kwast, F.H. Schider, G.J. Van Steenbrugge, Development of seven new human prostate tumor xenograft models and their histological characterization, Am. J. Pathol. 149 (1996) 1055-1062.

Abbreviations

AEC	3-amino-9-ethyl-carbazole
ANOVA	Analysis of Variance
AR	Androgen Receptor
BPA	Bisphenol A
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumine
BW	Body weight
CDC6	Cell Division Cycle 6
CDK2/4	Cyclin Dependent Kinase 2/4
CV	Crystal Violet Assay
CWR22	androgen-dependent human prostate cancer cells
DDT	Dichlordiphenyltrichlormethan
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DU-145	Human prostate carcinoma, epithelial-like cell line
Earle's BSS	Earle's Balanced Salt Solution
EC	Epicatechin
ECG	Epicatechingallate
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EGC	Epigallocatechin
EGCG	(-)-Epigallocatechin-3-gallate
EGF	epidermal growth factor
EPA	Environmental Protection Agency
EPC	Early Prostate Cancer

Erb B1-Shc-ERK1/2	epidermal growth factor receptor-Src homologous and collagen-extracellular signal-regulated protein kinase 1/2
FACS	fluorescence activated cell sorting
FBS	Fetal bovine serum
FL	Flutamide®
H & E	Hematoxylin & Eosin
HGPIN	High grade prostatic intraepithelial neoplasia
IGF-1	Insulin-like Growth Factor-1
ITS-G	Insulin-transferrin-selenium-G
Ki-76	antigen (345, 395 kDa) preferentially expressed during all active phases of the cell cycle (G_1, S, G_2 and M phases), but absent in resting (G_0) cells
KG	Körpergewicht
LADY	umbrella term for several transgenic mice for prostate cancer
LGPIN	Low grade prostatic intraepithelial neoplasia
LH	Luteinizing hormone
LNCaP	Lymph Node Carcinoma of Prostate
LPB-Tag	Large probasin-large T-antigen
MEM	Minimum Essential Medium
MIB-5	antibody for demonstration of the Ki-67 antigen
NGS	Normal goat serum
OD	Optical density
OX	Orchiectomized rats
PBS	Phosphate buffered saline
PC	Prostate Cancer
PC-3	Prostate Cancer-3
PC-346C	Prostate Cancer-346 Cancer
PCNA	Proliferating Cell Nuclear Antigen
PC-SPES	Prostate Cancer-Hope

PI	Propidium Iodide
PSA	Prostate Specific Antigen
RF-C	Reduced Folate-Carrier
SAB	StreptAvidin Biotin
SD	Standard Deviation
SE	Standard Error
SV40	Simian Virus 40
T3	Testosterone
TP	Testosterone Propionate
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TUNEL	TdT-mediated dUTP-biotin nick end labeling
TX	Triton X
UV	Ultraviolet

Scientific Communication

Publications

NISHINO T, WEDEL T, SCHMITT O, BÜHLMEYER K, SCHÖNFELDER M, HIRTREITER C, SCHULZ T, KÜHNEL W, MICHNA H: ANDROGEN-DEPENDENT MORPHOLOGY OF PROSTATES AND SEMINAL VESICLES IN THE HERSHBERGER ASSAY: EVALUATION OF IMMUNOHISTOCHEMICAL AND MORPHOMETRIC PARAMETERS. ANNALS OF ANATOMY 186, P 247-253 (2004).

NISHINO T, WEDEL T, SCHMITT O, SCHÖNFELDER M, HIRTREITER C, SCHULZ T, KÜHNEL W, MICHNA H: THE XENOESTROGEN BISPHENOL A IN THE HERSHBERGER ASSAY: ANDROGEN RECEPTOR REGULATION AND MORPHOMETRICAL REACTIONS INDICATE NO MAJOR EFFECTS. JOURNAL OF STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY 98, P 155-163 (2006).

西野津之 [1]、トーステン シュルツ [1]、西野幸重 [2]、ホルスト ミヒナ [1]: ラットの前立腺及び精嚢の形態及び免疫化学反応のアンドロゲン感受性について：殊にアンドロゲン受容体に及ぼすビスノール-A の影響 (*in progress*)

MICHNA H, HILBER K, NISHINO T, PETERS C, SELG PJ, SCHULZ T: WARUM MÄNNER UNGESUND LEBEN UND FRAUEN GESÜNDER STERBEN. I. INTERDISZIPLINÄRER KONGRESS JUNGE NATURWISSENSCHAFT UND PRAXIS, 11. – 13. JUNI 2003 IN MÜNCHEN, GESUNDHEIT FÖRDERN – KRANKHEIT HEILEN, HANNS MARTIN SCHLEYER-STIFTUNG, PROCEEDINGS 62 P 45-68.

NISHINO T, BÜHLMEYER K, PETERS C, SCHÖNFELDER M, SCHULZ T, MICHNA H: PHYTO- UND XENOÖSTROGENE: ENDOKRINE UND IMMUNOLOGISCHE WIRKUNGEN-ZUR BEDEUTUNG EINER RATIONALEN ABSCHÄTZUNG VON ERWÜNSCHTEN EFFEKTEN UND RISIKEN. 2. WISSENSCHAFTSTAGUNG WEIHENSTEPHAN „LEBENSMITTEL UND GESUNDHEIT“ PROCEEDINGS P 97-109 (2003).

NISHINO T, MICHNA H: ZENTRALE UND PERIPHERE WIRKUNGEN VON NATÜRLICHEN UND SYNTHETISCHEN GLUKOKORTIKOIDEN UND DEREN RELEVANZ IM DOPING. GLUKOKORTIKOSTEROIDE IN DER DOPINGFORSCHUNG BUNDESINSTITUT FÜR SPORTWISSENSCHAFT P BAND 06 P 57-74 (2007).

KAUFMANN K, POJAROVÁ M, VOGEL S, LIEBL R, GASTPAR R, GROSS D, NISHINO T, PFALLER T, VON ANGERER E: ANTIMITOTIC ACTIVITIES OF 2-PHENYLINDOLE-3-CARBALDEHYDES IN HUMAN BREAST CANCER CELLS. BIOORGANIC & MEDICINAL CHEMISTRY, VOL. 15, ISSUE 15, P 5122-5136 (2007).

Oral Presentations

NISHINO T, SCHÖNFELDER M, SCHULZ T, MICHNA H, TENNISWOOD M: MAJOR GREEN TEA POLYPHENOLIC PLANT CONSTITUENT (-)–EPIGALLOCATECHIN-3-GALLATE (EGCG) INDUCES RAPID CELL DEATH IN HUMAN PROSTATE CANCER CELL LINES (LNCAP, PC-346C AND PC-3) AND PC-346C / PC-3 XENOGRAFT. 101. VERSAMMLUNG DER ANATOMISCHEN GESELLSCHAFT AM 07.-10. APRIL 2006 IN FREIBURG, ANN ANAT. 101 (IM DRUCK).

MICHNA H, HILBER K, NISHINO T, PETERS C, SELG PJ, SCHULZ T: WARUM MÄNNER UNGESUND LEBEN UND FRAUEN GESÜNDER STERBEN. I. INTERDISZIPLINÄRER KONGRESS JUNGE NATURWISSENSCHAFT UND PRAXIS, 11. – 13. JUNI 2003 IN MÜNCHEN, GESUNDHEIT FÖRDERN – KRANKHEIT HEILEN, HANNS MARTIN SCHLEYER-STIFTUNG, PROCEEDINGS 62 P 45-68.

MICHNA H, BÜHLMEYER K, PETERS C, NISHINO T, SCHULZ T: PHYTO- UND XENOÖSTROGENE: ENDOKRINE UND IMMUNOLOGISCHE WIRKUNGEN-ZUR BEDEUTUNG EINER RATIONALEN ABSCHÄTZUNG VON ERWÜNSCHTEN EFFEKTEN UND RISIKEN. 2. WISSENSCHAFTSTAGUNG WEIHENSTEPHAN „LEBENSMITTEL UND GESUNDHEIT“ PROCEEDINGS P 97-109 (2003).

MICHNA H, NISHINO T: HORMONWIRKSAME UMWELT(SCHAD)STOFFE. POSTGRADUALSTUDIUM TOXIKOLOGIE, UNIVERSITÄT LEIPZIG (2005).

Posters and Abstracts

NISHINO T, MASKUS M, LAUDENBACH U, BÜHLMEYER K, VOβ A, SCHULZ T, MICHNA H: ANDROGEN RECEPTOR REGULATION AND THE INFLUENCE OF BISPHENOL A IN MALE REPRODUCTIVE ORGANS IN RATS (WISTAR): MOLECULAR AND IMMUNOHISTOCHEMICAL, MORPHOMETRIC ANALYSIS. 15TH INTERNATIONAL SYMPOSIUM OF THE JOURNAL OF STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY AM 17.-20 MAI 2002 IN MŰNCHEN, ABSTRACTBAND 257-P.

NISHINO T, SCHMITT O, WEDEL T, STRAUCHMANN H, BÜHLMEYER K, SCHULZ T, MICHNA H: REGULATION OF (ANTI-) ANDROGENIC PARAMETERS AND THE EFFECT OF BPA ON PROSTATE AND SEMINAL VESICLE IN RATS: IMMUNOHISTOLOGICAL, MORPHOMETRIC AND MOLECULAR STUDIES. 19. ARBEITSTAGUNG DER ANATOMISCHEN GESELLSCHAFT AM 01.-04.OKTOBER 2002 IN WŰRZBURG, ABSTRACT BAND P 5.

NISHINO T, SCHULZ T, BÜHLMEYER K, MICHNA H: IMMUNOHISTOCHEMICAL AND MORPHOMETRIC EVALUATION OF ANDROGEN RECEPTOR REGULATION IN PROSTATE AND SEMINAL VESICLE OF BPA-TREATED MALE WISTAR RATS. PROCEEDINGS OF THE INTERNATIONAL CONGRESS ON HORMONAL STEROIDS AND HORMONES AND CANCER (11TH ICHS AND 7TH ICHC) AM 21.-25. OKTOBER 2002 IN FUKUOKA, ABSTRACT BAND P 136.

NISHINO T, SCHMITT T, WEDEL T, STRAUCHMANN H, BÜHLMEYER K, SCHULZ T, MICHNA H: EFFEKTE VON BISPHENOL A AUF DEN MÄNNLICHEN GENITALTRAKT: MORPHOLOGISCHE, IMMUNHISTOCHEMISCHE UND MOLEKULARE STUDIEN. 2. WISSENSCHAFTSTAGUNG WEIHENSTEPHAN „LEBENSMITTEL UND GESUNDHEIT“ AM 24.- 26. FEBRUAR 2003 IN FREISING, ABSTRACT BAND P 146.

NISHINO T, BÜHLMEYER K, SCHULZ T, MICHNA H: DIE INDUSTRIECHEMIKALIE BISPHENOL A: ANWENDUNGSGEBIETE, RISIKOPOTENTIAL UND PRÜFUNG AUF (ANTI-) ANDROGENE WIRKUNG. 2. WISSENSCHAFTSTAGUNG WEIHENSTEPHAN „LEBENSMITTEL UND GESUNDHEIT“ AM 24.- 26. FEBRUAR 2003 IN FREISING, ABSTRACT BAND P 148.

NISHINO T, SCHMITT O, WEDEL T, STRAUCHMANN H, BÜHLMEYER K, SCHULZ T, MICHNA H: ANDROGEN-DEPENDENT MORPHOLOGY OF PROSTATE AND SEMINAL VESICLE: IMMUNOHISTOCHEMICAL AND MORPHOMETRIC PARAMETERS. 98. VERSAMMLUNG DER ANATOMISCHEN GESELLSCHAFT AM 28.-31. MÄRZ 2003 IN DRESDEN, ANN ANAT 98, (2003).

NISHINO T, WEDEL T, SCHMITT O, STRAUCHMANN H, BÜHLMEYER K, SCHÖNFELDER M, SCHULZ T, KÜHNEL W, MICHNA H: ANDROGENIC REACTIONS IN THE RAT PROSTATE: VALIDATION OF PROLIFERATION MARKERS AND ANDROGEN RECEPTOR STAINING PATTERN AND THE EFFECTS OF THE "XENOESTROGEN" BPA. 20. ARBEITSTAGUNG DER ANATOMISCHEN GESELLSCHAFT AM 01.-03.OKTOBER 2003 IN WŰRZBURG, ABSTRACT BAND P 6.

NISHINO T, BÜHLMEYER K, SCHÖNFELDER M, SCHULZ T, KÜHNEL W, MICHNA H: (ANTI-) ANDROGENIC REACTIONS IN THE RAT PROSTATE AND SEMINAL VESICLE: VALIDATION OF PROLIFERATION MARKERS AND ANDROGEN RECEPTOR STAINING PATTERN AND THE EFFECTS OF THE „XENOESTROGEN" BPA IN THE WISTAR RAT. 48.SYMPOSIUM DER DGE AM 03.-06. MÄRZ 2004 IN DRESDEN, ABSTRACT BAND P 263.

NISHINO T, WEDEL T, SCHMITT O, BÜHLMEYER K, SCHÖNFELDER M, SCHULZ T, KÜHNEL W, MICHNA H: EVALUATION OF THE (ANTI-) ANDROGENIC POTENTIAL OF THE XENOESTROGEN BPA ON THE REPRODUCTIVE TRACT IN WISTAR RATS. 45. FRŰHJAHRSTAGUNG DER DGPT AM 09.-11. MÄRZ 2004 IN MAINZ, ABSTRACT BAND P 215.

MICHNA H, NISHINO T, WEDEL T, SCHMITT O, BÜHLMEYER K, SCHÖNFELDER M, SCHULZ T, KÜHNEL W: HOW THE XENOESTROGEN BPA INFLUENCES GENE ACTIVITY IN (FE-) MALE ENDOCRINE TARGET ORGANS. PROCEEDINGS (POSTER) OF THE ANNUAL SPRING MEETING, GERMAN SOCIETY FOR BIOCHMISTRY AND MOLECULAR BIOLOGY (GBM) AM 01.-03. APRIL 2004 IN MOSBACH (BADEN), MOSBACHER KOLLOQUIUM (WWW.GBM-ONLINE.DE) P 37.

NISHINO T, BÜHLMEYER K, SCHÖNFELDER M, SCHULZ T, MICHNA H: (ANTI-) ANDROGENIC EFFECTS OF THE "XENOESTROGEN" BPA IN THE RAT PROSTATE: VALIDATION OF PROLIFERATION MARKERS AND ANDROGEN RECEPTOR STAINING PATTERN. 16TH INTERNATIONAL SYMPOSIUM OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY AM 05.-08. JUNI 2004 IN SEEFELD, ABSTRACT BAND P 51.

NISHINO T, WEDEL T, SCHMITT O, BÜHLMEYER K, SCHÖNFELDER M, SCHULZ T, KÜHNEL W, MICHNA H: THE (ANTI-) ANDROGENIC POTENTIAL OF THE XENOESTROGEN BISPHENOL A: EVALUATION OF "ULTRALOW" AND (SUPRA-) PHARMACOLOGICAL DOSES OF BPA IN THE REPRODUCTIVE TRACT OF WISTAR RATS. 86TH ANNUAL MEETING OF THE ENDOCRINE SOCIETY AM 16.-19. JUNI 2004 IN NEW ORLEANS, ABSTRACT BAND P 230.

NISHINO T, SCHULZ T, BÜHLMEYER K, MICHNA H: IMMUNOHISTOCHEMICAL AND MORPHOMETRIC EVALUATION OF ANDROGEN RECEPTOR REGULATION AND MORPHOMETRIC PARAMETERS IN PROSTATE AND SEMINAL VESICLE FOLLOWING TREATMENT WITH BPA. 12TH INTERNATIONAL SOCIETY OF ENDOCRINOLOGY AM 01.-09.-SEPTEMBER 2004 IN LISSABON, ABSTRACT BAND P 163.

NISHINO T, ROY S, WHYTE L, DENIS C, JEFFREY R, BÜHLMEYER K, SCHÖNFELDER M, SCHULZ T, MICHNA H, TENNISWOOD M: TREATMENT WITH (-)–EPIGALLOCATECHIN-3-GALLATE (EGCG) INDUCES CELL CYCLE ARREST AND APOPTOSIS IN HUMAN PROSTATE CANCER CELL LINES (LNCAP, PC-346C). 100TH VERSAMMLUNG DER ANATOMISCHEN GESELLSCHAFT AM 11.-14.MÄRZ 2005 IN LEIPZIG, ABSTRACT BAND P 146.

NISHINO T, ROY S, WHYTE L, DENIS C, JEFFREY R, BÜHLMEYER K, SCHULZ T, MICHNA H, TENNISWOOD M: THE GREEN TEA FLAVONOID (POLYPHENOLIC PLANT CONSTITUENT) (-)–EPIGALLOCATECHIN-3-GALLATE (EGCG) INDUCES RAPID CELL DEATH IN HUMAN PROSTATE CANCER CELL LINES (LNCAP, PC-346C). 13TH AEK/AIO CANCER CONGRESS AM 13.-16. MÄRZ 2005 IN WŰRZBURG, ABSTRACT BAND P 36.

BÜHLMEYER K, DANIEL H, DÖRING F, NISHINO T, SCHÖNFELDER M, SCHULZ T, MICHNA H: COLON CANCER AND EXERCISE: MORPHOLOGICAL AND MOLECULAR REACTIONS IN THE COLON MUCOSA FOLLOWING PHYSICAL EXERCISE. 13TH INTERNATIONAL AEK/AIO CANCER CONGRESS OF THE GERMAN CANCER SOCIETY AM 13.-16.03.2005 IN WÜRZBURG, ONKOLOGIE - INTERNATIONAL JOURNAL FOR CANCER RESEARCH AND TREATMENT 28(2), P 50.

MICHNA H, NISHINO T, SELG PJ: ENDOCRINE PHARMACOLOGICAL CHARACTERIZATION OF THE "DESIGNER DRUG" TETRAHYDROGESTRINONE (THG) 10TH ANNUAL CONGRESS OF THE EUROPEAN COLLEGE OF SPORT SCIENCE, 13.-16.07.2005 IN BELGRAD, SERBIEN-MONTENEGRO, ABSTRACT BOOK P 259.

NISHINO T, ROY S, WHYTE L, DENIS C, JEFFREY R, BÜHLMEYER K, SCHULZ T, MICHNA H, TENNISWOOD M: THE GREEN TEA FLAVONOID (POLYPHENOLIC PLANT CONSTITUENT) (-)–EPIGALLOCATECHIN-3-GALLATE (EGCG) INDUCES RAPID CELL DEATH IN HUMAN PROSTATE CANCER CELL LINES (LNCAP, PC-346C AND PC-3). 7TH EUROPEAN CONGRESS OF ENDOCRINOLOGY AM 03.-07. SEPTEMBER 2005 IN GÖTEBORG, ABSTRACT BOOK P 235, P2-253.

MICHNA H, SCHULZ T, NISHINO T: THE XENOESTROGEN BISPHENOL A: EFFECTS OF (ULTRA-)LOW AND HIGH DOSES ON PROLIFERATION MARKERS AND CELL GROWTH IN PROSTATE AND SEMINAL VESICLE. 7TH EUROPEAN CONGRESS OF ENDOCRINOLOGY AM 03.-07. SEPTEMBER 2005 IN GÖTEBORG, ABSTRACT BOOK P 124, P1-171.

MICHNA H, SELG PJ, NISHINO T: ENDOKRINPHARMAKOLOGISCHE CHARAKTERISIERUNG VON DESOXYMETHYLTESTOSTERON (DMT). 39. KONGRESS FÜR SPORTMEDIZIN UND PRÄVENTION - BEWEGUNG IST LEBEN - AM 14. BIS 17.09.2005 IN HAMBURG, DT Z SPORTMED 56 (7/8), 210 (2005).

SCHULZ T, MOUGIOS V, NISHINO T, NISHINO Y, SCHÖNFELDER M, MICHNA H: EIN PHARMAKOLOGISCHES TESTSYSTEM ZUR PRÜFUNG ZENTRALER EFFEKT VON (ANTI-) HORMONEN: DAS LAUFTRAININGSMODELL IN DER RATTE. 39. KONGRESS FÜR SPORTMEDIZIN UND PRÄVENTION - BEWEGUNG IST LEBEN - AM 14. BIS 17. SEPTEMBER 2005 IN HAMBURG, DT Z SPORTMED 56 (7/8), 200 (2005).

BUEHLMEYER K, DANIEL H, DOERING F, NISHINO T, SCHOENFELDER M, SCHULZ T, MICHNA H: HORMONAL SERUM CHANGES, MORPHOLOGICAL AND MOLECULAR REACTIONS IN THE COLON FOLLOWING PHYSICAL EXERCISE. 22. ARBEITSTAGUNG DER ANATOMISCHEN GESELLSCHAFT AM 28.-30. SEPTEMBER 2005 IN WÜRZBURG, (IM DRUCK).

NISHINO T, SCHULZ T, SCHÖNFELDER M, MICHNA H, TENNISWOOD M: CELL CYCLE ARREST AND APOPTOSIS INDUCTION IN HUMAN PROSTATE CANCER CELL LINES (PC-346C, LNCaP AND PC-3) BY THE GREEN TEA INGREDIENT EGCG (EPIGALLOCATECHINGALLATE). 50. SYMPOSIUM DER DEUTSCHEN GESELLSCHAFT FÜR ENDOKRINOLOGIE & 23RD MEETING OF THE DUTCH ENDOCRINE SOCIETY AM 01.-04. MÄRZ 2006 IN ESSEN, ABSTRACTBAND (IM DRUCK).

NISHINO T, SCHÖNFELDER M, SCHULZ T, MICHNA H, TENNISWOOD M: GREEN TEA POLYPHENOLIC PLANT CONSTITUENT (-)-EPIGALLOCATECHIN-3-GALLATE (EGCG) INDUCES RAPID CELL DEATH IN HUMAN PROSTATE CANCER CELL LINES (LNCaP, PC-346C AND PC-3) AND PC-346C / PC-3 XENOGRAFTS. THE ENDOCRINE SOCIETY´S 88TH ANNUAL MEETING, 2006 JUNE 24 – 27 IN BOSTON, MASSACHUSETTS, ABSTRACT BOOK P3-224, P 721.

MICHNA H, SELG PJ AND NISHINO T: ENDOCRINE PHARMACOLOGICAL CHARACTERIZATION OF THE DOPING „DESIGNER“ STEROIDS NORBOLETHONE, DESOXYMETHYLTESTOSTERONE (DMT) AND TETRAHYDROGESTRINONE (THG). 17TH INTERNATIONAL SYMPOSIUM OF THE JOURNAL OF STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY, 2006 MAY 31ST – JUNE 3RD IN SEEFELD, TYROL, AUSTRIA, ABSTRACT BOOK P 21.

NISHINO T, SCHÖNFELDER M, SCHULZ T, MICHNA H: TREATMENT WITH THE XENOESTROGEN BPA IN WISTAR RATS INDICATE NO MAJOR EFFECTS. 17TH INTERNATIONAL SYMPOSIUM OF THE JOURNAL OF STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY, 2006 MAY 31ST – JUNE 3RD IN SEEFELD, TYROL, AUSTRIA, ABSTRACT BOOK P 16.

NISHINO T, SCHÖNFELDER M, SCHULZ T, MICHNA H, TENNISWOOD M: EFFECTS OF GREEN TEA ON EARLY STAGE PROSTATE CANCER IN PC-346C/PC-3 XENOGRAFTS. 17TH INTERNATIONAL SYMPOSIUM OF THE JOURNAL OF STEROID BIOCHEMISTRY & MOLECULAR BIOLOGY, 2006 MAY 31ST – JUNE 3RD IN SEEFELD, TYROL, AUSTRIA, ABSTRACT BOOK P 15.

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MICHNA H, NISHINO T, KÜHNEL W: GLUCOCORTICOIDS INDUCE ANDROGENIC ACTIVITY IN PROSTATE (CANCER CELLS). 23. ARBEITSTAGUNG DER ANATOMISCHEN GESELLSCHAFT IN WÜRZBURG 2006 SEPTEMBER 27TH – 29TH P 29, 121.

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Appendix

Appendix I. Nishino T, Wedel T, Schmitt O, Bühlmeyer K, Schönfelder M, Hirtreiter C, Schulz T, Kühnel W, Michna H: Androgen-dependent morphology of prostates and seminal vesicles in the Hershberger assay: Evaluation of immunohistochemical and morphometric parameters. Annals of Anatomy 186, p 247-253 (2004).

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Appendix III. Nishino T, Bühlmeyer K, Peters C, Schönfelder M, Schulz T, Michna H, Phyto- und Xenoöstrogene: Endokrine und immunologische Wirkungen – Zur Bedeutung einer rationalen Abschätzung von erwünschten Effekten und Risiken, 2. Wissenschaftstagung Weihenstephan „Lebensmittel und Gesundheit“ Proceedings P 97-109 (2003).

Appendix IV. Michna H, Hilber K, Nishino T, Peters C, Selg PJ, Schulz T: Warum Männer ungesund leben und Frauen gesünder sterben. i. Interdisziplinärer Kongress junge Naturwissenschaft und Praxis, 11. – 13. Juni 2003 in München, Gesundheit fördern – Krankheit heilen, Hanns Martin Schleyer-Stiftung, Proceedings 62 P 45-68.

Appendix V. Nishino T, Michna H: Zentrale und periphere Wirkungen von natürlichen und synthetischen Glukokortikoiden und deren Relevanz im Doping. Glukokortikosteroide in der Dopingforschung Bundesinstitut für Sportwissenschaft Band 06 P 57-74 (2007).

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Appendix I.

Nishino T, Wedel T, Schmitt O, Bühlmeyer K, Schönfelder M, Hirtreiter C, Schulz T, Kühnel W, Michna H: Androgen-dependent morphology of prostates and seminal vesicles in the Hershberger Assay: Evaluation of immunohistochemical and morphometric parameters.

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ANNALS OF ANATOMY

Androgen-dependent morphology of prostates and seminal vesicles in the Hershberger Assay: Evaluation of immunohistochemical and morphometric parameters*

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Summary. The aim of this study was to evaluate androgen-like effects using immunohistochemical and morphometric methods. Therefore, orchiectomized Wistar rats ($n \geq 13$) were treated s.c. with 1 mg/kg bw/day testosterone propionate (TP) for 7 days and compared to orchiectomized rats without TP substitution (OX) and to an untreated intact control group. Sections obtained from prostates and seminal vesicles were stained with polyclonal and monoclonal antibodies against the androgen receptor (AR) and assessed densitometrically (intensity of the immunoreaction) and morphometrically (epithelial height, luminal area). TP caused an enhancement of staining intensity and an increase in organ weights, epithelial height and luminal area. The use of proliferation markers (PCNA, MIB-5) showed also a highly significant increase of immunoreactive cells in TP-substituted orchiectomized rats compared with the OX group. Based on the present data, the densitometric analysis of AR-immunoreactivity as well as the assessment of proliferation markers, epithelial height and luminal area proved to be sensitive parameters for the evaluation of androgen effects on prostates and seminal vesicles. In further studies these parameters will be used to test several industrial xenooestrogens as well as phytooestrogens on their possible androgenic capacity.

Key words: Androgenic reactions – Androgen receptor – Prostate – Seminal vesicle – Densitometry – Morphometry – Proliferation markers

Introduction

The rodent Hershberger Assay using castrated peripubertal male rats measures the androgenic or anti-androgenic effects of test chemicals on several accessory glands/tissues (e.g. ventral and dorso-lateral prostate and seminal vesicles with coagulating glands) by means of organ weight measurements (Hershberger et al. 1953). Generally, accessory sex glands and tissues are dependent upon androgen stimulation to gain and maintain weight during or after puberty. If endogenous testicular sources of androgens are removed, exogenous sources of androgens are necessary to increase or maintain the weights of these tissues (Ashby and Lefevre 2000; Kim et al. 2002; Breckwoldt et al. 1991). All anabolic/(anti-) androgenic compounds (drugs) in clinical use have been screened by means of this *in vivo* bioassay (Kühnel 1970; Kühnel 1974). This assay is one of the assays in the proposed

* Dedicated to Professor Dr. med. Bernhard Tillmann on the occasion of his 65th birthday.

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Tier I screening battery by EDSTAC (Endocrine Disruptor Screening and Testing Advisory Committee). Tier I sites are those that have greater potential to require long-term or emergency cleanup work under the Federal Superfund program. These are sites which have a release of a hazardous substance, pollutant or contaminant that has caused, or is likely to cause exposure to humans or contamination of a sensitive environment. They typically involve contamination of drinking water, surface water, air or soils which have either caused or are likely to cause exposure to nearby populations, or have contaminated, or are likely to contaminate sensitive environments such as for example wetlands, national parks and habitats of endangered species (EDSTAC, EPA 1998).

However, the measurements of body and organ weight deliver only a limited amount of information, especially if this assay will be used to carry out a more subtle assessment of the androgenic properties of an unknown substance. Additional parameters such as morphometric and qualitative data are therefore required to determine the androgenicity of a given substance, in particular if the effects are of low degree.

The present study was undertaken to analyze these additional parameters and to assess their sensitivity for the evaluation of androgen-like effects using immunohistochemical and morphometric techniques.

Material and methods

Animals and housing. Wistar rats (male HdrBrHan from Harlan Winkelmann, Borchen, Germany), weighing about 150 g (age of 2 weeks) were randomly separated into different groups. They received tap water and ssniff R 10, laboratory standard rat diet (in pellet form) *ad libitum* (ssniff Spezialdiäten GmbH, Soest, Germany). Groups of 2–4 animals were kept in Makrolon cages type IV with ssniff bedding (3/4 Faser) at 22 ± 3 °C, a relative humidity of 30–70% and artificial 12 h light. After acclimatization, animals were orchiectomized under Ketanest/Rompun-anesthesia (Ketanest 10 mg/kg bw from Parke-Davis, Berlin, Germany and Rompun 2 mg/kg bw from Bayer AG, Leverkusen, Germany).

Treatment of animals. Animals were partitioned into three groups ($n \geq 13$ in each group). Two groups were orchiectomized. Seven days after orchiectomy one group was treated subcutaneously with testosterone propionate (TP) in arachis oil for seven days (kindly provided by Schering AG) and compared to vehicle treated, orchiectomized rats without TP substitution (OX) and to a vehicle treated intact control group (intact). After the treatment animals were sacrificed by decapitation. Seminal vesicles and prostates were harvested surgically, weighed and immediately fixed in 4% neutral buffered paraformaldehyde.

Immunohistochemistry. After fixation the specimens (prostate, seminal vesicle) were dehydrated in ascending series of alcohol, embedded in paraffin and cut in sections of 5 µm thickness (10 sections per specimen). For the immunohistochemical visualization of androgen receptor and proliferation markers the following primary antibodies were applied: anti-androgen-receptor (1:100, sc-815, rabbit polyclonal, Santa Cruz Biotechnology, California, USA), anti-androgen-receptor (1:100, 554224, mouse monoclonal, BD PharMingen, Germany), anti-PCNA (1:200, PC-10, mouse monoclonal, Novocastra, New Castle, United Kingdom) and anti-MIB-5 (1:100, M 7248 mouse monoclonal, DakoCytomation, Denmark).

After deparaffinization in xylene and descending series of alcohol the sections were treated in 0.3% hydrogen peroxide in distilled water to quench endogenous peroxidase activity, rinsed 2 times in 0.01 M phosphate buffered saline (PBS) containing 0.1% Triton X-100 (PBS-TX), incubated for 10 min in 10% normal goat serum (NGS) in PBS-TX to block nonspecific antigen binding sites, incubated with the above mentioned primary antibodies diluted in PBS containing 1% NGS overnight, rinsed in PBS, incubated with biotinylated secondary antibodies (1:200, Dako, Denmark) for 1 h, rinsed in PBS and incubated with StreptAvidin-Biotin (SAB)-Complex (Biocare Medical, USA) conjugated to horseradish peroxidase for 1 h. Peroxidase activity was revealed with the chromogen 3-amino-9-ethyl-carbazole (AEC, Zymed, USA) for 2 minutes. Sections stained with proliferation markers were counterstained with Mayer`s hematoxylin for 10 minutes. Omission of primary antibodies resulted in the absence of a specific labelling.

Morphometry and densitometry. Intensity of immunohistochemical staining was determined densitometrically, while epithelial height and luminal area of the glandular ducts were measured morphometrically (KS 100, KS RUN, Zeiss-Vision, Jena, Germany). Microscopy was performed with an Axiophot light microscope (Zeiss, Jena, Germany) equipped with a high resolution scanner camera (Axiocam, Zeiss, Germany).

All images have a uniform size of 1300 × 1030 pixel. Since the images were generated by using a 20× objective and 1.0 Optovar the final resolution of the edge lengths of one pixel in the resulting image is 0.32 µm. This resolution was adequate for deciding which profile of glandular ductus in the field of vision is suitable for densitometric measurements. By using densitometry, gray values were transformed pixel by pixel into optical densities (Oberholzer 1996), followed by calculation of the sum, mean and standard deviation. Densitometry was performed in gray level images with a dynamic range of 256 intensities. Each pixel had a value between 0 and 255, where 0 is darkest (black) and 255 is lightest (white). We used the software package KS 400 for image analysis.

The glandular duct to be measured was centered into the optical field at the resolution mentioned above. After focusing and adaption of light intensity (3200 K) the section was removed and a white image (w) was generated iteratively. The adaption of light intensity was carried out by the following procedure. Firstly the illumination was adjusted, so that the objects of interest could be clearly identified. Thereafter, an image without any histologic object was grabed and the mean pixel intensity was calculated. If the mean pixel intensity differed much from the pixel intensity obtained from preceding measurements, the light intensity was adapted until the mean pixel intensity adapted similar values to those in the previous measurements. The mean gray value of the white image was determined for each section in order to obtain the same illumination condition within each specimen examined. After white image calibration the glandular duct of interest was grabed and its immunoreactive epithelium was surrounded interactively. Within this defined epithelial area the mean optical density (OD) and the variance of OD were determined. Five measurements were performed within each section. Five sections were examined per animal resulting in 25 measurements for each animal. The mean values were calculated and compared between the different groups.

For morphometric measurements the software package KS 100 3.0 (Zeiss-Vision, Jena, Germany) was used. The epithelial height was determined by using a 40× objective and 1.0 optovar. For the

determination of the luminal area a 10× objective and 1.0 optovar were used. The quantitative assessment of proliferating cells was performed using a 40× objective and 1.0 optovar. We have counted 1000 of those cells per section exhibiting nuclei.

A two-sided t-test at a significance level of $p < 0.05$ was applied for statistical comparison. Data were depicted as means ± standard deviation (SD).

Results

Hershberger Assay. Measurement of the body weight yielded no significant differences between the intact group ($n \geq 13$), OX group ($n \geq 13$) and TP group ($n \geq 13$). However, examination of wet weights (absolute and relative) of prostates and seminal vesicles showed that the orchiectomy in the OX group induced a significant (t-test, $p \leq 0.05$, $n \geq 13$) decrease in organ wet weights in comparison to the intact group, which was nearly reversed by an administration of a pharmacological dose of TP in the TP group (Fig. 1).

Densitometric analysis. Using the monoclonal antibody the staining intensity of AR in the prostates revealed the following optical density values: 104 ± 20 (intact), 56 ± 17 (OX), and 77 ± 22 (TP). Statistical analysis confirmed that the intensity of staining was significantly higher in both the intact and TP group (t-test, $p \leq 0.05$, $n \geq 13$) compared to the OX group (Figs. 2, 7 A–C).

The staining intensity after incubation with the polyclonal antibody against AR was 75 ± 21 (intact), 43 ± 12 (OX) and 61 ± 24 (TP) for the prostates and 42 ± 17 (intact), 11 ± 10 (OX) and 19 ± 11 (TP) 11 ± 10 for seminal vesicles, respectively. In the prostate tissues the intensity of staining obtained by the polyclonal antibody was significantly higher in both the intact and TP group (t-test, $p \leq 0.05$, $n \geq 13$) compared to the OX group.

The data provide evidence that orchiectomy results in a reduced staining intensity of AR, whereas substitution with TP enhances the immunoreactive signal of AR.

Morphometric analysis. The mean epithelial height of prostate glands measured 17 ± 2 μm in both the intact and TP group, whereas the mean epithelial height was significantly (t-test, $p \leq 0.05$, $n \geq 13$) decreased to 11 ± 1 μm in the OX group. Similar significant (t-test, $p \leq 0.05$, $n \geq 13$) data were obtained for the epithelial height of seminal vesicle glands: 18 ± 3 μm (intact), 9 ± 2 μm (OX) and 15 ± 2 μm (TP), respectively (Fig. 3).

The mean luminal area of prostate glands was 131.206 ± 40.000 μm^2 in the intact group, 6.559 ± 5 000 μm^2 in the OX group and 86.892 ± 30.000 μm^2 in the TP group. The luminal area in the OX group was significantly (t-test, $p \leq 0.05$, $n \geq 13$) reduced compared to the intact und TP groups. In seminal vesicles the mean luminal area measured 113.188 ± 80.000 μm^2 (intact), 7.476 ± 5.000 μm^2 (OX) and 150.769 ± 86.000 μm^2 (TP), respectively. Similarly as observed for the prostates, the luminal area in the OX group was significantly (t-test, $p \leq 0.05$, $n \geq 13$) reduced compared to the intact and TP group (Fig. 4).

These morphologic observations clearly reveal that orchiectomy causes a substantial reduction of both epithelial height and luminal area of prostates and seminal vesicles. If TP is substituted both parameters return to values similar to those found in the intact group.

Cell proliferation. The assessment of cell proliferation markers yielded the following data in the rat prostates: The percentage of immunoreactive epithelial cells for MIB-5 was 85 ± 9% in the intact group, 9 ± 1% in the OX group and 90 ± 2% in the TP group. The percentage of

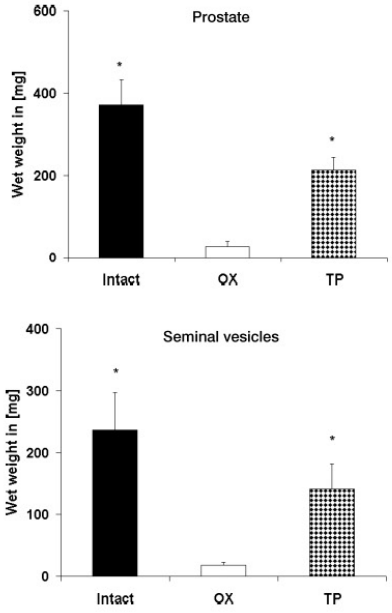


Fig. 1. Comparison of absolute wet weights of prostate and seminal vesicles between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

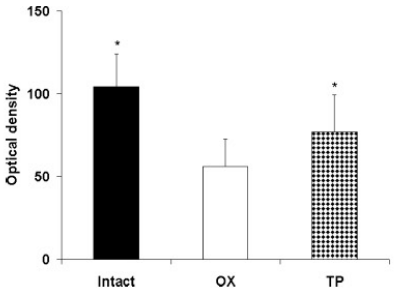
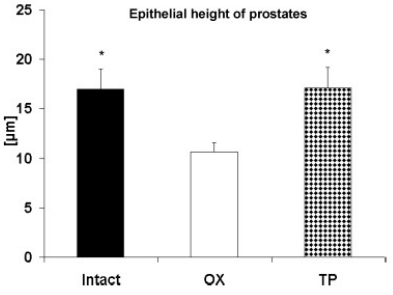


Fig. 2. Comparison of densitometric values between the intact group, OX group and TP group after immunohistochemical staining of androgen receptor in the prostate using a monoclonal antibody. Asterisks indicate statistically significant differences.



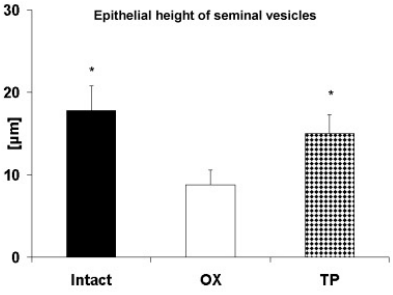


Fig. 3. Comparison of epithelial height of prostate and seminal vesicles between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

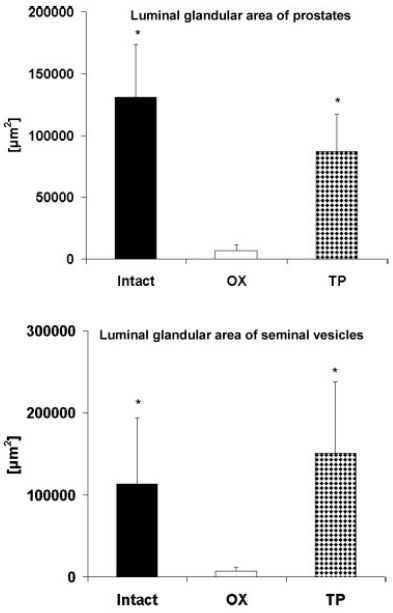


Fig. 4. Comparison of luminal glandular area of prostate and seminal vesicles between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

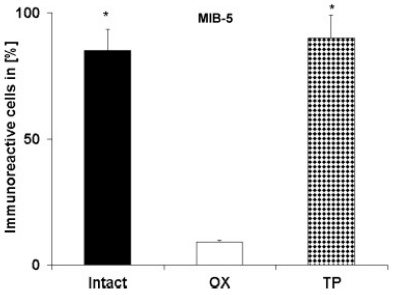


Fig. 5. Quantitative comparison of MIB-5-immunoreactive epithelial prostatic cells between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

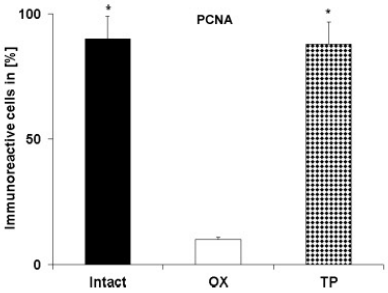


Fig. 6. Quantitative comparison of PCNA-immunoreactive epithelial prostatic cells between the intact group, OX group and TP group. Asterisks indicate statistically significant differences.

immunoreactive epithelial cells for MIB-5 in the OX group was significantly (t-test, $p \leq 0.05$, $n \geq 13$) reduced compared to the intact und TP group (Figs. 5, 7 D–F). Similar results were obtained for the relative amount of cells immunoreactive for PCNA: $90 \pm 9\%$ in the intact group, $10 \pm 2\%$ in the OX group and $88 \pm 9\%$ in the TP group. The percentage of epithelial cells immunoreactive for PCNA in the OX group was significantly (t-test, $p \leq 0.05$, $n \geq 13$) reduced compared to the intact und TP group (Figs. 6, 7 G–I).

Thus, whereas orchiectomy caused a considerable decrease in cell proliferation, administration of TP could reverse this effect and induced a cell proliferation index similar to the intact group.

Discussion

Hershberger Assay. Although the Hershberger assay (Hershberger et al. 1953) is a valid quantitative method for evaluating androgenic or anti-androgenic properties of substances by measuring the organ weight of seminal vesicles and prostates, the findings obtained by this assay provide only limited information on the specifity of the

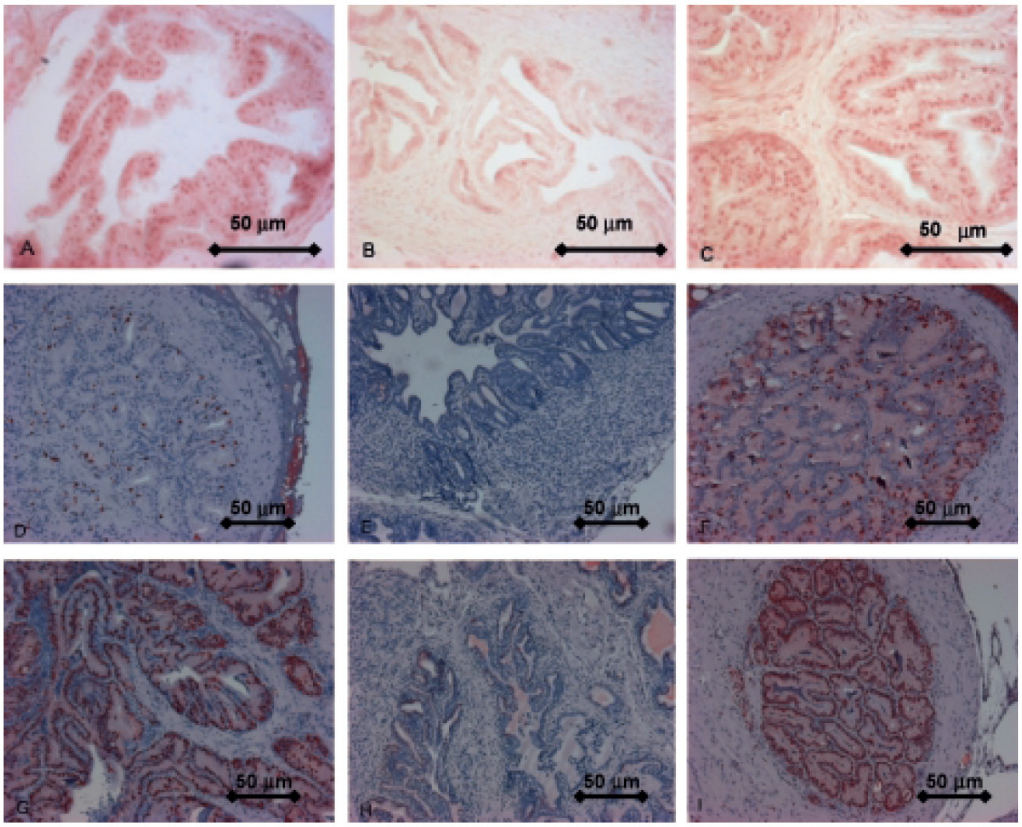


Fig. 7. All panels show photographs of the prostate. Zones are described according to McNeal (McNeal 1988).
A–C: Immunohistochemical staining of AR (monoclonal antibody) showing the transition zone of the intact group (A), OX group (B) and TP group (C). Original magnificaton 20×.
D–F: Immunohistochemical staining of MIB-5 showing the peripheral zone of the intact group (D), OX group (E) and TP group (F). Original magnification 10×.
G–I: Immunohistochemical staining of PCNA showing the transiton zone of he intact group (G), OX group (H) and TP group (I). Original magnificaton 10×.

observed effects. For example, the growth of seminal vesicles can be stimulated not only by androgens but also by oestrogenic substances, well known as a paradoxical effect of oestrogens (de Jongh 1935; de Jongh 1937; Freud 1933).

Morphologic and functional analysis of cellular parameters in male accessory organs may allow a more subtle and reliable assessment of the (anti-) androgenicity of substances; in previous studies we analyzed the regulation of tenascin expression (Vollmer et al. 1994). Since the amount of nuclear AR present in the rat prostate has been demonstrated to be influenced by androgens (Moore et al. 1979; Blondeau et al. 1982), a densitometric analysis of AR-immunoreactive cells in prostates and seminal vesicles was performed by using immunohistochemical methods (Huggins and Hodges 2002; Rolf and Nieschlag 1998).

Densitometric analysis. It is known (Nabors et al. 1988; Mize et al. 1988) that the concentrations of biochemically active substances can be estimated from the optical density of the immunoreactive signal. As a result, we found a marked decrease in staining intensity of AR-positive cells after orchiectomy in comparison to the control group. This effect of orchiectomy was mostly reversed by an administration of a pharmacological dose of TP. The advantages of a computer-assisted densitometry are a faster scoring procedure of sections from large series and a higher reliability. However, the disadvantage of a semiquantitative approach is the possibility that relevant signals can easily be missed, so that comparative studies should be based on rather robust signals (De Boer et al. 2001).

Morphologic alterations. Morphologic or functional changes in the accessory reproductive organs have mainly

been used as hormone indicators. The main accessory glands of the male rat are the prostate and the seminal vesicle. The induction of tremendous size changes in these glands in castrated immature animals has been used for the assay of androgenic activity by Tschopp (Tschopp 1936).

The ventral prostate is the most sensitive region among the accessory glands in rats. After castration at birth the accessory glands are most sensitive to exogenous androgen like testosterone at 40–60 days of age (Hooker 1937). In contrast, in the mouse the prostate is much less prominent and the seminal vesicles are comparatively insensitive (Voss and Loewe 1931; Deanesly and Parkes 1933).

Changes in the glandular epithelium under the influence of androgen administration take place before any substantial change in weight occurs. These changes were screened in a semi-quantitative approach by Moore and Gallagher (Moore and Gallagher 1930) and the quantitative assessment by Hansen (Hansen 1933) suggested that epithelial cell height in both prostate and seminal vesicles may serve as an androgen assay. A detailed dose-response curve of the effect of 14 daily injections of androsterone on the height of the vesicular epithelium in the castrated rat was given by Jacobsen (Jacobsen 1938).

Markers of cell proliferation. In order to further characterize androgenic effects two proliferation markers were used in this study. The proliferation associated antigen Ki-67 (MIB-5) and proliferating cell nuclear antigen (PCNA) have been used as proliferation and prognostic markers in a large number of studies, in particular in malignancies (Korkolopoulou et al. 1993; Kawai et al. 1994). PCNA is a highly conserved 36 kDa acidic nuclear protein that is expressed during cell replication and DNA repair (Shivji et al. 1992; Hall et al. 1990). PCNA interacts with DNA-polymerase delta and with RF-C protein to bind at DNA primer-template junctions. Immunostaining of S-phase nuclei will detect PCNA in sites of DNA synthesis. Ki-67 antigen is expressed during the G_1, S, G_2 and M phases of the cell cycle, but is not expressed during the G_0 (resting) phase. Because Ki-67 antigen has a short half-life, it can be used as a marker of actively proliferating cells (McCormick et al. 1993; Pinder et al. 1995).

Endothelial cell proliferation in male reproductive organs of adult rat is high and regulated by testicular factors. In the epididymis, the ventral and dorsolateral prostate lobes, and the seminal vesicles, endothelial cell proliferation decreased after testosterone withdrawal and increased following testosterone treatment (Franck Lissbrant et al. 2002).

In our study orchiectomy caused a dramatic decrease in the percentage of proliferating epithelial cells – an effect which could be almost completely reversed by the administration of testosterone.

Conclusion

Based on the present data, the densitometric analysis of AR-immunoreactivity and the assessment of both cell morphology and cell proliferation proved to be independent and sensitive parameters for the evaluation of androgen effects on prostates and seminal vesicles. The combined application of these parameters may provide an additional tool to test the broad spectrum of endocrine active substances, such as endocrine disruptors which are currently being discussed as to their potential risk to the environment and humans.

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References

Ashby J, Lefevre PA (2000) The peripubertal male rat assay as an alternative to the hershberger castrated male rat assay for the detection of anti-androgens, oestrogens and metabolic modulators. J Appl Toxicol 20: 35–47

Blondeau JP, Baulieu EE, Robel P (1982) Androgen-dependent regulation of androgen nuclear receptor in the rat ventral prostate. Endocrinology 110: 1926–1932

Breckwoldt M, Neumann F, Bräuer H (1991) Exempla endokrinologica: Bildatlas zur Physiologie und Morphologie des endokrinen Systems, Bd. 1, [Hrsg.: Schering AG, Berlin]. Medical Service München

Deanesly R, Parkes AS (1933) Size changes in the seminal vesicles of the mouse during development and after castration. J Physiol 78: 442–446

De Boer WI, Hiemstra PS, Sont JK, De Heer E, Rabe KF, Van Krieken JHJM, Sterk PJ (2001) Image analysis and quantification in lung tissue. Clin Exp Allergy 31: 504–508

De Jongh SE (1935) Paradoxe Wirkungen von Follikelhormon (Menformon) bei männlichen Tieren; ihre Beeinflussbarkeit durch männliches Hormon. Arch Int Pharmacodyn 50: 348–355

De Jongh SE (1937) De beinvloeding van het mannelijk genitaal apparaat door hormonen. Hormoon VII: 42–51

Endocrine Disruptor Screening and Testing Advisory Committee, Environmental Protection Agency (1998) Final report. Washington D. C.: Environmental Protection Agency

Franck Lissbrant I, Lissbrant E, Persson A, Damber JE, Bergh A (2003) Endothelial cell proliferation in male reproductive organs of adult rat is high and regulated by testicular factors. Biol Reprod 68: 1107–1111

Freud J (1933) Conditions of hypertrophy of the seminal vesicles in rats. Biochem J 27: 1438–1445

Hall PA, Levison DA, Woods AL, Yu CCW, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, Waseem NH, Lane DP (1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 162: 285–294

Hansen IB (1933) Rat seminal vesicles and prostate glands as quantitative indicators of testicular hormone. Endocrinology 17: 163–170

Hershberger L, Shipley E, Meyer R (1953) Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. Proc Soc Exp Biol Med 83: 175–180

Hooker CW (1937) Pubertal increase in responsiveness to androgen in the male rat. Endocrinology 30: 77–81

Huggins C, Hodges CV (2002) Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. J Urol 167: 948–951

Jacobsen E (1938) Die Wirkung der männlichen Hormone auf die Vesiculardrüse der Ratte. Skand Arch Physiol 78: 170–175

Kawai T, Suzuki M, Kono S, Shinomiya N, Rokutanda M, Takagi K, Ogata T, Tamai S (1994) Proliferating cell nuclear antigen and Ki-67 in lung carcinoma. Correlation with DNA flow cytometric analysis. Cancer 74: 2468–2475

Kim HS, Han SY, Kim TS, Kwack SJ, Lee RD, Kim IY, Seok JH, Lee BM, Yoo SD, Park KL (2002) No androgenic/anti-androgenic effects of bisphenol A in Hershberger Assay using immature castrated rats. Toxicol Lett 135: 111–123

Korkolopoulou P, Oates J, Crocker J, Edwards C (1993) p53 expression in oat and non-oat small cell lung carcinomas: correlation with proliferating cell nuclear antigen. J Clin Pathol 46: 1093–1096

Kühnel W (1970) Antiandrogeneffekte an den Geschlechtsorganen männlicher Ratten. Anat Anz Suppl 126: 149–160

Kühnel W (1974) Der Einfluß von Antiandrogenen auf die testosteronabhängige Morphologie des männlichen Genitalapparates. Anat Anz 136: 261–265

McCormick D, Chong H, Hobbs C, Datta C, Hall PA (1993) Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB-1. Pathol 22: 355–360

McNeal JE (1988) Normal histology of the prostate. Am J Surg Pathol 12: 619–633

Mize RR, Holdefer RN, Nabors LB (1988) Quantitative immunocytochemistry using an image analyzer. I. Hardware evaluation, image processing and data analysis. J Neurosci Methods 26: 1–23.

Moore CR, Gallagher TF (1930) Rat prostate cytology as a testis-hormone indicator and the prevention of castration changes by testis-extract injections. Am J Anat 45: 71–80.

Moore RJ, Gazak JM, Wilson JD (1979) Regulation of cytoplasmic dihydrotestosterone binding in dog prostate by 17β-estradiol. J Clin Invest 63: 351–357

Nabors LB, Songu-Mize E, Mize RR (1988) Quantitative immunocytochemistry using an image analyzer. II. Concentration standards for transmitter immunocytochemistry. J Neurosci Methods 26: 25–34

Oberholzer M, Oestreicher M, Christen H, Bruehlmann M (1996) Methods in quantitative image analysis. Histochem Cell Biol 105: 333–355

Pinder SE, Wencyk P, Sibbering DM, Bell JA, Elston CW, Nicholson R, Robertson JFR, Blamey RW, Ellis IO (1995) Assessment of the new proliferation marker MIB-1 in breast carcinoma using image analysis: Associations with other prognostic factors and survival. Br J Cancer 71: 146–149

Rolf C, Nieschlag E (1998) Potential adverse effects of long-term testosterone therapy. Baillieres Clin Endocrinol Metab 12: 521–534

Shivji MKK, Kenny MK, Wood RD (1992) Proliferating cell nuclear antigen is required for DNA excision repair. Cell 69: 367–374

Tschopp E (1936) Untersuchungen über die Wirkung der männlichen Sexualhormone und ihrer Derivate. Arch Int Pharmacodyn 52: 381–390

Vollmer G, Michna H, Ebert K, Knuppen R (1994) Androgen ablation induces tenascin expression in the rat prostate. Prostate 25: 81–90

Voss HE, Loewe S (1931) Zur Wertbestimmung männlichen Sexualhormones an den Vesikulardrüsen des Nagermännchens. Arch Exp Path Pharmak 159: 532–536

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Appendix II.

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The xenoestrogen bisphenol A in the Hershberger assay: Androgen receptor regulation and morphometrical reactions indicate no major effects

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Abstract

We evaluated androgen-like effects of bisphenol A (BPA) using orchiectomized Wistar rats. Animals were treated p.o. either with vehicle or with 3, 50, 200, 500 mg/kg bw/day BPA ($n = 13$) for 7 days. One group was treated s.c. with 1 mg/kg bw/day testosterone propionate (TP). Flutamide (FL) (3 mg/kg bw/day, p.o.) was used to antagonize androgen effects of the supraphysiological dose (500 mg/kg bw/day) of BPA. Androgen-like effects of BPA on prostates and seminal vesicles were assessed by the Hershberger assay, densitometric analysis of androgen receptor (AR) immunoreactivity, cell proliferation-index and a morphometric analysis. Absolute weights of prostates and seminal vesicles were not increased by BPA, whereas the relative weights were increased at higher doses of BPA, most likely due to a decrease in body weight. Staining intensity for AR immunoreactivity was increased at low but not at higher doses of BPA in comparison to the orchiectomized rats. BPA at all doses tested did not cause an increase of the cell proliferation-index. Epithelial height and glandular luminal area were increased by low doses of BPA, whereas higher doses caused a decrease of these parameters. The data provide evidence that BPA does not exert major androgenic effects.

Keywords: Bisphenol A; Prostate; Seminal vesicle; Immunohistochemistry; Morphometry; Densitometry; Androgen receptor regulation; PCNA; MIB-5; Proliferation markers

1. Introduction

BPA is a chemical monomer used primarily to make epoxy-resins, polycarbonate (PC) plastic products and the flame retardant tetrabromobisphenol A [1]. A number of so-called xenobiotics, including pesticides (*p,p′*-DDT), plasticizers (BPA) and a variety of other industrial chemicals (polychlorinated biphenyls) contain a phenolic ring that mimics the A-ring of estradiol and have been reported to have hormonal or antihormonal activity [2,3]. Although the level of exposure to these xenobiotics may be, if any, very low, they may exert their potential toxicity or endocrine disturbance in human beings and wildlife.

Recent in vitro studies demonstrate, in fact, that xenobiotics can bind with estrogen receptors and activate them, resulting in gene expression [4–6]. BPA slightly induced MCF-7 cell proliferation at a level of 0.1 μM and maximum proliferation at 10 μM [6]. Also in in vivo studies, BPA showed estrogenic activity. Plasma free testosterone levels were dramatically decreased following 8 weeks of BPA treatment [7]. It was claimed by vom Saal that, the exposure of pregnant mice to extremely low concentrations of certain xenobiotics, for instance, results in offspring with lower sperm production, increased prostate size or alters maternal behaviour, postnatal growth rate and reproductive function in female mice [8–11]. Other work groups, in

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contrast, found an uterotrophic response (increase in uterine wet weight) at doses up to 100 mg/kg BPA for 3 days [12–14].

Also Gupta [15] claimed an enhancement of the anogenital distance and the prostate size of fetuses, when pregnant CD-1 mice were treated with BPA in the microgram range per kg bw/day. BPA induces inappropriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells (LNCaP). Takao reported a significant decrease in plasma free testosterone levels at 50 μg BPA/ml in drinking water (14 mg/kg/day). No significant effect (although a trend in the same direction) was observed neither after 4 weeks of exposure nor after 4 and 8 weeks exposure to 5 μg of BPA/ml drinking water (0.14 mg/kg/day) [7].

In addition, Kim et al. did not detect any androgenic or anti-androgenic activities of BPA in Hershberger assay [16]: the BPA doses used were 10–1000 mg/kg/day.

The present study was carried out to clarify the androgenic potential of BPA in a broad dose range from "ultralow", "pharmacological" to "suprapharmacological" in rats using the standard Hershberger assay with additionally androgen-sensitive parameters.

Additional parameters such as morphometric and qualitative data are therefore required to determine the androgenicity of a given substance, in particular if the expected effects are of lower degree.

2. Materials and methods

2.1. Animals and housing

Wistar rats (male HdrBrHan from Harlan Winkelmann, Borchen, Germany), weighing about 150 g (age of 2 weeks) were separated into different groups by randomized procedure. They received tap water and ssniff R 10, laboratory standard rat diet (in pellet form) ad libitum (ssniff Spezialdiäten GmbH, Soest, Germany). Groups of 2–4 animals were kept in Makrolon cages type IV with ssniff bedding (3/4 Faser) at 22 ± 3 °C, a relative humidity of 30–70% and artificial 24 h light. After acclimatization animals were orchiectomized under Ketanest/Rompun—anesthesia (Ketanest 10 mg/kg bw from Parke-Davis, Berlin, Germany and Rompun 2 mg/kg bw from Bayer AG, Leverkusen, Germany).

2.2. Treatment of animals

Seven days after orchiectomy animals were partitioned into eight groups ($n = 13$ in each group). They were treated p.o. with 3, 50, 200 and 500 mg BPA/kg/day dissolved in propylene glycol for 7 days. BPA was purchased from Fa. Bayer (PtNr. 97.001/Prod.Nr. 04111095, CasNr. 80-05, Leverkusen, Germany). Another group of orchiectomized animals was treated s.c. with testosterone propionate (TP) 1 mg/kg bw in arachis oil. Flutamide (FL) 3 mg/kg bw p.o. in combination with 500 mg BPA was used to antagonize possible androgen effects of the "suprapharmacological" dose of BPA. These groups were compared to vehicle (propylene glycol) treated, orchiectomized rats without any other substitution (OX) and to a vehicle treated intact control group (Intact). Propylene glycol was purchased from Merck, Darmstadt, Germany. TP and FL were kindly provided by Schering AG, Berlin, Germany. After the treatment animals were sacrificed by decapitation and seminal vesicles and prostates were harvested surgically, weighed and immediately fixed in 4% neutral buffered paraformaldehyde for 24 h.

2.3. Immunohistochemistry

After fixation the specimens (prostate, seminal vesicle) were dehydrated in ascending series of alcohol, embedded in paraffin and cut in sections of 5 μm thickness (10 sections per specimen). For the immunohistochemical visualization of androgen receptors and proliferation markers the following primary antibodies were applied using the standard procedure protocols provided by the manufacturer: anti-androgen-receptor (1:100, sc-815, rabbit polyclonal, Santa Cruz Biotechnology, CA, USA), anti-androgen-receptor (1:100, 554224, mouse monoclonal, BD PharMingen, Germany), anti-PCNA (1:200, PC-10, mouse monoclonal, Novocastra, New Castle, United Kingdom) and anti-MIB-5 (1:100, M 7248 mouse monoclonal, DakoCytomation, Denmark).

2.4. Densitometry and morphometry

Intensity of immunohistochemical staining was determined densitometrically, while epithelial height and luminal area of the glandular ducts were measured morphometrically (KS 100, KS RUN, Zeiss-Vision, Jena, Germany). Microscopy was performed with an Axiophot light microscope (Zeiss, Jena, Germany) equipped with a high resolution scanner camera (Axiocam, Zeiss, Germany).

All images had a uniform size of 1300 × 1030 pixel. Since the images were generated by using a 20× objective and 1.0 optovar the final resolution of the edge lengths of one pixel in the resulting image is 0.32 μm. This resolution was large enough for deciding which profile of glandular ductus in the field of vision was suitable for densitometric measurements. Gray values were transformed pixel by pixel into optical densities [17].

Five measurements were performed within each section. Five sections were examined per animal resulting in 25 measurements for each animal. The mean values were calculated and compared between the different groups.

For morphometric measurements the software package KS 100 3.0 (Zeiss-Vision, Jena, Germany) was used. The epithelial height was determined by using a 40× objective and 1.0 optovar. For the determination of the luminal area a 10× objective and 1.0 optovar was used. The quantitative assessment of proliferating cells was performed using a 40× objective and 1.0 optovar. One thousand cells per section

were counted excluding those which due to the section did not show a nucleus to avoid overestimation of the total cell number. A two-sided t-test at a significance level of $p < 0.05$ was applied for statistical comparison. Data were depicted as mean ± standard deviation (S.D.).

3. Results

3.1. Hershberger assay

In contrast to TP, BPA induced no effect on absolute weights of prostate (Fig. 1) and seminal vesicle (not shown). BPA at high doses of 200 and 500 mg/kg bw caused a decrease in body weights (Fig. 2) and a significant increase in relative weights of prostate and seminal vesicle (not shown). A simultaneous administration of FL had no further effect in BPA treated animals. Animals treated with 200, 500 and 500 + FL BPA showed severe signs of gastro-intestinal toxicity.

3.2. Densitometric analysis

The staining intensity after incubation with the polyclonal antibody (1:100, sc-815, rabbit polyclonal, Santa Cruz Biotechnology, CA, USA) directed against AR was 75 ± 21

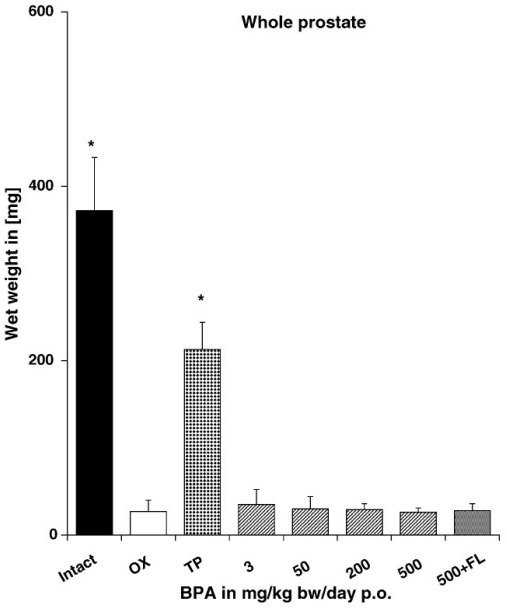


Fig. 1. Comparison of absolute wet weights of the whole prostate in (mg) between the Intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

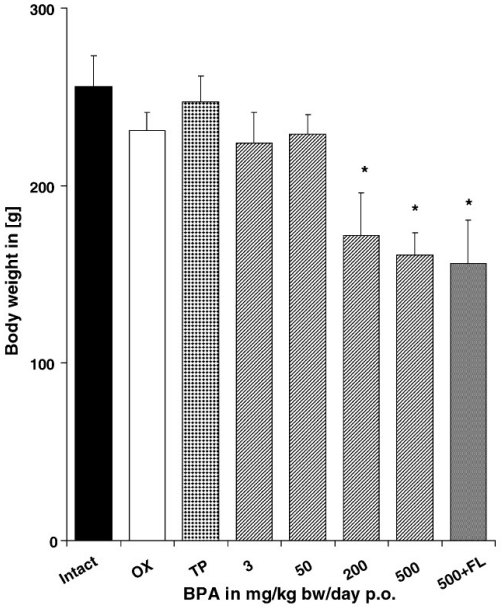


Fig. 2. Comparison of body weights in (g) between the Intact group, orchiectomized group (OX) and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

(Intact), 43 ± 12 (OX), 61 ± 24 (TP), 65 ± 27 (BPA 3), 57 ± 19 (BPA 50), 48 ± 18 (BPA 200), 39 ± 9 (BPA 500) and 33 ± 10 (BPA 500*FL) for the prostates and 42 ± 17 (Intact), 11 ± 10 (OX), 19 ± 11 (TP), 8 ± 5 (BPA 3), 11 ± 15 (BPA 50), 7 ± 3 (BPA 200), 3 ± 1 (BPA 500) and 15 ± 17 (BPA 500 + FL) for seminal vesicles, respectively. In the prostate tissue the intensity of staining was significantly higher in both the Intact and TP group (t-test, $p < 0.05$, $n = 13$) compared to the OX group. BPA at lower doses (3 and 50 mg/kg bw) increased AR immunoreactivity and staining intensity of prostate tissue, but reduced them at higher doses (200 and 500 mg/kg bw). In seminal vesicles, the intensity of AR staining was reduced by orchiectomy in comparison with the Intact control. The treatment of orchiectomized animals with BPA showed no dose-dependent effects (not shown).

Using the anti-androgen-receptor monoclonal antibody (1:100, 554224, mouse monoclonal, Novocastra, New castle, United Kingdom) the staining intensity of AR in the prostates revealed the following optical density values: 104 ± 20 (Intact), 56 ± 17 (OX), 77 ± 22 (TP), 99 ± 7 (BPA 3), 103 ± 23 (BPA 50), 78 ± 16 (BPA 200), 49 ± 23 (BPA 500) and 36 ± 6 (BPA 500 + FL). Statistical analysis confirmed that the intensity of staining was significantly higher in both the intact and TP group (t-test, $p < 0.05$, $n = 13$) compared to the OX group (Figs. 3 and 4A–H). Thus, the data obtained for both antibodies used provide evi-

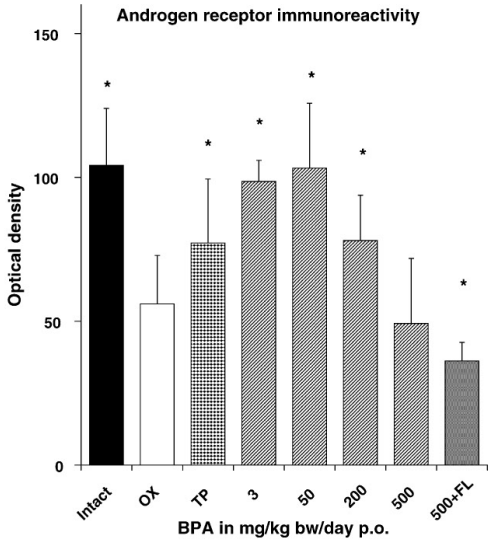


Fig. 3. Densitometric values of the Intact group, orchiectomized group (OX), TP group and the BPA-treated groups (3, 50, 200, 500 and 500 + FL) after immunohistochemical staining (monoclonal antibody) of the androgen receptor in the prostate. Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

dence that orchiectomy results in a reduced staining intensity of AR, whereas substitution with TP enhances the immunoreactive signal of AR. The intensity of staining was significantly increased in prostate after treatment with lower doses of BPA (3 and 50 mg/kg bw). At 500 mg/kg bw staining intensity was similar to the castrated control, but the combination of BPA (500 mg/kg bw) with FL significantly reduced the staining intensity (Figs. 3 and 4A–H). Absolute organ weights (prostate, seminal vesicle) at 200 and 500 mg/kg/day (40/100 fold the NOAEL (no-observed-adverse-effect-level)) were not significantly altered (Fig. 1). The NOAEL is the greatest concentration or amount of a substance e.g. BPA, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure.

3.3. Cell proliferation

The assessment of cell proliferation markers yielded the following data in rat prostates: The percentage of immunoreactive epithelial cells for MIB-5 was 85 ± 9% in the Intact group, 9 ± 1% in the OX group and 90 ± 2% in the TP group. BPA treated groups displayed: 10 ± 1% (BPA 3), 8 ± 1% (BPA 50), 2% (BPA 200), 1% (BPA 500) and 1% (BPA 500 + FL). The percentage of immunoreactive epithelial cells for MIB-5 in the OX group was significantly (*t*-test, $p < 0.05$, $n = 13$) reduced compared to the intact and TP group (Figs. 4I–P and 5). Similar results were obtained for the relative amount of cells immunoreactive for PCNA: 90 ± 9% in the Intact group, 10 ± 2% in the OX group and 88 ± 9% in the TP group, 2% (BPA 3), 4% (BPA 50), 2% (BPA 200), 5 ± 1% (BPA 500) and 6 ± 1% (BPA 500 + FL). The percentage of epithelial cells immunoreactive for PCNA in the OX group was significantly (*t*-test, $p < 0.05$, $n = 13$) reduced compared to the Intact and TP group (Figs. 4Q–X and 5). Whereas orchiectomy caused a considerable decrease of cell proliferation, administration of TP could reverse this effect and induced a cell proliferation index similar to the Intact group. The assessment of both proliferation markers revealed that BPA showed at all doses tested no stimulation of proliferating activity in prostate.

3.4. Morphometry

The mean epithelial height of prostate glands measured 17 ± 2 μm in both the Intact and TP group, whereas the mean epithelial height was significantly (*t*-test, $p < 0.05$, $n = 13$) decreased to 11 ± 1 μm in the OX group. BPA treated groups displayed following data: 14 ± 2 μm (BPA 3), 14 ± 2 μm (BPA 50), 10 ± 1 μm (BPA 200), 9 ± 1 μm (BPA 500) and 8 ± 1 μm (BPA 500 + FL). Similar significant (*t*-test, $p < 0.05$, $n = 13$) data were obtained for the epithelial height of seminal vesicle glands: 18 ± 3 μm (Intact), 9 ± 2 μm (OX) and 15 ± 2 μm (TP), 11 ± 1 μm (BPA 3), 11 ± 1 μm (BPA 50), 8 ± 1 μm (BPA 200), 8 ± 1 μm (BPA 500) and 8 ± 1 μm (BPA 500 + FL), respectively (Fig. 6).

The mean luminal area of prostate glands was 131 000 ± 40 000 μm^2 in the Intact group, 7000 ± 5000 μm^2 in the OX group and 87 000 ± 30 000 μm^2 in the TP group. BPA treated groups showed following data: 23 000 ± 16 000 μm^2 (BPA 3), 25 000 ± 12 000 μm^2 (BPA 50), 15 000 ± 10 000 μm^2 (BPA 200), 4000 ± 500 μm^2 (BPA 500) and 4000 ± 3000 μm^2 (BPA 500 + FL). The luminal area in the OX group was significantly (*t*-test, $p < 0.05$, $n = 13$) reduced compared to the Intact and TP group. In seminal vesicles the mean luminal area measured 113 000 ± 80 000 μm^2 (Intact), 7000 ± 5000 μm^2 (OX) and 151 000 ± 86 000 μm^2 (TP), 35 000 ± 43 000 μm^2 (BPA 3), 27 000 ± 20 000 μm^2 (BPA 50), 8000 ± 3000 μm^2 (BPA 200), 4000 ± 1000 μm^2 (BPA 500) and 3000 ± 2000 μm^2 (BPA 500 + FL), respectively. Similarly as observed for the prostates the luminal area in the OX group was significantly (*t*-test, $p < 0.05$, $n = 13$) reduced compared to the Intact and TP group (Fig. 7).

These morphologic observations clearly reveal that orchiectomy causes a substantial reduction of both epithelial height and luminal area of the prostate gland and seminal vesicles. If TP is substituted both parameters return to values similar to those found in the Intact group. Lower doses of BPA caused an increase in epithelial height and luminal area of prostate and seminal vesicle, while high doses reduced the

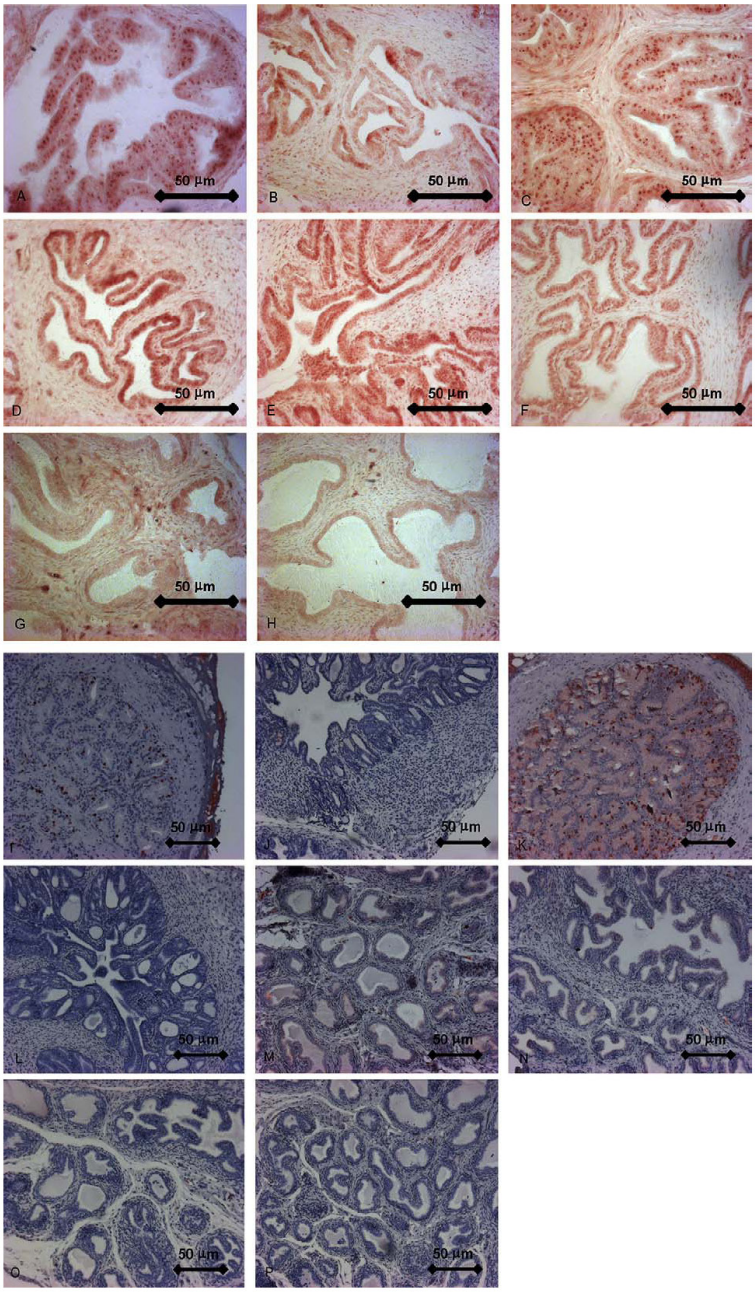


Fig. 4. All panels show photographs of the prostate. Zones are described according to McNeal [35]. A–H: Immunohistochemical staining of androgen receptor (monoclonal antibody) showing the transition zone of the Intact group (A), OX group (B) and TP group (C) in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Original magnification 20×. I–P: Immunohistochemical staining of MIB-5 showing the peripheral zone of the Intact group (I), OX group (J) and TP group (K) in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Original magnification 10×. Q–X: Immunohistochemical staining of PCNA showing the transition zone of the intact group (Q), OX group (R) and TP group (S) in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Original magnification 10×.

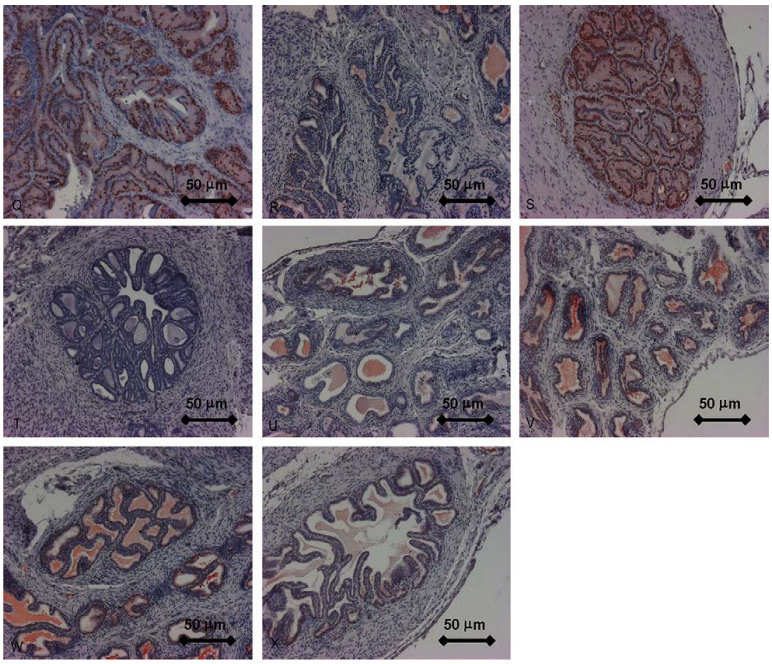


Fig. 4. (*Continued*).

epithelial height of prostate significantly in comparison to the orchiectomized group.

4. Discussion

4.1. Methodologic approaches

4.1.1. Hershberger assay

Although the Hershberger assay is a valid quantitative method for evaluating androgenic or anti-androgenic properties of substances by measuring the organ weight of seminal vesicles and prostates, the findings obtained by this assay provide only limited information on the specificity of the observed effects when only the reactions of the organ weights are judged: For example, the growth of seminal vesicles can be stimulated not only by androgens but also by estrogenic substances, well known as a paradoxical effect of estrogens [18–20].

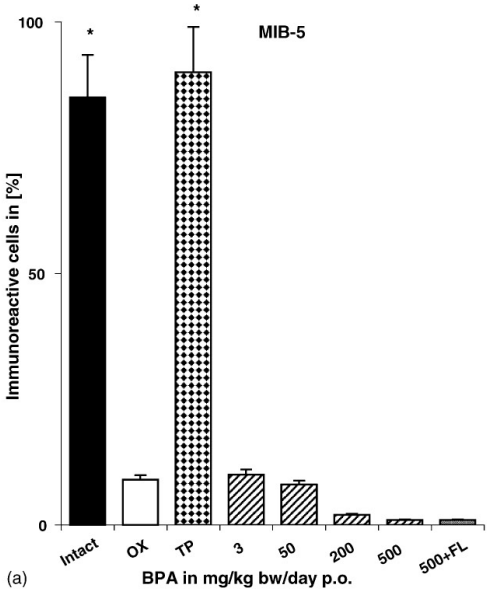
Morphologic and functional analysis of cellular parameters in male accessory organs may allow a more subtle and reliable assessment of the (anti-) androgenicity of substances; in previous studies we analyzed the regulation of tenascin expression [21]. Since the amount of nuclear AR present in the rat prostate has been demonstrated to be influenced by androgens [22,23], a densitometric analysis of AR-immunoreactive cells in prostates and seminal vesicles was performed by using immunohistochemical methods [24,25].

4.2. Densitometric analysis

It has been previously described that the concentrations of biochemically active substances can be estimated from the optical density of the immunoreactive signal [26,27]. As a result, we found a marked decrease in staining intensity of AR-positive cells after orchiectomy in comparison to the control group. This effect of orchiectomy was mostly reversed by an administration of a pharmacological dose of TP. The advantages of a computer-assisted densitometry are a faster scoring procedure of sections from large series and a higher reliability. However, the disadvantage of a semiquantitative approach is the possibility that relevant signals can easily be missed, so that comparative studies should be based on rather robust signals [28].

4.3. Influence of BPA

In this study lower doses of BPA (3 and 50 mg/kg bw/day) were found to cause an enhancement in staining intensity of AR in rat prostate. Morphometric data showed that lower doses of BPA cause an increase in epithelial height and luminal area of prostate and seminal vesicle, while high doses significantly reduce the epithelial height of prostate. These findings are similar to those observed in testosterone propionate substituted castrated rats. Gupta [15] proposed androgen-like effects of BPA in pregnant CD-1 mice at 0.05 mg/kg/day by observing an enhancement of the



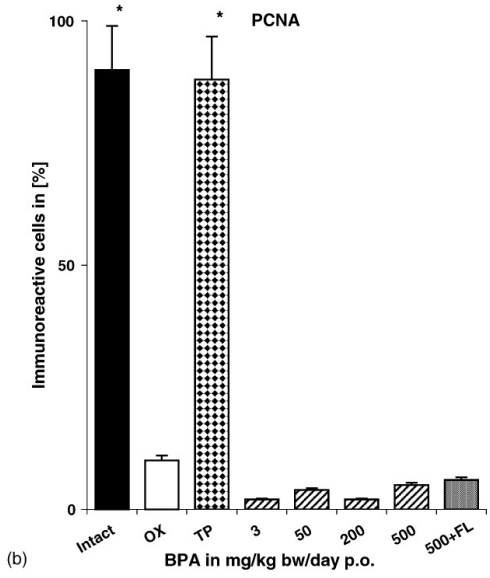
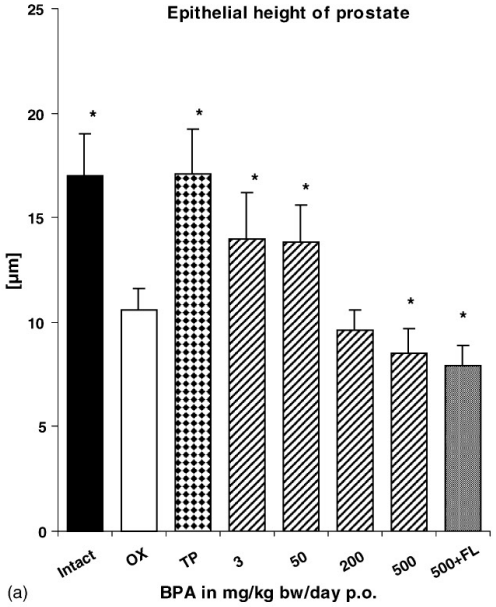


Fig. 5. Quantitative comparison of (a) MIB-5-immunoreactive and (b) PCNA-immunoreactive epithelial prostatic cells between the Intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.



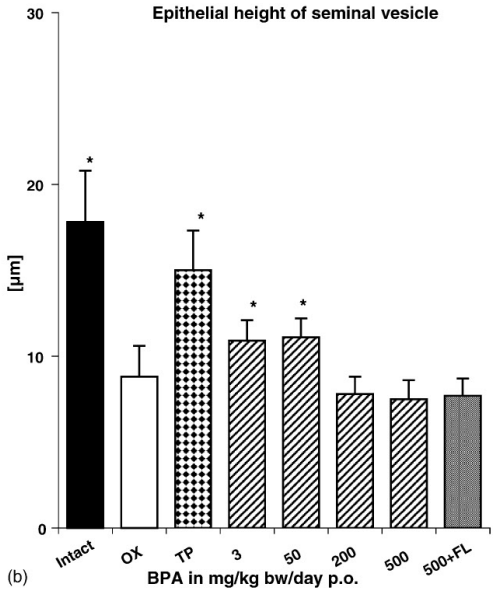


Fig. 6. Comparison of epithelial height of (a) prostate and (b) seminal vesicle between the Intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

anogenital distance and the size of the prostate in fetuses. The androgen receptor (AR) binding affinity in prostate of fetuses was also increased significantly. In contrast, Kim et al. did not found any androgenic or anti-androgenic activities of BPA in Hershberger assay at 10–1000 mg/kg/day [16].

BPA at all doses tested exerted no significant effects on absolute weights of prostate and seminal vesicle. High doses

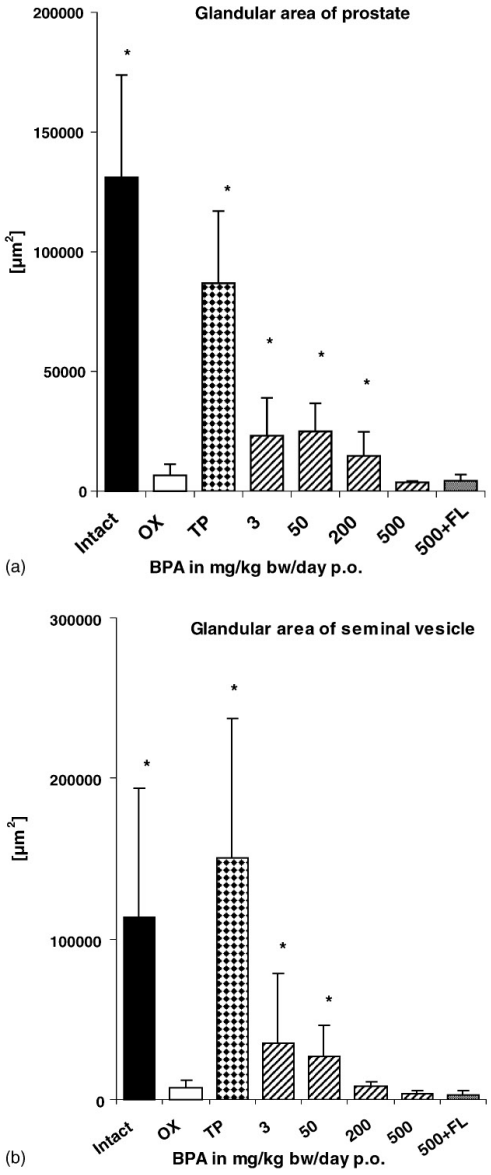


Fig. 7. Comparison of luminal glandular area of (a) prostate (μm^2) and (b) seminal vesicle (μm^2) between the Intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500 + FL). Asterisks indicate statistically significant differences ($p < 0.05$), which refer to the castrated control group.

of BPA (200 and 500 mg/kg bw/day) caused a significant increase in relative weights of prostates and seminal vesicles. These effects of BPA may be due rather to a toxicity-related significant decrease in body weights and well known general side effects, e.g. loss of appetite and diarrhea [29–31], than to an androgenic effect of this substance. An oral predictable no effect concentration ($PNEC_{oral}$) of 33 mg/kg food has been derived for the secondary poisoning assessment from a NOAEL of 50 mg/kg bw (based on a reduction in litter size) from a three-generation multi-dose level feeding study in rats [31]. The PNEC represents the concentration below which an unacceptable risk should not occur. At 3 and 50 mg BPA/kg bw/day no effect on body weight, organ weight, cell proliferation in epithelial prostatic cells, but effects on androgen receptor immunoreactivity and epithelial height of prostate and seminal vesicles, glandular area of prostates and seminal vesicles was observed. Overall, in standard developmental studies in rodents, there is no convincing evidence that BPA is a developmental toxicant [32].

The estrogenic activity of BPA has been mostly observed in higher doses up to 100 mg/kg bw/day [12–14]: the mechanism concerning the androgen-like effect of the low-doses of BPA on the epithelial cells of the prostate is not clear at the present time. It may be difficult to correlate this BPA-effect with the known estrogenic property of this compound [12–14], since BPA had almost no effect on the seminal vesicle. Estrogens have been known to stimulate the development of the fibrous tissue and muscular walls of both prostate and seminal vesicle [20], without stimulating the epithelium and secretory activity [33]. Moreover, it seems very improbable, that the increase of the immunoreactive AR in the prostatic epithelial cells (not in the seminal vesicle) is up-regulated by estrogens. It is also unlikely that BPA, via an activation of adrenal androgen synthesis (the production of corticosteroid-binding globulin CBG and an activation of adrenal functions, leads to an increased steroidogenesis including androgen production), stimulates the prostate selectively, although estrogens have been considered to be one of the controllers of adrenal androgen secretion [34]. Further studies on the low-dose effects of BPA using an antiandrogen may be necessary to elucidate the selective effects on the prostate in the rat.

Based on the present data, the densitometric analysis of AR-immunoreactivity and the assessment of both cell morphology and cell proliferation proved to be independent and sensitive parameters for the evaluation of androgen effects on prostates and seminal vesicles. The combined application of these parameters may provide an additional tool to test the broad spectrum of endocrine active substances such as endocrine disruptors, which are actually discussed on their potential risk to the environment and humans.

Acknowledgements

The authors wish to thank the director of the Institute of Anatomy (Univ. Prof. Dr. med. J. Westermann) for his generous support. We gratefully acknowledge Mrs. H. Strauchmann, Mrs. K. Budler, Mrs. L. Gutjahr, and Mrs. U. Müller-Horn for their excellent technical assistance.

References

[1] T. Zincke, Mittheilungen aus dem chemischen Laboratorium der Universitat Marburg, Justus Liebigs Annalen Chemie 343 (1905) 75–99.
[2] S. Krimski, Hormonal Chaos, The Johns Hopkins University Press, Baltimore-London, 2000, 1–54.
[3] J. Ashby, J. Odum, D. Paton, P.A. Lefevre, N. Beresford, J.P. Sumpter, Re-evaluation of the first synthetic estrogen, 1-keto-1,2,3,4-tetrahydrophenantrene, and bisphenol A, using both the ovariectomized rat model used in 1933 and additional assays, Toxicol. Lett. 115 (2000) 231–238.
[4] F. Paris, P. Balaguer, B. Terouanne, N. Servant, C. Lacoste, J.P. Cravedi, J.C. Nicolas, C. Sultan, Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines, Mol. Cell Endocrinol. 193 (2002) 43–49.
[5] S.C. Nagel, F.S. vom Saal, K.A. Thayer, M.G. Dhar, M. Boechler, W.V. Welshons, Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol, Environ. Health Perspect. 105 (1997) 10–16.
[6] H.S. Kim, S.Y. Han, S.D. Yoo, B.M. Lee, K.L. Park, Potential estrogenic effects of bisphenol-A estimated by in vitro and in vivo combination assays, J. Toxicol. Sci. 26 (2001) 111–118.
[7] T. Takao, W. Nanamiya, I. Nagano, K. Asaba, K. Kawabata, K. Hashimoto, Exposure with the environmental estrogen bisphenol A disrupts the male reproductive tract in young mice, Life Sci. 65 (1999) 2351–2357.
[8] W.V. Welshons, S.C. Nagel, K.A. Thayer, B.M. Judy, F.S. vom Saal, Low-dose bioactivity of xenoestrogens in animals: fetal exposure to low doses of methoxychlor and other xenoestrogens increases adult prostate size in mice, Toxicol. Ind. Health 15 (1999) 12–25.
[9] F.S. vom Saal, P.S. Cooke, D.L. Buchanan, P. Palanza, K.A. Thayer, S.C. Nagel, S. Parmigiani, W.V. Welshons, A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production and behaviour, Toxicol. Ind. Health 14 (1998) 239–260.
[10] P.L. Palanza, K.L. Howdeshell, S. Parmigiani, F.S. vom Saal, Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice, Environ. Health Perspect. 110 (2002) 415–422.
[11] K.L. Howdeshell, A.K. Hotchkiss, K.A. Thayer, J.G. Vandenbergh, F.S. vom Saal, Environmental toxins: exposure to bisphenol A advances puberty, Nature 401 (1999) 763–764.
[12] P. Diel, T. Schulz, K. Smolnikar, E. Strunck, G. Vollmer, H. Michna, Ability of xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotrophic activity, J. Steroid Biochem. Mol. Biol. 73 (2000) 1–10.
[13] C.M. Markey, C.L. Michaelson, E.C. Veson, C. Sonnenschein, A.M. Soto, The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A, Environ. Health Perspect. 109 (2001) 55–60.
[14] H. Tinwell, R. Joiner, I. Pate, A. Soames, J. Foster, J. Ashby, Uterotrophic activity of bisphenol A in the immature mouse, Regul. Toxicol. Pharmacol. 32 (2000) 118–126.
[15] C. Gupta, Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals (44516), Proc. Soc. Exp. Biol. Med. 224 (2000) 61–68.
[16] H. Kim, S. Han, T. Kim, S. Kwack, R. Lee, I. Kim, J. Seok, B. Lee, S. Yoo, K. Park, No androgenic/anti-androgenic effects of bisphenol-A in Hershberger assay using immature castrated rats, Toxicol. Lett. 5 (135) (2002) 111, 1–2.
[17] M. Oberholzer, M. Oestreicher, H. Christen, M. Bruehlmann, Methods in quantitative image analysis, Histochem. Cell Biol. 105 (1996) 333–355.
[18] S.E. De Jongh, Paradoxe Wirkungen von Follikelhormon (Menformon) bei männlichen Tieren; ihre Beeinflussbarkeit durch männliches Hormon, Arch. Int. Pharmacodyn. 50 (1935) 348–355.
[19] S.E. De Jongh, De beinvloeding van het mannelijk genitaal apparaat door hormonen, Hormoon VII (1937) 42–51.
[20] J. Freud, Conditions of hypertrophy of the seminal vesicles in rats, Biochem. J. 27 (1933) 1438–1445.
[21] G. Vollmer, H. Michna, K. Ebert, R. Knuppen, Androgen ablation induces tenascin expression in the rat prostate, Prostate 25 (1994) 81–90.
[22] R.J. Moore, J.M. Gazak, J.D. Wilson, Regulation of cytoplasmic dihydrotestosterone binding in dog prostate by 17β-estradiol, J. Clin. Invest. 63 (1979) 351–357.
[23] J.P. Blondeau, E.E. Baulieu, P. Robel, Androgen-dependent regulation of androgen nuclear receptor in the rat ventral prostate, Endocrinology 110 (1982) 1926–1932.
[24] C. Huggins, C.V. Hodges, Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate, J. Urol. 167 (2002) 948–951.
[25] C. Rolf, E. Nieschlag, Potential adverse effects of long-term testosterone therapy, Baillieres Clin. Endocrinol. Metab. 12 (1998) 521–534.
[26] L.B. Nabors, E. Songu-Mize, R.R. Mize, Quantitative immunocytochemistry using an image analyzer. II. Concentration standards for transmitter immunocytochemistry, J. Neurosci. Methods 26 (1) (1988) 25–34.
[27] R.R. Mize, R.N. Holdefer, L.B. Nabors, Quantitative immunocytochemistry using an image analyzer. I. Hardware evaluation, image processing and data analysis, J. Neurosci. Methods 26 (1) (1988) 1–23.
[28] W.I. De Boer, P.S. Hiemstra, J.K. Sont, E. De Heer, K.F. Rabe, J.H.J.M. Van Krieken, P.J. Sterk, Image analysis and quantification in lung tissue, Clin. Exp. Allergy 31 (2001) 504–508.
[29] R.E. Morissey, J.D. George, C.J. Price, R.W. Tyl, M.C. Marr, C.A. Kimmel, The developmental toxicity of bisphenol A in rats and mice, Fundam. Appl. Toxicol. 8 (1987) 571–582.
[30] K. Yamasaki, M. Sawaki, S. Noda, N. Imatanaka, M. Takatsuki, Subacute oral toxicity study of ethynylestradiol and bisphenol A, based on the draft protocol for the "Enhanced OECD Test Guideline no. 407", Arch. Toxicol. 76 (2002) 65–74.
[31] R.W. Tyl, C.B. Myers, M.C. Marr, B.F. Thomas, A.R. Keimowitz, D.R. Brine, M.M. Veselica, P.A. Fail, T.Y. Chang, J.C. Seely, R.L. Joiner, J.H. Butala, S.S. Dimond, S.Z. Cagen, R.N. Shiotsuka, G.D. Stropp, J.M. Waechter, Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats, Toxicol. Sci. 68 (2002) 121–146.
[32] European Chemicals Bureau: EU-Risk-Assessment Summary Vol. 37 (2003) on 4,4'-isopropylidenephenol (bisphenol-A), CAS#: 80-05-7, EINECS#: 201-245-8.
[33] K. David, J. Freud, S.E. de Jongh, Conditions of hypertrophy of seminal vesicle in rats II. The effect of derivatives of oestrone (Menformon), Biochem. J. 28 (1934) 1360–1367.
[34] W.D. Odell, L.N. Parker, Control of adrenal androgen production, Endocr. Res. 10 (1984) 617–630.
[35] J.E. McNeal, Normal histology of the prostate, Am. J. Surg. Pathol. 12 (1988) 619–633, Publication I.03.149.

Appendix III.

Nishino T, Bühlmeyer K, Peters C, Schönfelder M, Schulz T, Michna H, Phyto- und Xenoöstrogene: Endokrine und immunologische Wirkungen – Zur Bedeutung einer rationalen Abschätzung von erwünschten Effekten und Risiken.

2. Wissenschaftstagung Weihenstephan „Lebensmittel und Gesundheit“
Proceedings P 97-109 (2003)

Technische Universität München

Wissenschaftszentrum für Ernährung, Landnutzung und Umwelt

2. Wissenschaftstagung "Lebensmittel und Gesundheit"

Proceedings

H. Graßmann, E. Elstner, U. Kulozik (Hrsg.)

Vorwort

Der Zusammenhang zwischen dem Gesundheitsstatus und den Eigenschaften der heute verfügbaren Lebensmittel steht immer mehr im Brenpunkt einer öffentlichen Debatte. Ernährungsbedingte Erkrankungen verschlingen Milliardensummen. Der ebenfalls heute fast zum Normalfall gewordene Bewegungsmangel und inaktive Lebensstil steht weniger im Vordergrund, trägt aber dazu seinen Teil in erheblichem Umfang bei.

Diesen Fragen stellt sich die Fakultät für Ernährung, Landnutzung und Umwelt der Technischen Universität München in seinen Forschungsprogrammen rund um Lebensmittel und Ernährung, aber auch Sport-, Wirtschafts- und Sozialwissenschaften sowie Medizin.

Die erste Tagung zum Thema „Lebensmittel und Gesundheit" fand im Jahr 2002 mit einer großen Resonanz statt. Daraus resultierte der Entschluss, diese Veranstaltung auch im Jahr 2003 weiterzuführen. Damit ergab sich ein besonderer Bezug zum Jahr 1803, als 200 Jahre zurück die Säkularisation in Bayern zu gewaltigen Umwälzungen des Staats- und Sozialgefüges führte. Auch wurde im Jahr 1803 Justus von Liebig geboren, der mit seinen Erfindungen und wissenschaftlichen Erkenntnissen erheblich zur Versorgung der Bevölkerung mit gesunden Lebensmitteln beigetragen hat. Man ist sich heute kaum mehr bewusst, dass seinerzeit die Mangel- und Unterversorgung der breiten Bevölkerung die Regel war, während heute aufgrund einer bisher nie da gewesenen Verfügbarkeit und Vielfalt von Lebensmitteln eine zeitlich wie örtlich gegebene lückenlose Versorgung festgestellt werden kann und eher sogar das Risiko der Überversorgung offensichtlich wird. Dennoch hat sich während der letzten 100 Jahre das durchschnittliche, statistisch zu erwartende Lebensalter verdoppelt. Auch heute befindet sich das durchschnittliche Lebensalter nach wie vor im Anstieg, wie aber die Perspektive zur weiteren Entwicklung dieses Trends aussieht, hängt nicht zuletzt vom individuell beeinflussbaren Ernährungs- und Bewegungsverhalten ab.

Ohne Anspruch auf eine umfassende Darstellung sollte die Tagung „Lebensmittel und Gesundheit 2003" diesen Bezügen durch Berücksichtigung historischer Aspekte gerecht werden. In einem Spektrum an Beiträgen wurde dabei der heute erreichte Status in der Lebensmittelqualität und –verfügbarkeit deutlicher, als wenn nur in einem kürzeren Rückblick die gleiche Betrachtung angestellt worden wäre. Mehr oder weniger fällt die betrachtete Periode von 200 Jahren zusammen mit dem Zeitraum, in dem sehr wesentliche Erkenntnisse zur Lebensmittelsicherheit gewonnen und die ersten Verfahren zur industriellen Haltbarmachung von Lebensmitteln eingeführt wurden. Heute stehen neue Möglichkeiten zur Verfügung, Lebensmittel sollen zunehmend eigenständig physiologische, teilweise präventiv wirkende Funktionen übernehmen. Erkenntnisse zur Physiologie oder epidemiologische Daten werden in einzelnen Beiträgen ebenfalls behandelt und spannen den Bogen in die heutige und vor uns liegende Zeit.

Dr. Johanna Graßmann
Prof. Dr. Erich Elstner
Prof. Dr. Ulrich Kulozik

Phyto- und Xenoöstrogene: Endokrine und immunologische Wirkungen

T. Nishino

Katja Bühlmeyer, Christiane Peters, Martin Schönfelder, Thorsten Schulz, Horst Michna

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Einleitung

Zur Bedeutung einer rationalen Abschätzung von erwünschten Effekten und Risiken

Seit über 70 Jahren beschäftigt man sich in der Endokrinologie mit synthetischen Östrogenen, den sogenannten Xenoöstrogenen. Es stehen inzwischen Hunderte von Substanzen in Verdacht, Schäden am Hormonsystem hervorzurufen; mit weitreichenden Folgen wie Immunstörungen, Missbildungen, Unfruchtbarkeit oder auch Verhaltensänderungen. Dabei handelt es sich sowohl um bewusst hergestellte östrogen-wirkende Pharmazeutika, als auch um Substanzen, die ungewollt diese Wirkung hervorrufen.
Neben diesen synthetischen Chemikalien, wie z.B. Bisphenol A, Dichlordiphenyltrichlorethan (DDT) und Oktylphenol sind auch natürliche, im Pflanzenreich weit verbreitete, östrogen-wirksame Substanzen, wie Naringenin, Genistein, Daidzein und Zearalenon isoliert worden. Diese sogenannten Phytoöstrogene besitzen ein ebenfalls nicht zu unterschätzendes östrogenes Potential.
Diese auch allgemein als endokrine Disruptoren bezeichneten Chemikalien gilt es nun in Zukunft auf ihre Toxizität bzw. ihr möglicherweise auch gesundheitsförderndes Potential hin zu untersuchen [13]. Dabei wird die Definition der „endocrine disruptors" noch weiter gefasst, indem zusätzlich zu den direkten hormonartigen Wirkungen ebenfalls indirekte agonistische und antagonistische Einflüsse – wie der Eingriff in die Hormonbiosynthese bzw. den Abbau der Hormone – mit dazu gezählt werden.

1. Phytoöstrogene

Die Phytoöstrogene, hauptsächlich Flavonoide, zählen zu einer der größten Gruppen der sekundären Pflanzeninhaltsstoffe. Sie kommen im Pflanzenreich ubiquitär vor (Abb. 1). Man trifft sie dort oft an Zuckerkomponenten wie D-Glukose, D-Galaktose oder L-Rhamnose gebunden an. In der Pflanze selbst sind sie hauptsächlich in den oberirdischen Pflanzenteilen lokalisiert.
Flavonoide sind chemisch gesehen Phenylchromanderivate, welche aus einem C_6-C_3-C_6-Grundgerüst mit 15 C-Atomen bestehen. Die nach einer Schätzung über 2000 verschiedenen Derivate [26] sind über vielfältige Substitutionen an den beiden aromatischen Ringen A und C und über den Oxidationsgrad der C-Atome 2, 3 und 4 des Chromangerüstes zu erklären. Aglyka kennt man alleine ca. 500 verschiedene [26]. Die Biosynthese der Phenylchromanderivate geht von drei Acetat-Einheiten

(Malonyl-CoA) und einer Phenylpropan-Einheit (Cinnamoyl-CoA) aus, die zu einem C_{15}-Zwischenprodukt kondensieren, welches schließlich zu einem primär gebildeten Chalkon reagiert. Dieses Chalkon wird enzymatisch zu Flavanonen zyklisiert oder aber es geht spontan in solche über. Durch chemische Modifikationen entstehen die einzelnen Untergruppen. Beispiele dieser Untergruppen sind die Flavone, Flavonole oder Flavan-3,4-diole.
Die biologischen Bedeutungen der Flavonoide für die Pflanze sind zum einen Schutz vor Insektenbefall bzw. Bakterien-, Virus- oder Pilzinfektionen und zum anderen eine Anlockung von Bestäubern durch die charakteristischen Farben und Fluoreszenzen. Weiterhin haben sie Kontrollfunktionen bei Wachstumsvorgängen. Ihr Name leitet sich vom lateinischen Wort *flavus* (= gelb) ab und beruht auf der u.a. intensiven Gelbfärbung einiger ihrer Vertreter, jedoch sind auch leuchtend rote und blaue Derivate zu finden. Eine wichtige Rolle bei der Farbgebung spielen neben der chemischen Struktur (Chromophore) der Substanzen auch der vorherrschende pH-Wert in den jeweiligen Pflanzenteilen sowie eine mögliche Chelatbildung der Flavonoide mit verschiedenen Metallionen (z.B. Fe^{3+} oder Al^{3+}).

Von besonderer Bedeutung in Hinblick auf eine mögliche östrogene Wirksamkeit der Flavonoide ist die Untergruppe der Isoflavonoide, die man im Gegensatz zu den weit verbreiteten Flavonoiden nur in wenigen Pflanzenarten findet. Im Mittelpunkt dieser Pflanzen steht hierbei die Familie der *Fabaceae* (Schmetterlingsblütengewächse), die zu der Ordnung der *Fabales* (Leguminosen) zählen und als Hülsenfrüchte bezeichnet werden. Chemisch betrachtet, handelt es sich bei dieser Untergruppe um 3-Phenylchromane, die sich aus den Flavonoiden ableiten und aus ihnen durch Wanderung des Arylrestes von der Position 2 des Chromanringes in die Position 3 hervorgehen. So entsteht z. B. Genistein aus dem Flavanon Naringenin. Diese Reaktion wird durch das Cytochrom-P-450 Enzym Isoflavon-Synthase katalysiert [10], (Abb. 2).

Wirkung von Phytoöstrogenen

Genistein und Daidzein, ebenfalls Flavonoide (3,4`,5-Trihydroxyflavon und 3,4`-Dihydroxyflavon), wurden aus Sojaprodukten isoliert. Die Sojabohne *Glycine max (L.) MERR (Fabaceae)* ist ein fett- und eiweißreiches, einjähriges, behaartes Kraut, welches in ostasiatischen Regionen beheimatet ist. Heutzutage wird sie jedoch auch in subtropischen Gebieten Ostasiens, Nordamerikas, Brasiliens, Osteuropas und Südafrikas kultiviert [27]. Interessanterweise deuten Studien darauf hin, dass in asiatischen Ländern ein vermindertes Risiko an Mammakarzinom zu erkranken besteht, was mit der erhöhten und regelmäßig eingenommenen Menge an Soja in Verbindung gebracht wird [Übersichtsartikel: Wuttke et al.: Phytoestrogens for hormone replacement therapy?, JSBMB (2002)].
Naringenin, ein Flavonoid (4`,5,7-Trihydroxyflavon), kommt u.a. im Hopfen und demzufolge in vielen Biersorten vor. Konzentrate von Orangen bzw. Grapefruits sowie Naringenin, das Hauptflavonoid von Grapefruits, hemmen die Proliferation von Krebszellen, indem sie die Signalübertragung durch Proteinkinasen, insbesondere Tyrosinkinasen, wie Proteinkinase C, beeinträchtigen [Frydoonfar et al., 2003].
Zearalenon, ein RAL (resorcyclic acid lactone), ist eine aus Schimmelpilzgiften isolierte östrogene Substanz, die eine leicht anabole Wirksamkeit besitzt. Sie wird von bestimmten Pilzarten (Fusarium-Arten) in Heu, Futtermitteln und Getreiden (Mais) gebildet, insbesondere bei kühlen Temperaturen (12-14 °C). Wegen seiner Giftigkeit zählt es zu den Mykotoxinen, wobei die im Körper aus Zearalenon

gebildeten Metaboliten um ein Vielfaches stärker wirken als Zearalenon selbst. Es ist verantwortlich für viele östrogenbedingte Syndrome bei diversen Farmtieren [Bauer et al., 1987].

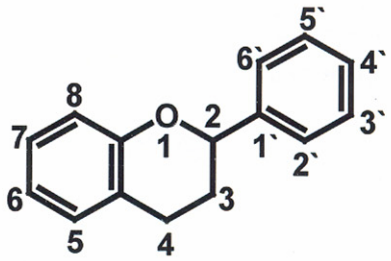
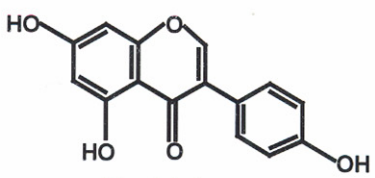
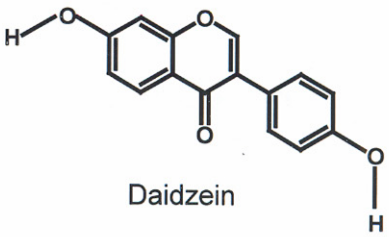


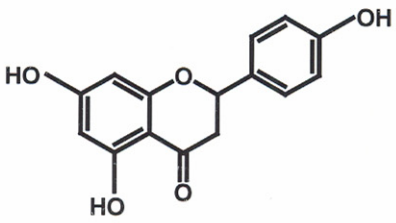
Abb. 1: Flavangrundgerüst



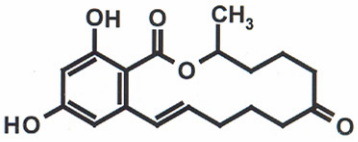
Genistein



Daidzein



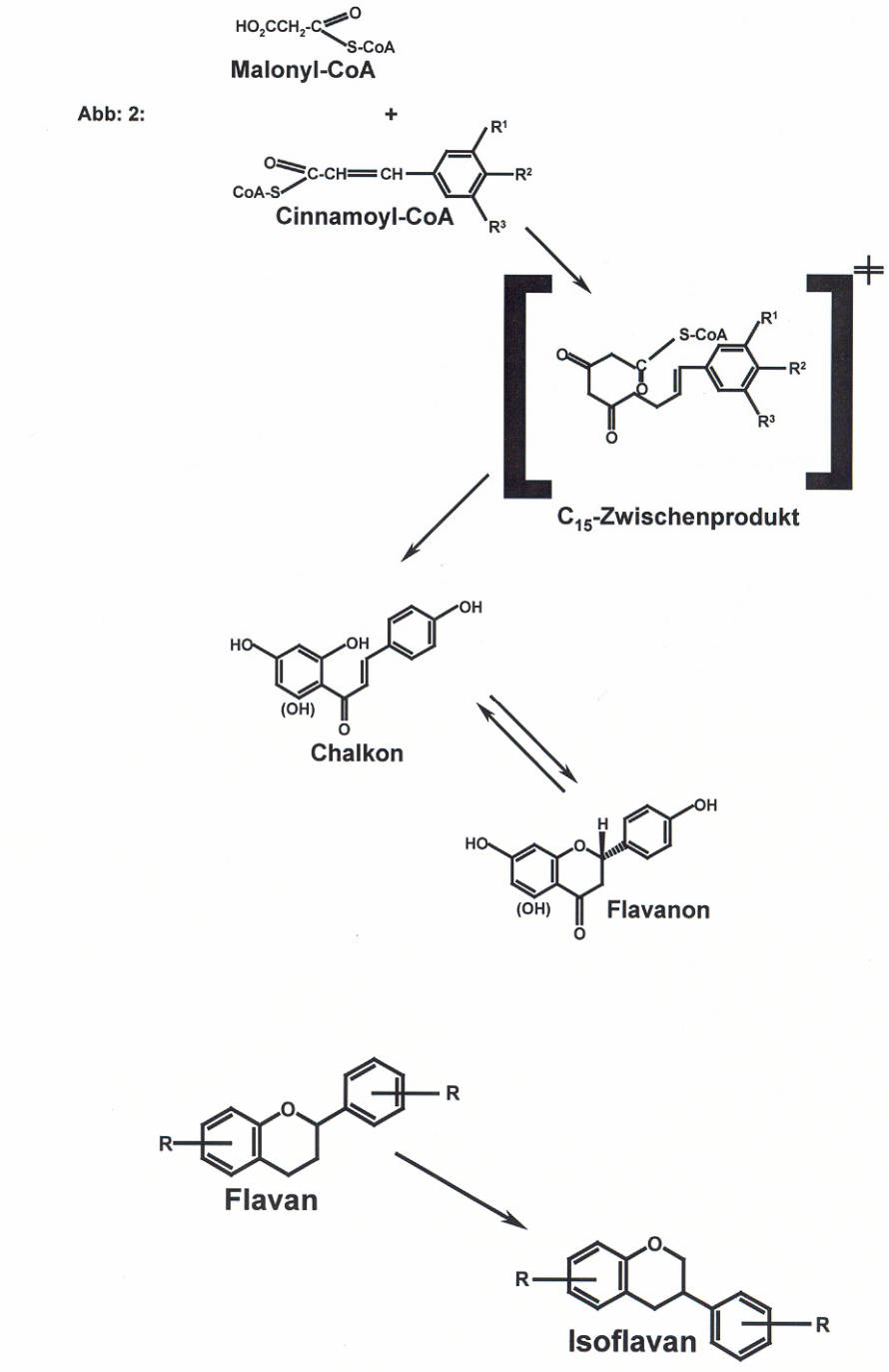
Naringenin



Zearalenon

99

Abb: 2:


Malonyl-CoA
+
Cinnamoyl-CoA
C_{15}-Zwischenprodukt
Chalkon
Flavanon
Flavan
Isoflavan

100

2. Xenoöstrogene (Abb. 3)

Allgemein definiert werden Xenoöstrogene als in der Umwelt vorkommende Chemikalien, die an körpereigene Hormonrezeptoren binden können und hormonähnliche Wirkungen entfalten. Bisphenol A (BPA) ist eine der wichtigsten und meistproduzierten Chemikalien weltweit. Die Verarbeitung erfolgt hauptsächlich zu Polykarbonat, Epoxidharzen und in geringer Menge auch zu dem Flammschutzmittel Tetrabromobisphenol A. BPA wird seit etwa 40 Jahren im großtechnischen Maßstab (über 2.0 Mio. t/Jahr weltweit) hergestellt. Dabei wird es z.B. bei Zahnfüllungen und Thermopapier in seiner Grundform benutzt, wogegen es viele weitere Anwendungen gibt, die auf vernetztem BPA / Polykarbonat beruhen: CDs, Armaturen, Plastikteile im Auto, Haushaltsgegenstände, transparente Babyplastikflaschen, Nahrungs- und Getränkeverpackungen. Auch Epoxidharze und Kleber enthalten BPA. Nahrungsmittel- und Getränkedosen werden in der Regel auf der Innenseite mit einem BPA-haltigen Epoxidharz überzogen [13, 18]. Der jährliche Verbrauch von BPA in der EU wird auf über 600.000 t jährlich geschätzt. BPA wird häufig in kommunalen und industriellen Klärschlämmen nachgewiesen. Die Werte liegen meist unterhalb von 1 mg/kg TR (Trockenrückstand), können jedoch durchaus auch bis über 30 mg/kg TR erreichen. In Abwasser, Deponiesickerwasser und in durch Kläranlagenabläufe beeinflussten Oberflächengewässern wird BPA ebenfalls häufig nachgewiesen [11, 12, 14, 24, 28, 29]. BPA wurde in den dreißiger Jahren gezielt als künstliches Östrogen synthetisiert. Erst in den fünfziger Jahren entdeckte man es als Monomer zur Herstellung von Polymeren, also Kunststoffen, wieder [13, 18].

Experimentelle Untersuchungen zur Abschätzung der Risiken auf Mensch und Umwelt

Es besteht der Verdacht, dass die Industriechemikalie BPA auch in geringen Konzentrationen eine schädigende Wirkung zeigen kann. BPA wirkt ähnlich dem Hormon Östrogen und kann eine Vergrößerung der Prostata, Reduktion der Spermienkonzentration oder eine verfrühte Pubertät hervorrufen. Die Gefahren für den Menschen sind noch nicht ausreichend nachgewiesen.

Ein Abbauprodukt von Tensiden aus Reinigungsmitteln ist das Oktylphenol, welches in *in vitro* Studien ebenfalls als „schwach östrogen" eingestuft wurde.

Bei DDT, früher ein in großen Mengen genutztes Pestizid, handelt es sich in erster Linie um ein starkes und wirksames Kontaktgift, das im Gegensatz zu einem Fraßgift durch bloße Berührung seine Giftwirkung auslösen kann. Großtechnisch lässt sich dieses Insektizid relativ einfach durch eine sauer katalysierte Kondensationsreaktion von Chlorbenzol mit dem chlorierten Aldehyd Chloral (1,1,1-Trichlorethan) gewinnen. DDT wurde erstmals 1874 in Deutschland synthetisiert. Seine Wirkung als Schädlingsbekämpfungsmittel erkannte jedoch erst 1939 der Schweizer Chemiker Paul Müller. Für seine Arbeiten erhielt Müller 1948 den Nobelpreis für Medizin. Jahrzehntelang war DDT das erfolgreichste Insektizid. Man erkannte jedoch das einige Schadinsekten DDT-resistente Stämme entwickelten, deren Populationen sich unkontrolliert vermehren konnten, während ihre natürlichen Feinde wie Wespen durch die Besprühung ausgerottet wurden. Aus diesem Grund wurde der Einsatz von DDT in fast allen Industrieländern – in der Bundesrepublik Anfang der 70er Jahre – verboten. In einigen Entwicklungs- und Schwellenländern sowie Ländern des ehemaligen Ostblocks wird DDT noch heute produziert und eingesetzt.

Diese hormonell wirksamen Phyto- und Xenoöstrogene greifen in vielfältiger Weise in die natürlichen Körperfunktionen ein. Auch unsere Immunabwehr wird beeinflusst, was zu einer erhöhten Krankheitsanfälligkeit führen kann. Möglich ist auch die

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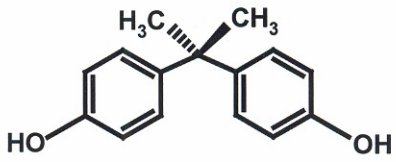
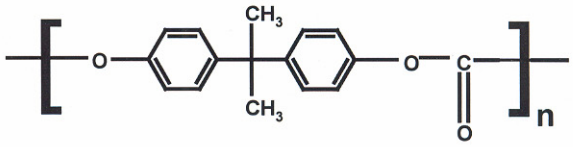
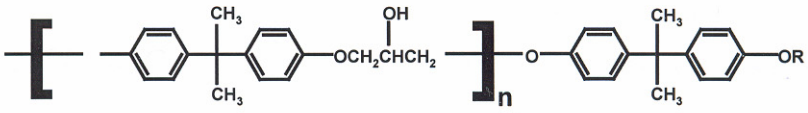
Förderung bestimmter östrogen-abhängiger Krebsarten wie Brustkrebs sowie die Verminderung der Fertilität bis hin zur Beeinflussung der Spermatogenese beim Mann [1, 3, 13, 18].



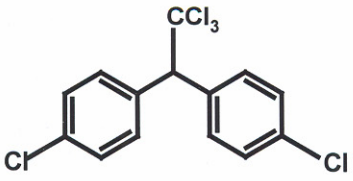
Bisphenol A



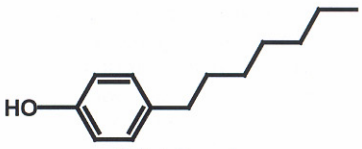
Polykarbonat



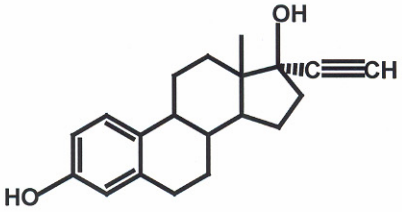
Epoxidharz



DDT



p-Oktylphenol



Ethinylestradiol

Abb. 3:

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Experimentelle Hinweise und Methoden zur rationalen Abschätzung der hormonellen Wirkungen von Phyto- und Xenoöstrogenen

1. *In vitro* Untersuchungen

Zur Untersuchung hormoneller Aktivitäten der beschriebenen weit verbreiteten Substanzen werden verschiedene *in vitro* Test-Assays angewendet. Dazu gehören *in vitro* Zellkultursysteme wie Reportergen-Assays oder Proliferations-Assays.

Der Proliferations-Assay erfasst den Endpunkt des Zellwachstums und erlaubt eine zwar sensitive Abschätzung einer Wirkung, aber eine relativ unspezifische Aussage über den Mechanismus. In eigenen Untersuchungen konnten wir bei östrogen-abhängigen Krebszelllinien beobachten, dass bei Entzug von Östrogenen eine Hemmung des Zellwachstums um bis zu 20 % auftreten kann. Gleichzeitig kann bei Gabe von Antiöstrogenen je nach Krebszelllinie eine Proliferationshemmung von bis zu 60% beobachtet werden. Diese enormen Effekte macht man sich bei der Untersuchung von Substanzen zunutze, bei denen eine potentielle Hormonwirkung vermutet wird.

Über ein Tunel-Assay kann eine weitere Abschätzung zur Wirkung der Substanzen gemacht werden: der Apoptoseinduktion. Über wirkmechanistische Untersuchungen *in vitro* ist letztendlich festgestellt worden, dass ein Entzug östrogener Substanzen zum programmierten Zelltod, der Apoptose, führt.

Ein weiteres *in vitro* Testsystem zur Untersuchung der Östrogenität diverser Chemikalien ist der CAT-Assay (Chloramphenicol-Acetyltransferase-Assay). Chloramphenicol wird oft als Antibiotikum eingesetzt. Im CAT-Assay wird die Aktivität eines Chloramphenicolacetyltransferase-Reportergens nachgewiesen. CAT wird nur von Prokaryonten exprimiert, so dass eine in eukaryontischen Zellen nachgewiesene CAT-Aktivität ausschließlich auf ein durch Transfektion eingebrachtes CAT-Protein zurückzuführen ist. Das als Reportergen bezeichnete CAT-Gen wird hinter den zu untersuchenden Promotor kloniert und das Plasmid in Zellen durch Transfektion eingebracht. Die transfizierten Kulturen werden nach einigen Stunden in Kultur stimuliert, um den Promotor zu aktivieren und damit die Expression des Reportergens zu induzieren. Die Zellen werden anschließend, in der Regel nach 24-48 Stunden aufgeschlossen. Für den Nachweis der Aktivierung eines Promotors wird das Reportergen (CAT) genutzt. Das Enzym acetyliert *in vitro*, mit Acetyl-CoA als Donor für die Acetylgruppen, das Substrat, ^{14}C-markiertes Chloramphenicol (CLA), zu 1-Acetyl-CLA, 3-Acetyl-CLA und 1,3-Diacetyl-CLA. Die Reaktionsprodukte können anschließend dünnschichtchromatographisch aufgetrennt und detektiert werden. Dieses Resistenzsystem kann sowohl zur Qualifizierung (Klonselektion) als auch zur Quantifizierung von Proteinsynthesen genutzt werden [5, 6, 30]. Auch in diesem Zusammenhang zeigen Untersuchungen, dass in Abhängigkeit von der Benutzung verschiedener Reportergen-Assays und auch verschiedener Zellkulturlinien unterschiedliche Ergebnisse bei der Behandlung mit Hormonen und Antihormonen auftreten können. Trotzdem sind Reporter-Gene-Assays wirkungsvolle Testsysteme, um Substanzen auf ihr östrogenes Wirkungspotential hin zu untersuchen [8].

2. *In vivo* Untersuchungen

2.1 Gewebetypische Wirkungen

In vivo gibt es ebenfalls eine Reihe validierter Testsysteme zur Untersuchung von (Anti-)Androgenität, (Anti-)Östrogenität und (Anti-)Gestagenität. Bei solchen Testsystemen werden Mäuse oder Ratten eines bestimmten Stammes mit Testsubstanzen behandelt und anschließend organspezifische Feuchtgewichtsanalysen, morphometrische und immunhistochemische

Untersuchungen sowie molekularbiologische Analysen auf mRNA- und Proteinebene durchgeführt.
Bei der Evaluierung von potentiellen Östrogenen kommt im speziellen der sogenannte Uteruswachstumstest zum Einsatz, wobei man sich verschiedener Arten und Stämme von weiblichen Nagern zur Risikoabschätzung bedient. Nach der Ovariektomie werden diese Tiere 3 Tage mit den Testsubstanzen behandelt und anschließend im Vergleich mit Positiv- und Negativkontrollen einer Gewichtsanalyse unterzogen.
In eigenen Untersuchungen mit NMRI-Mäusen zur Wirkungsabschätzung von BPA, Oktylphenol, DDT, Genistein und Daidzein zeigte sich die erwartete starke dosisabhängige Zunahme des Uterusgewichts nach Applikation von Ethinylöstradiol [9]. BPA dagegen zeigt erst bei einer Dosierung von 500 mg einen geringen Effekt auf das Uteruswachstum. Die Untersuchung der Uterus-Epithelhöhe zeigt ebenfalls nur bei der höchsten Dosierung von 500 mg/kg KG/Tag einen geringen Anstieg der Epithelhöhe. Ein ähnliches Ergebnis zeigt sich beim Proliferationsnachweis mittels immunhistochemischer Parameter wie dem PCNA (PCNA = proliferating cell nuclear antigen): Bei der höchsten Dosierung im Vergleich zur Negativkontrolle sind vermehrt exprimierte Proliferationsproteine (PCNA: 36 kDa) zu detektieren.
In weiblichen Wistar-Ratten zeigt sich in unseren Untersuchungen im Vergleich zur Ovariektomie kein signifikanter Anstieg des Uterusfeuchtgewichtes bei Behandlung weder mit Genistein, Bisphenol A noch Oktylphenol.
Andere Rattenstämme wie z.B. Sprague-Dawley oder DA/Han reagierten unterschiedlich sensitiv im Vergleich zu Wistar-Ratten und führten entsprechend zu anderen Ergebnissen. Auch interkontinentale Unterschiede gleicher Rattenstämme sind bei Wirkabschätzungen nicht zu vernachlässigen [16].
Wie die genannten Beispiele zeigen, entfalten endokrine Disruptoren sowohl gewebespezifische als auch konzentrationsabhängige Effekte. Bei der Whole Mount Präparation der Rattenmilchdrüsen zeigt sich z.B., dass DDT eine starke östrogene Wirksamkeit entfaltet, wobei es z.B. im Uteruswachstumstest im Vergleich zu weiteren Xenoöstrogenen nur geringfügig östrogen wirkt [15].
In der semiquantitativen Auswertung zeigt sich ein komplexes Bild, welches nochmals verdeutlicht, wie unterschiedlich die getesteten Substanzen auf unterschiedliche östrogen-abhängige Gewebe reagieren [2, 4, 7].

Uterus	Uterus Gewicht	+++	+	+	++	+	+
	Uterus Epithelhöhe	+++	-	-	+++	+	+
	Uterus ER	+++	-	++	+++	++	++
	Uterus PR	+++	-	++	+++	++	++
	Uterus C3-mRNA	+++	++	+	+++	+++	+
	Uterus CLU-mRNA	+++	-	++	+++	+	++

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Vagina	Vaginal Epithel	+++	-	+	+++	++	++
	Verhornung	+++	+	+	+++	++	+++
Mamma	***Brustdrüsen***	+++	-	-	++	-	++
	Mamma PR	+++	-	-	+++	++	++

Neben den vielfältigen Untersuchungen zur Östrogenität der beschriebenen Substanzen wurden die möglichen androgenen Effekte bisher weitgehend vernachlässigt: Bei der Untersuchung der androgenen Wirkungen von BPA kommt der sogenannte Hershberger Assay zum Einsatz. Dieser Assay wird in der pharmazeutischen Industrie seit Jahren in der Wirkstofffindung eingesetzt. Unter Verwendung von Positiv- (intakte Tiere) und Negativkontrollen (orchiektomierte Tiere) werden dabei orchiektomierte Tiere mit z.B. BPA in unterschiedlichen Dosierungen täglich einmal über 7 Tage behandelt, und klassische akzessorische Geschlechtsorgane der männlichen Ratte untersucht [17, 19].
In eigenen Untersuchungen zeigt die Gewichtsanalyse der Prostata im Hershberger-Assay eine deutliche Abnahme des Feuchtgewichts nach Orchiektomie. Werden die orchiektomierten Tiere mit BPA behandelt, bleibt das absolute Feuchtgewicht bei allen getesteten Dosierungen im Vergleich zur Negativkontrolle Orchiektomie unverändert. Dagegen ist eine deutliche Gewichtszunahme der Prostata bei den Positivkontrollen (Intakt und TP=Testosteronpropionat behandelt) im Vergleich zur Orchiektomie zu erkennen. Als weiteren geeigneten Untersuchungsparameter der Androgenität werden die Lumenflächen der Prostatadrüsen morphometrisch vermessen. Hierbei stellten wir einen signifikanten Anstieg der Lumenflächen bei den Dosierungen von 3-200 mg/kg KG/Tag fest, die jedoch im Vergleich zur orchiektomierten Positivkontrolle (TP) als biologisch nicht bedeutsam einzustufen ist [20, 21 22, 23].

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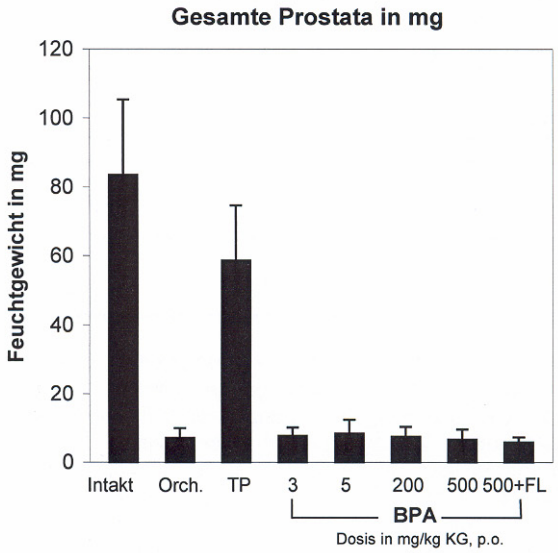

2.2 Immunologische Wirkungen

Seit Jahren ist bekannt, das (Anti-)Östrogene und (Anti-)Androgene die Entwicklung und Regulation des Immunsystems beeinflussen können. Die stimulierenden oder inhibierenden Effekte durch Östrogene auf die mitotische Aktivität aber auch auf spezielle Funktionen von Immunzellen bzw. lymphatischen Organen werden durch ihre Rezeptoren vermittelt. Inwiefern endocrine disruptors, hier im speziellen die Xenoöstrogene Einfluss auf das Immunsystem und damit die Gesundheit nehmen, ist bisher nur gering beschrieben. Zum Teil besitzen die Xeno- und Phytoöstrogene im Gegensatz zum Östrogen eine Selektivität für die Östrogenrezeptoren -alpha oder -beta. Je nach Rezeptor-Selektivität können Phyto- oder Xenoöstrogene damit potentiell Einfluss auf das Immunsystem bzw. auf einzelne Funktionen der zellulären Immunabwehr nehmen. Bisher ist nur wenig über die Wirkungen und die zugrunde liegenden Mechanismen bekannt. Im Gegensatz zur dosisabhängigen östrogeninduzierten Suppression der NK-Aktivität (Nilsson und Carlsten, 1994) sollen Daidzein und Genistein die NK-Zellaktivität in physiologischen Dosen erhöhen und sogar einen additiven Effekt nach einer Stimulation mit IL-2 entfalten. In eigenen immunologischen Untersuchungen konnten wir ebenfalls zum Teil einen stimulierenden Effekt von BPA und Genistein zeigen, im Vergleich zur Positivkontrolle Ethinylöstradiol [25].
Neben diesen immunstimulierenden Auswirkungen der Phytoöstrogene sollen Xenoöstrogene wie Bisphenol A (BPA), PCBs oder Oktylphenol supprimierend auf das Immunsystem wirken. PCBs z.B. hemmen die Phagozytoseaktivität von Peritonealmakrophagen *in vitro*. Auch hier konnten wir in eigenen Untersuchungen zum Teil einen gering supprimierenden Einfluss von Bisphenol A auf die Phagozytoseaktivität von Granulozyten und Monozyten nach einer Behandlung von 3 Tagen zeigen, der sich aber nur in der Gruppe mit einer Behandlung von 2mg/kg/Körpergewicht Bisphenol A niederschlug.

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	Parameter	EE	BIS 2µg	BIS **2 mg**	BIS 50 mg	GEN 50	GEN 100
Milz Blut	NK-Zell Aktivität	-	-	+	-	+	-
	Phagozytose Granulozyten	-	-	-	-	-	-
	Phagozytose Monozyten	-	-	-	-	-	-
	Phagozytose / einzelnem	-	-	--	-	-	-
	Phagozytose / einzelnem	-	-	---	-	-	-
Blut	CD11b positive Monozyten	-	-	-	-	-	-
	NK-Zellen	-	-	-	-	-	-

Viele weitere Wirkungen von Xenoöstrogenen auf weitere Parameter des Immunsystems sind bisher nicht untersucht und daher gänzlich unbekannt, und das sowohl für die kurz- als auch für die langfristigen Effekte. Diese Datenlage macht deutlich, dass ein dringender Bedarf besteht das organspezifische Wirkpotential von Phyto- und Xenoöstrogenen auf das Immunsystem und die molekularen Grundlagen dieser Wirkung noch näher zu charakterisieren.

Fazit

Abhängig von den gewählten Testsystemen *in vitro* als auch *in vivo* können unterschiedliche Ergebnisse erhalten werden, die sogar gegensätzlich sein können.
Phyto- und Xenoöstrogene entfalten endokrine Wirkungen, wie z.B. Prostatawachstum, Brustdrüsenwachstum, die mit entsprechenden Testsystemen erkannt werden können.
Der Hershberger Assay und der Uteruswachstumstest sowie das Uterus-Gen-Fingerprint-Expressionsmuster sind validierte und sensitive Testsysteme und Parameter zur Untersuchung von Androgenität und Östrogenität.
Phyto- und Xenoöstrogene entfalten außer der hormonellen Wirksamkeit auch immunologisches Potential.
Viele mögliche Wirkungen von Phyto- und Xenoöstrogenen auf verschiedene Zellsysteme und Organe sind noch nicht abschließend untersucht, z.B. das Immunsystem, und bedürfen einer weiteren Risikoabschätzung.

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Es besteht die Notwendigkeit und die Pflicht, potentiell unerwünschte Wirkungen sowie die frühzeitige Erkennung der potentiellen Risiken von endogenen Disruptoren zu untersuchen.

Literatur

BOLT, H. M., JANNING, P., MICHNA, H., DEGEN, G. H.: Comparative assessment of endocrine modulators with oestrogenic activity: I. Definition of a hygiene-based margin of safety (HBMOS) for xeno-oestrogens against the background of European developments. Arch Toxicol 74: pp. 649-662 (2001)

DEGEN, G. H., JANNING, P., DIEL, P., MICHNA, H., BOLT, H. M.: Transplacental transfer of the phytoestrogen daidzein in DA/Han rats. Arch Toxicol 76 (1): 9-23 (2002)

DEGEN, G. H., JANNING, P., MICHNA, H., BOLT, H. M.: Comparative assessment of endocrine modulators with oestrogenic activity (xenooestrogens). Arch Pharmacol 363, Suppl. 4: R 172 (2001)

DEGEN, G. H., JANNING, P., UPMEIER, A., DIEL, P., MICHNA, H., BOLT, H. M.: Comparative toxicokinetics of bisphenol A in pregnant and non-pregnant DA/Han rats. Reprod Toxicol (2001)

DIEL, P., OLFF, S., SCHMIDT, S., MICHNA, H.: Effects of the environmental estrogens bisphenol A, o,p`-DDT, p-tert octylphenol and coumestrol on progesterone receptor, estrogen receptor alpha and androgen receptor expression in the human breast cancer cell line MCF-7. J Steroid Biochem Mol Biol 80: pp. 61-70 (2002)

DIEL, P., OLFF, S., SCHMIDT, S., MICHNA, H.: Molecular identification of potential selective estrogen receptor modulator (SERM) like properties of phytoestrogens in the human breast cancer cell line MCF-7. Planta Med 67(6): pp. 510-514 (2001)

DIEL, P., SCHULZ, T., SMOLNIKAR, K., STRUNCK, E., VOLLMER, G., MICHNA, H.: Ability of xeno- and phytoestrogens to modulate the expression of estrogen-sensitive genes in the rat uterus: estrogenicity profiles and uterotrophic activity. J Steroid Biochem Mol Biol 73: pp. 1-10 (2000)

DIEL, P., SMOLNIKAR, K., MICHNA, H.: In vitro test systems for the evaluation of the estrogenic activity of natural products. Planta Med 65(3): pp. 197-203 (1999)

DIEL, P., SMOLNIKAR K., SCHULZ, T., LAUDENBACH, U., DEGEN, G., SCHUHMACHER, U., BOLT, H., MICHNA, H.: Molecular and morphological analysis of the endocrine profile of the xenoestrogens bisphenol A, p-tert-octylphenol, o,p´-DDT and the phytoestrogen daidzein. Arch Pharmacol 358, Suppl. 3: R 770 (1998)

DIXON, D.A. & FERREIRA, D.: Genistein. *Phytochemistry* 60 (3): 205-211 (2002)

GEHRING, M.; WELTIN, D.; TENNHARDT, L.; VOGEL, D.; BILITEWSKI, B. (2002): Elimination of Bisphenol A During Wastewater Treatment: Detailed Mass Balances Derived from Laboratory Experiments. Proceedings of the 2002 AWWA Endocrine Disruptors & the Water Industry Symposium, (2002)

HEEMKEN, O.P.; THEOBALD, N.; HEBBEL, H.; STACHEL, B.; REINCKE, H.: Endokrin wirksame Stoffe in der Elbe, in Nebenflüssen und in der Nordsee. http://www.arge-elbe.de/wge/Download/Berichte/Endokrin.pdf;(2000)

KRIMSKY; S.: Hormonal Chaos, The Johns Hopkins University Press, Baltimore. (2000)

KROIß, H.; (Hrsg.): Hormonell wirksame Substanzen in der Umwelt. Wiener Mitteilungen Wasser-Abwasser-Gewässer, Band 153, Wien: Technische Universität (1998)

MICHNA, H., DIEL, P., VOLLMER, G., DEGEN, G., LAUDENBACH, U., SCHULZ, T., SMOLNIKAR, K., WÜNSCHE, W., MUßLER, B., EISENBRAND, G., BOLT, H.: Analysis of the tissue type specific estrogenic potential of xeno- and phytoestrogens *in vitro* and *in vivo*. (DGE-Symposium „Breast and Prostate Cancer: Genes, hormones and the environment") (1999)

MICHNA, H., SCHMIDT, S., LAUDENBACH, U., MASKUS, M., SCHULZ, T., SMOLNIKAR, K., DIEL, P.: The effects of low and high doses of BPA on (anti-)androgenic and (anti-)estrogenic parameters in the reproductive tract of mice (NMRI) and rats (Wistar, Sprague Dawley, Da/Han). Reprod Toxicol (2001)

NISHINO, T., MASKUS, M., LAUDENBACH, U., BÜHLMEYER, K., VOß, A., SCHULZ, T., MICHNA, H.: Androgen Receptor regulation and the influence of bisphenol A in male reproductive organs in rats (wistar): Molecular and immunohistochemical, morphometric analysis. 15th International Symposium of the Journal of Steroid Biochemistry & Molecular Biology, (2002)

NISHINO, T., SCHULZ, T., BÜHLMEYER, K., MICHNA, H.: Die Industriechemikalie Bisphenol A: Anwendungsgebiete, Risikopotential und Prüfung auf (anti-) androgene Wirkung. Abstractband 2. Wissenschaftstagung „Lebensmittel und Gesundheit", Weihenstephan, (2003)

NISHINO, T., SCHULZ, T., BÜHLMEYER, K., MICHNA, H.: Immunohistochemical and morphometric evaluation of androgen receptor regulation in prostate and seminal vesicle of BPA-treated male wistar rats. 11th International Congress on Hormonal Steroids (ICHS)/7th International Congress on Hormones and Cancer (ICHC), Fukuoka, (2002)

NISHINO, T., SCHMITT, O., WEDEL, T., STRAUCHMANN, H., BÜHLMEYER, K., SCHULZ, T., KÜHNEL W., MICHNA, H.: Androgenic Reactions in the rat prostate: validation of proliferation markers and androgen receptor staining pattern and the effects of the "xenoestrogen" BPA. Verhandlungen der Anatomischen Gesellschaft, Würzburg (2003)

NISHINO, T., SCHMITT, O., WEDEL, T., STRAUCHMANN, H., BÜHLMEYER, K., SCHULZ, T., MICHNA, H.: Regulation of (anti-) androgenic parameters and the effect of BPA on prostate and seminal vesicle in rats: immunohistological, morphometric and molecular studies. Verhandlungen der Anatomischen Gesellschaft, Band 185, Würzburg (2002)

NISHINO, T., SCHMITT, O., WEDEL, T., STRAUCHMANN, H., BÜHLMEYER, K., SCHULZ, T., MICHNA, H.: Effekte von Bisphenol A auf den männlichen Genitaltrakt: Morphologische, immunhistochemische und molekulare Studien. Abstractband 2. Wissenschaftstagung „Lebensmittel und Gesundheit", Weihenstephan, (2003)

NISHINO, T., SCHMITT, O., WEDEL, T., STRAUCHMANN, H., BÜHLMEYER, K., SCHULZ, T., MICHNA, H.: Androgen-dependent morphology of prostate and seminal vesicle: Immunohistochemical and morphometric parameters. Verhandlungen der Anatomischen Gesellschaft, Band 185, Dresden (2003)

PURDOM, C.E.; HARDIMAN, P.A.; BYE, V.J.; ENO, N.C.; TYLER C.R.; SUMPTER J.P.: Estrogenic Effects of Effluents from Sewage Treatment Works. Chem. Ecol., 8, 275-285 (1994)

SCHULZ, T., LAUDENBACH, U., MASKUS, M., MATSAKAS, A., MICHNA H.: Wirkung von Xenoöstrogenen und Phytoöstrogenen auf die Phagozytoseaktivität von Monozyten und Granulozyten. Abstractband 2. Wissenschaftstagung „Lebensmittel und Gesundheit", Weihenstephan (2003)

TEUSCHER, E.: Pharmazeutische Biologie. Braunschweig, Wiesbaden: Vieweg (1990)

VON BRUCHHAUSEN, F.: Hagers Handbuch der pharmazeutischen Praxis. Berlin, Heidelberg, New York, London: Springer Band 5: Drogen E-O; R. Hänsel (Hrsg.) (1993)

YAMAMOTO, T.; YASUHARA, A.; SHIRAISHI, H.; NAKASUGI, O.: Bisphenol A in Hazardous Waste Landfill Leachates. Chemosphere, 42, 415-418 (2001)

YASUHARA, A.; SHIRAISHI, H.; NISHIKAWA, M.; YAMAMOTO T.; NAKASUGI, O.; OKUMURA, T.; KENMOTSU, K.; FUKUI, H.; NAGASE, M.; KAWAGOSHI, Y.: Organic Components in Leachates from Hazardous Waste Disposal Sites. Waste Manage. Res., 17, 186- 197 (1999)

ZIERAU, O., DIEL, P., WOBER, J., THOMAE, R., KRETSCHMAR, G., METZ, P., MICHNA, H., NISSLEIN, T., VOLLMER, G.: Molecular mechanisms of phytoestrogen function. 15th International Symposium of the Journal of Steroid Biochemistry & Molecular Biology, München 2002. J Steroid Biochem Molec Biol Abstractband: 6-L (2002)

Appendix IV.

Michna H, Hilber K, Nishino T, Peters C, Selg PJ, Schulz T: Warum Männer ungesund leben und Frauen gesünder sterben. i. Interdisziplinärer Kongress junge Naturwissenschaft und Praxis, 11. – 13. Juni 2003 in München.

Gesundheit fördern – Krankheit heilen, Hanns Martin Schleyer-Stiftung, Proceedings 62 P 45-68 (2003)

Gesundheit fördern – Krankheit heilen

Neue Wege im Zusammenwirken von Naturwissenschaft – Medizin – Technik

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Horst Michna

Warum Männer ungesund leben und Frauen gesünder sterben*

Ehrwürdiger Vizepräsident, oder lieber Ruderkamerad Melzer, dass darf man ja in diesem Zusammenhang hier sagen, Herr Vorsitzender Professor Dickhuth, es gibt sie ja noch die Professoren die gemeinsam Sport treiben, zumindest an der Technischen Universität München, erlauchtes Auditorium, vor allem: liebe Jungwissenschaftler!
Nun – ich habe noch nie so viel Zustimmung bei der Wahl eines Vortragsthemas erhalten und ich war dabei noch nie so unpräzise in der Formulierung des Themas meines Vortrages. Bekanntlich flehen die Menschen die Götter um Gesundheit an und ich werde versuchen Ihnen im Nachfolgenden aufzuzeigen, dass Sie die Macht darüber entsprechend der Lebenserfahrung von Demokrit (Abb. 1) eigentlich selber besitzen. Ich werde auch – mit Ihrer Erlaubnis – einen Spaziergang durch München unternehmen: das Motto wird eine medizinhistorische Eskapade vor dem Hintergrund

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* Verantwortlich für die schriftliche Fassung des Vortrags: Horst Michna, Katharina Hilber, Dipl.-Chemiker Tsuyuki Nishino, Dr. Christiane Peters, Dipl.-Sportwiss.cand. Peter Selg sowie Dr. Thorsten Schulz.
Die Autoren widmen diesen Vortrag in Verehrung dem Ordinarius für Präventive und Rehabilitative Sportmedizin Herrn Univ.-Prof. (em.) Dr. Dieter Jeschke und seinem „Team" in Anerkennung für 17 Jahre im Dienste der Gesundheitsförderung an der TU-München.

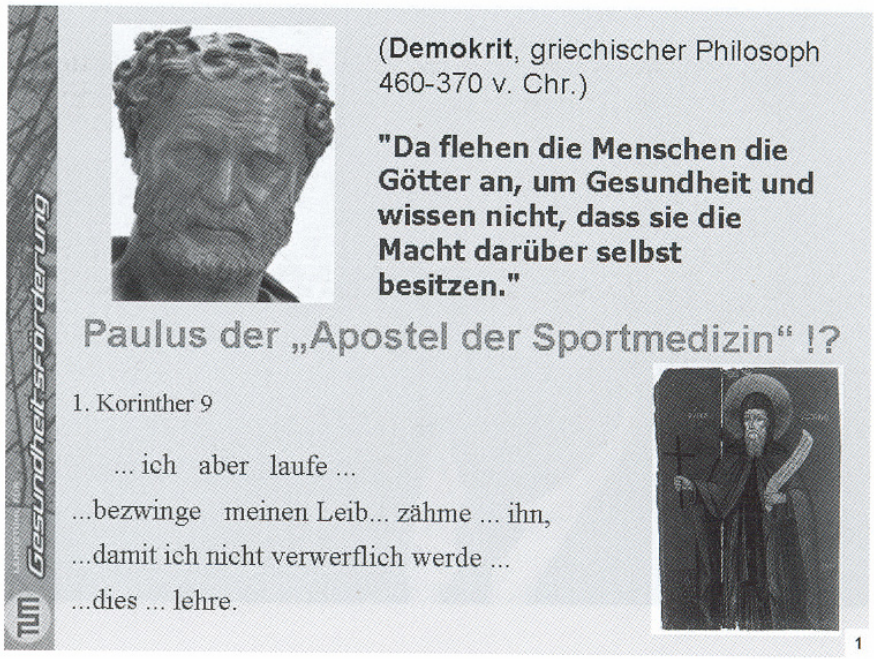

Abb. 1

des Themas sein. In diesem Zusammenhang: der Apostel der Sportmedizin könnte wohl Paulus sein – den wir eindrucksvoll dargestellt in der Alten Pinakothek finden: dieser im Korintherbrief formuliert, „ich aber laufe, ... bezwinge meinen Leib und zähme ihn..." (1. Kor. 9, 26) (Abb. 1).

Abb. 2

Abb. 3

Dies lehrt der Apostel Paulus, der auch in der Alten Pinakothek dargestellt ist. (Abb. 2)

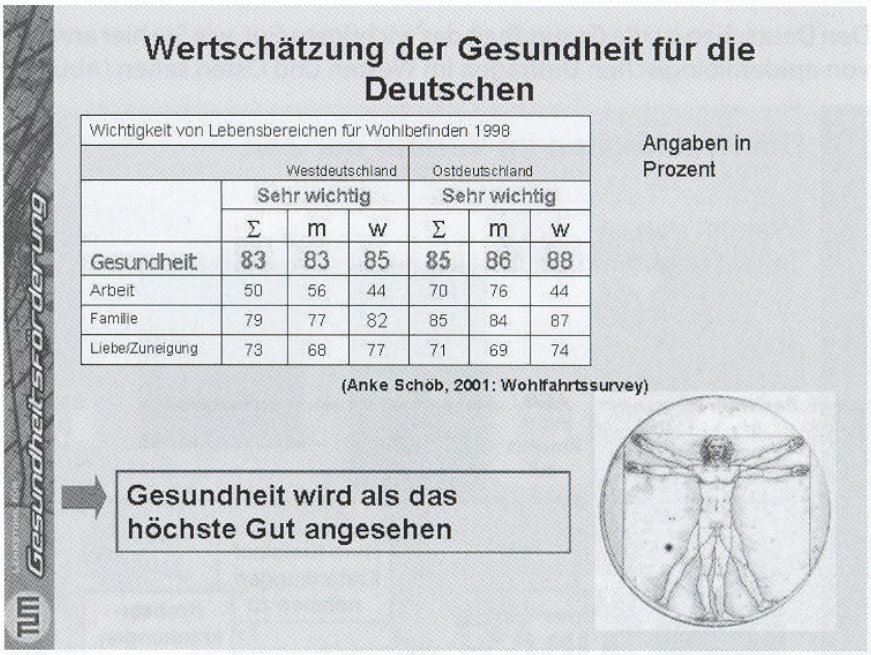

Abb. 4

Nun Gesundheit ist in aller Munde, nach der Definition der Weltgesundheitsorganisation ist Gesundheit „... ein Zustand des umfassenden kör-

perlichen, geistigen und sozialen Wohlbefindens und nicht lediglich das Freisein von Krankheit und Schwäche. ..." (Abb. 3).

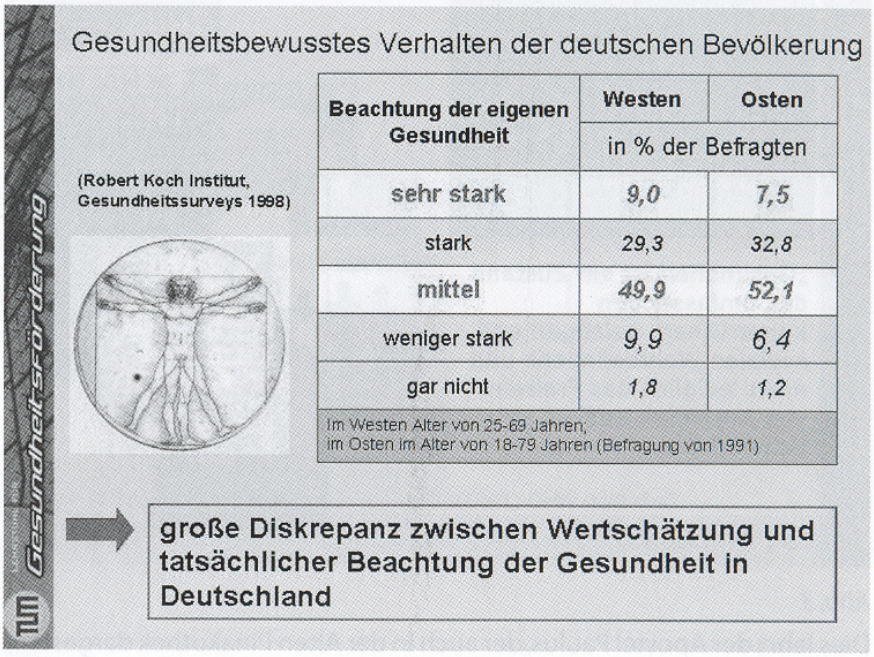

Abb. 5

Den Deutschen ist die Gesundheit das wichtigste Gut, wie Sie hier anhand von epidemiologischen Umfragen im Westen und Osten sehen (Abb. 4).

Abb. 6

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Gleichwohl befragt man die Menschen nach ihrem *Gesundheitsbewusstsein*, dann dokumentiert sich, dass offensichtlich ein Widerspruch vorliegt, eine große Diskrepanz zwischen der initial gezeigten *Wertschätzung* und der *tatsächlichen Beachtung* von Gesundheit (Abb. 5).

Die Krankheitshäufigkeiten haben sich im Laufe der Zeit gewandelt; Herz-Kreislauferkrankungen nahmen in den achtziger Jahren zu. Es zeichnet sich nun ab, dass sich eine vermehrte Inzidenz von Krebserkrankungen einstellt (Abb. 6).

Hält man sich die Häufigkeit der Mortalität in Deutschland vor Augen wird erkennbar, dass insbesondere Krankheiten des Herz-Kreislaufsystems Todesursache Nr.1 sind; an zweiter Stelle drohen die Krebserkrankungen, Todesfälle aufgrund von Krankheiten des Muskel-, Skelett- und Bindegewebssystems, auf die ich später noch zu sprechen kommen werde, tauchen hier weniger auf. Krebserkrankungen bedrohen also mehr und mehr unser Leben (Abb. 7).

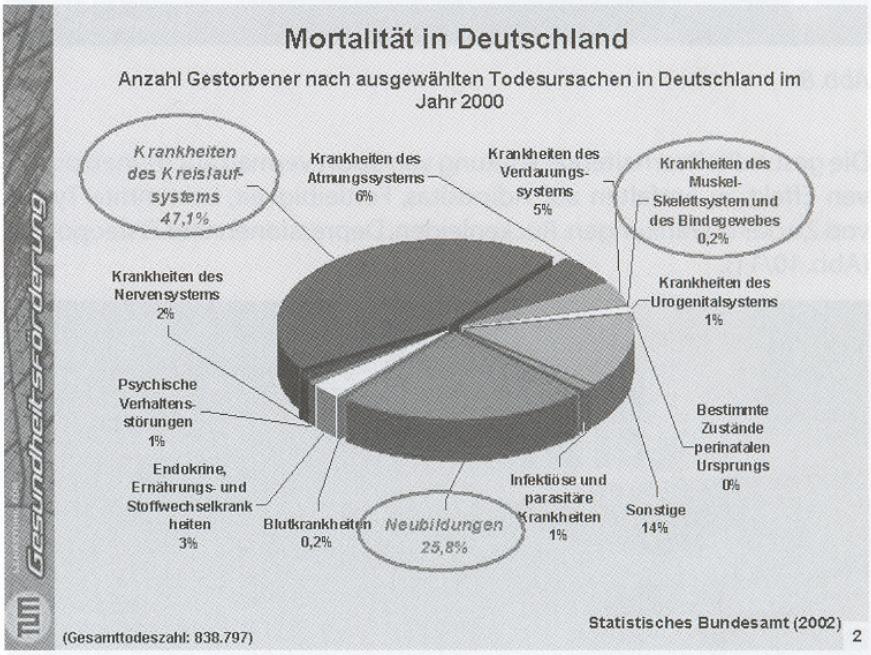


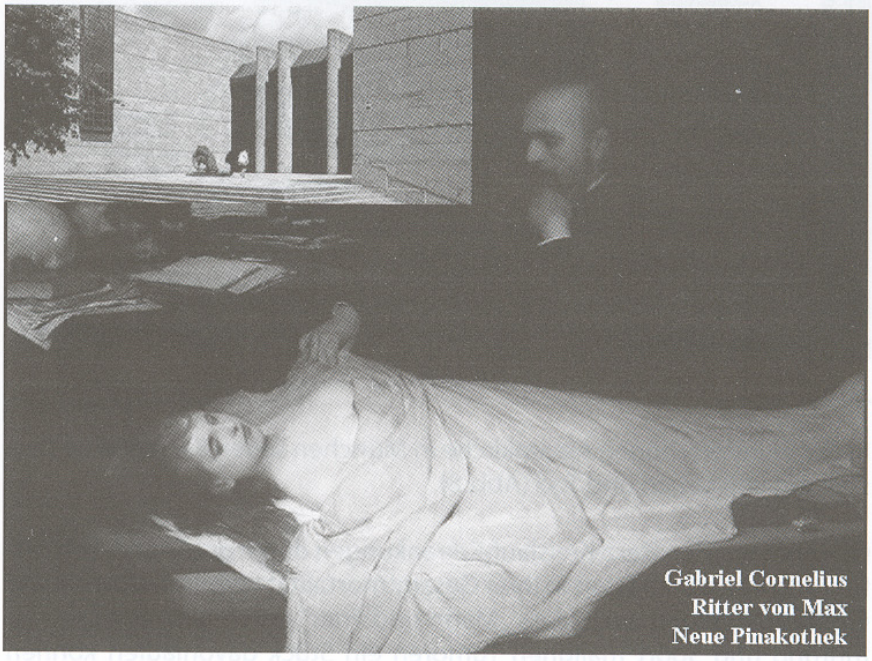
Abb. 7

In der wunderschönen Assamkirche in München sehen Sie hier eine Darstellung von Leben und Tod (Abb. 8).

Offenbar wurden Herz-Kreislauf-Erkrankungen durch gesündere Lebensführung und durch Sport in Ihrer Gesamtmortalität vermindert; und erste Ergebnisse zeigen auch, dass „Mann und Frau" durch gesunde Lebensführung und Sport malignen Tumoren ein Stück davonlaufen können (Abb. 9).

Abb. 8

Die gesundheitserhaltende Wirkung von Sport vermag auch einen positiven Effekt zu entfalten auf Adipositas, Fettleibigkeit, bestimmte Typen von Zuckererkrankungen, Rückenleiden, Depressionen und Osteoporose (Abb. 10/11).



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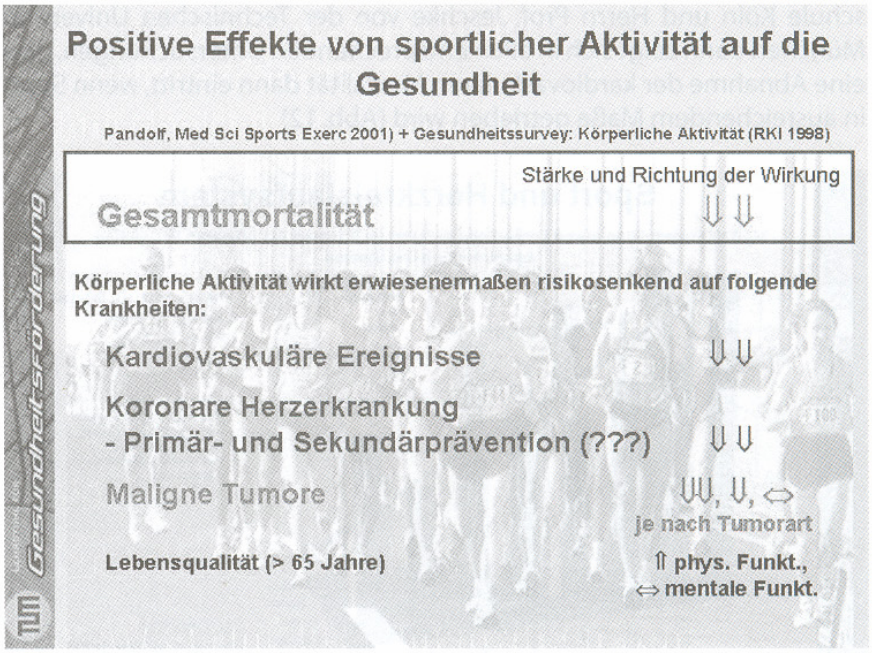


Abb. 10

Sieht man sich die Reduktion der Mortalität durch körperliches Training in Abhängigkeit von Intensitätsskalierungen an, aus der Arbeitsgruppe unseres Vorsitzenden Herrn Prof. Dickhuth, des Spiritus Rectors der deutschen Sportmedizin Herrn Prof. Hollmann von der Deutschen Sporthoch-

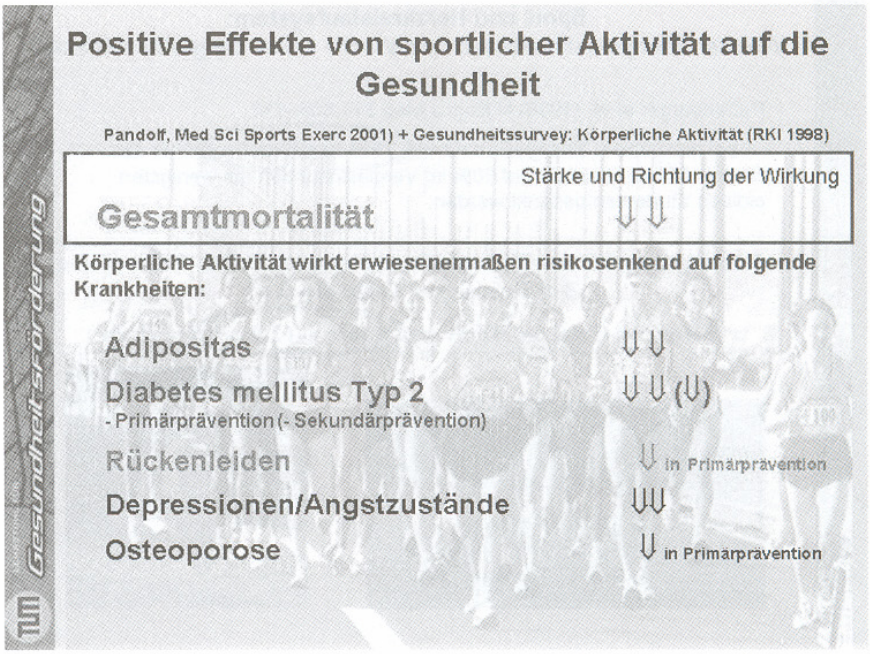
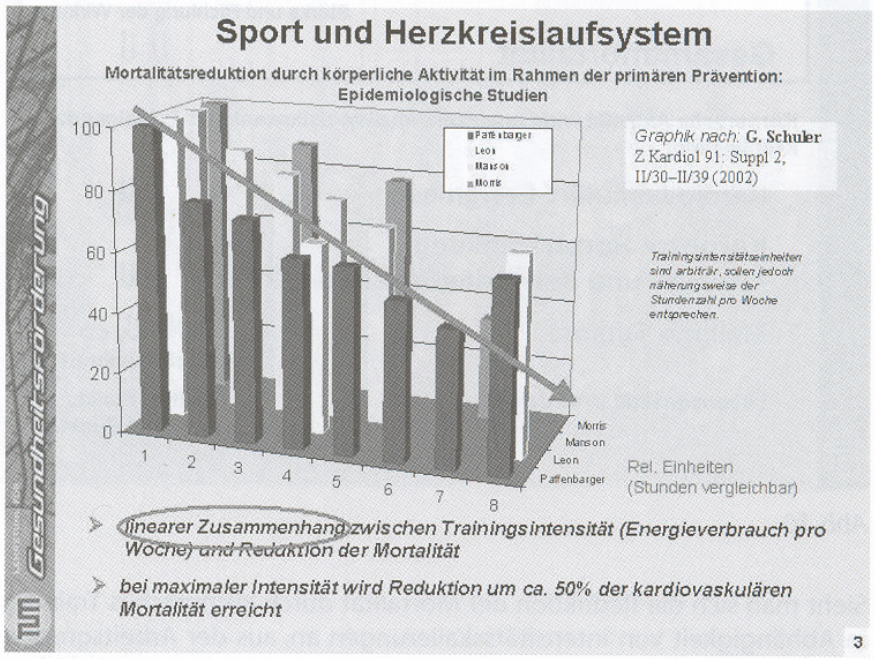


Abb. 11

schule Köln und Herrn Prof. Jeschke von der Technischen Universität München dann zeigt sich in allen entsprechenden Untersuchungen, dass eine Abnahme der kardiovaskulären Mortalität dann eintritt, wenn Sport in ausreichendem Maße getrieben wird (Abb. 12)



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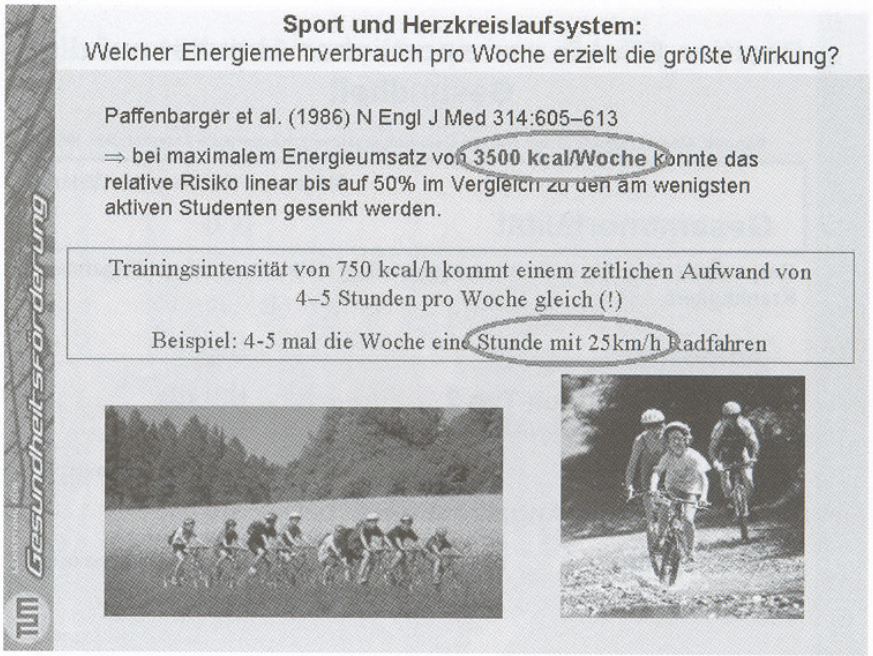


Abb. 13

Hier handelt es sich um zahlreiche epidemiologische Studien, die eigentlich auf den bahnbrechenden Arbeiten der internistischen Sportmediziner in Deutschland beruhen, u.a. auch aus der Arbeitsgruppe von Herrn Prof. Dickhuth.
Was empfehlen die Experten?
Die deutsche Gesellschaft für Kardiologie empfiehlt einen Energiemehrverbrauch von 3500 Kilokalorien pro Woche, das sind ungefähr „über den unwissenschaftlichen Daumen gepeilt", vier- bis fünfmal die Woche intensives Radfahren (Abb. 13).

Ein anderes Extrem empfiehlt 1000 Kilokalorien pro Woche, dazu würde es schon reichen, viermal die Woche nur zu putzen (Abb. 14).

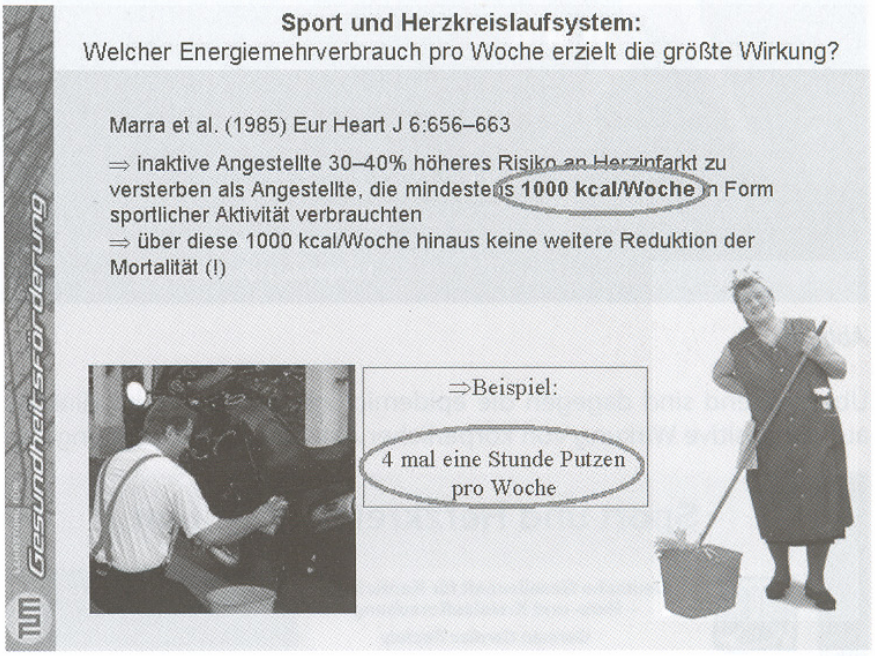


Abb. 14

Die Wahrheit liegt wahrscheinlich dazwischen. Was empfehlen die Experten, Deutsche Gesellschaft für Kardiologie, vier- bis fünfmal wöchentlich körperliche Aktivität über 30-45 Minuten mit mäßiger Intensität (Abb. 15).

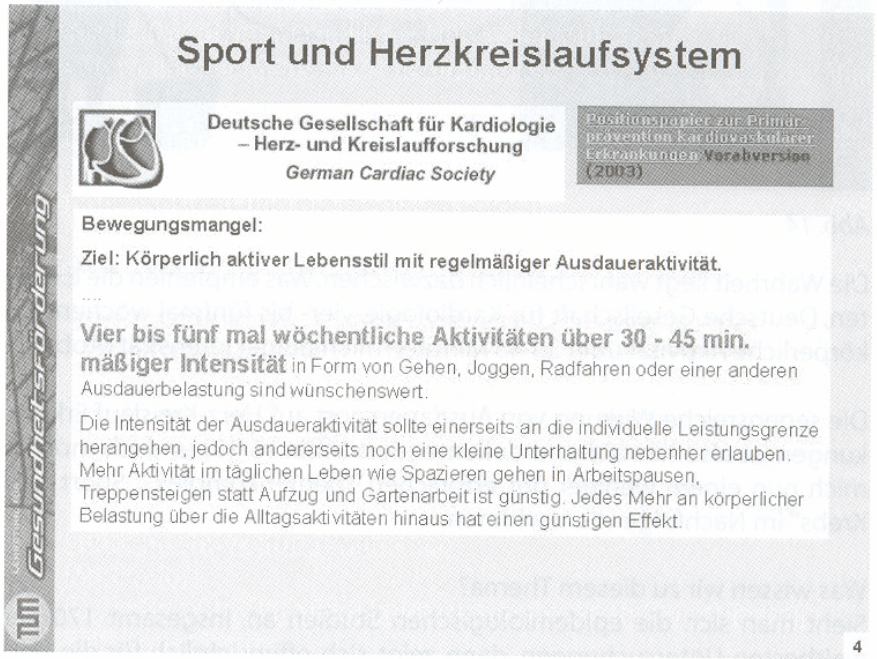
Die segensreiche Wirkung von Ausdauersport auf Herz-Kreislauf Erkrankungen ist also hinreichend dokumentiert (Abb. 16/17) und ich möchte mich nun einem weniger gut erforschen Themenkomplex „ Sport und Krebs" im Nachfolgendem widmen.

Was wissen wir zu diesem Thema?
Sieht man sich die epidemiologischen Studien an, insgesamt 170 der weltbesten Untersuchungen, dann zeigt sich offensichtlich für die hormonabhängigen Krebsformen wie Brustkrebs bei der Frau, eine überzeu-

gende Reduktion. Für das Prostatakarzinom zeigt sich eine weniger ausreichend analysierte Studiensituation (Abb. 18).

Abb. 15

Überzeugend sind dagegen die epidemiologischen Daten im Hinblick auf die positive Wirkung von körperlicher Aktivität zur Vorbeugung von



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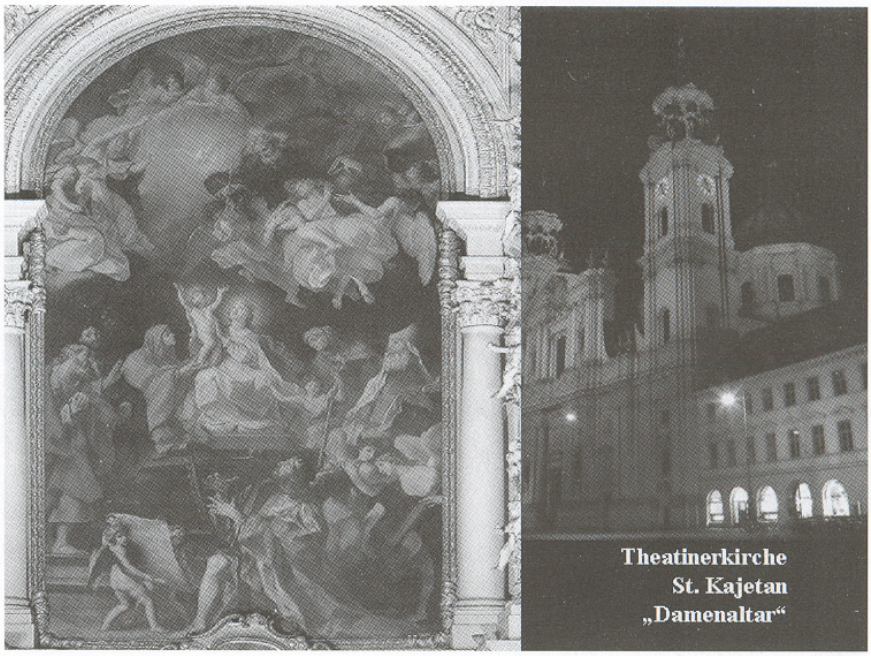

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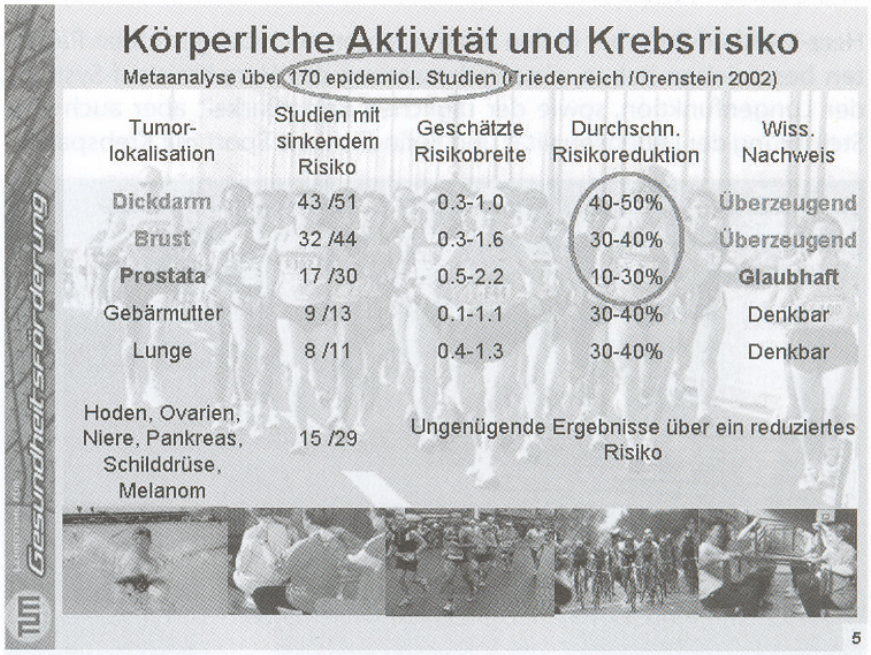

Abb. 18

Dickdarmkrebs. Quintessenz: wir können also durch Sport dem Krebs – *wenigstens in einigen Krebsformen* – zumindest ein Stück davonlaufen. Die therapeutische Wirkung von Sport bei den Krebspatienten ist zum einen eine Verbesserung der physischen Funktionen, das, was die

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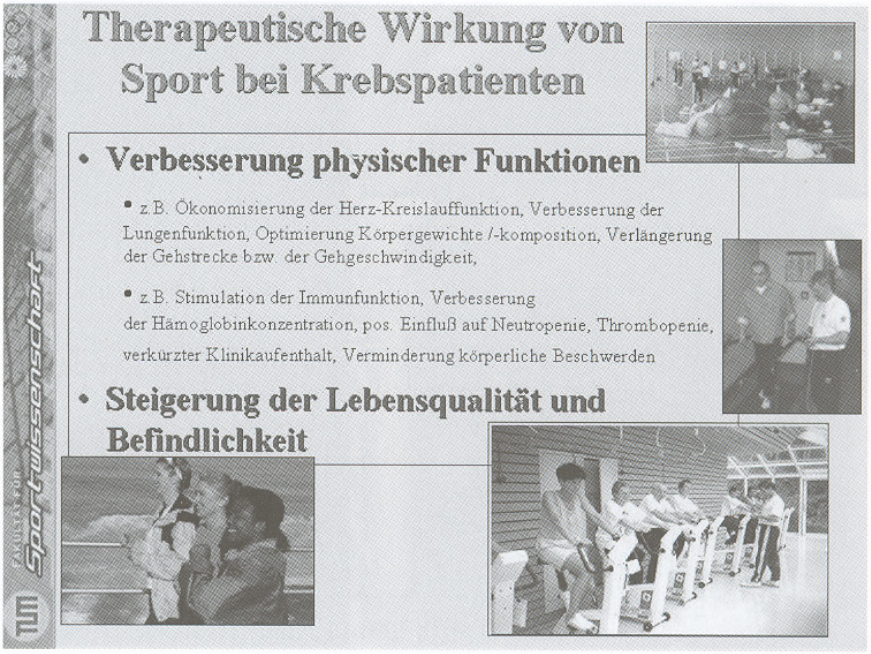
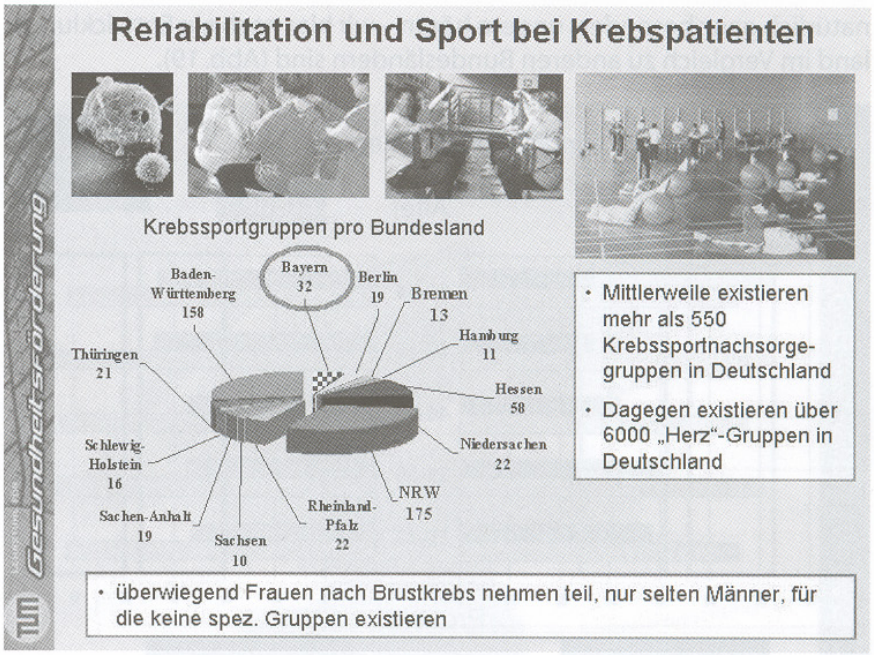


Abb. 19

Herz-Kreislauf-Forscher in den letzten Jahren auch bei gesunden Patienten beschrieben haben, eine Verbesserung des Herz-Kreislauf-Systems, der Lungenfunktion, sowie der täglichen Belastbarkeit aber auch eine Steigerung der Lebensqualität und Befindlichkeit. Sport mit Krebspatien-

Abb. 20



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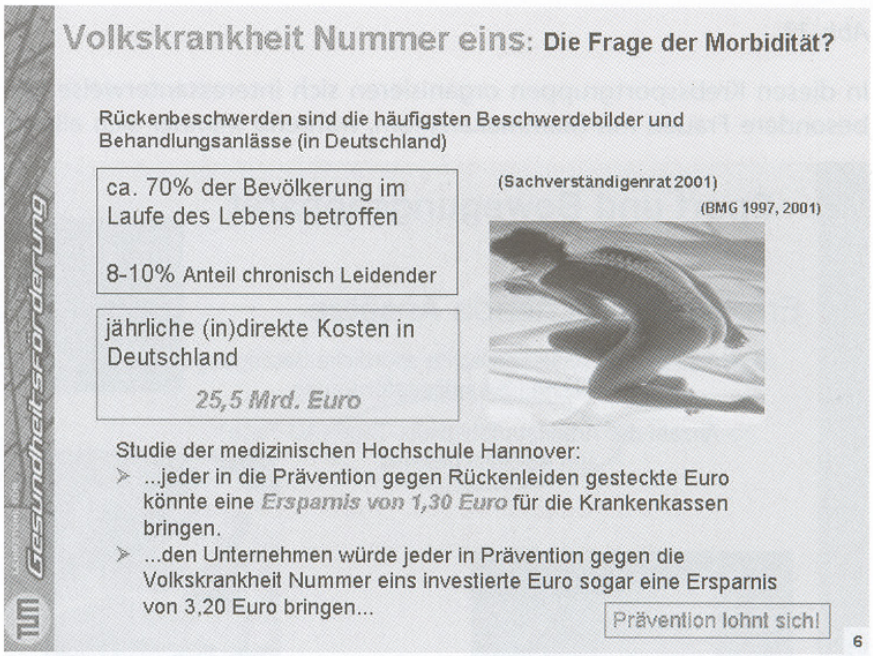

Abb. 22

ten findet in sogenannten Krebssportgruppen statt und ich möchte Ihnen hier vor Augen halten, dass in Bezug auf die Anzahl der Krebsport-gruppen in den Bundesländern – und das ist etwas, was wir „Bayern"

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natürlich ganz besonders ungern hören – wir hier noch ein Entwicklungsland im Vergleich zu anderen Bundesländern sind (Abb. 19).

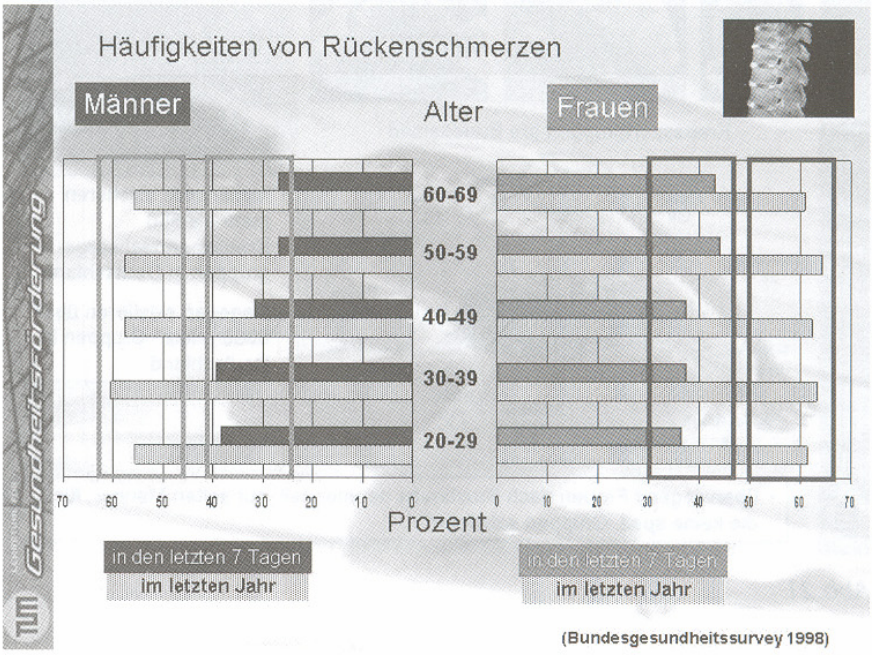

Abb. 23

In diesen Krebssportgruppen organisieren sich interessanterweise insbesondere Frauen mit Mammakarzinom, während Männer sich alleine

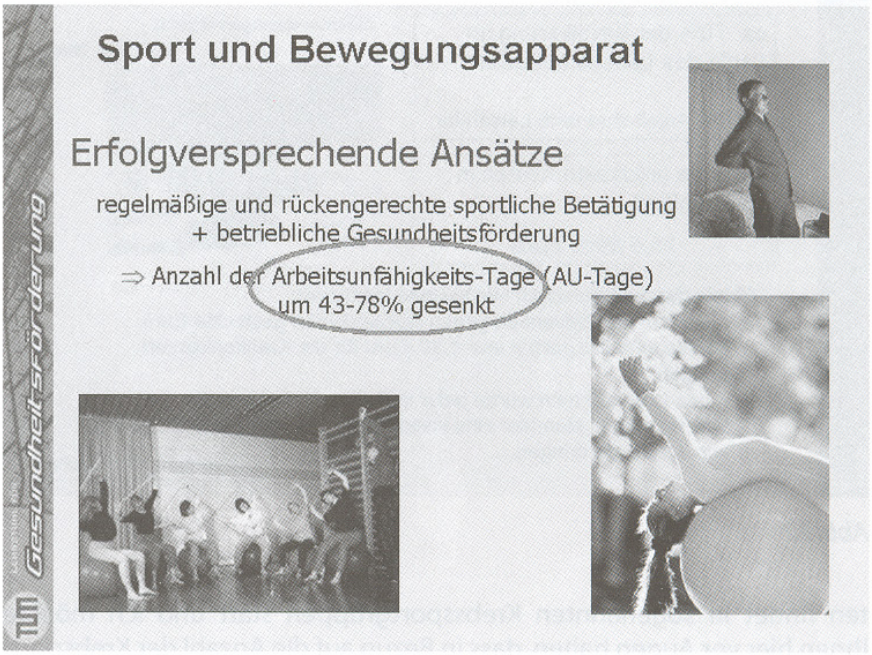

Abb. 24

„hinter dem Ofenrohr" mit ihrem Prostatakarzinom „zurückziehen". Als Illustration dafür habe ich das Beispiel des armen Poeten von Karl Spitzweg aus der neuen Pinakothek gewählt.(Abb. 20).

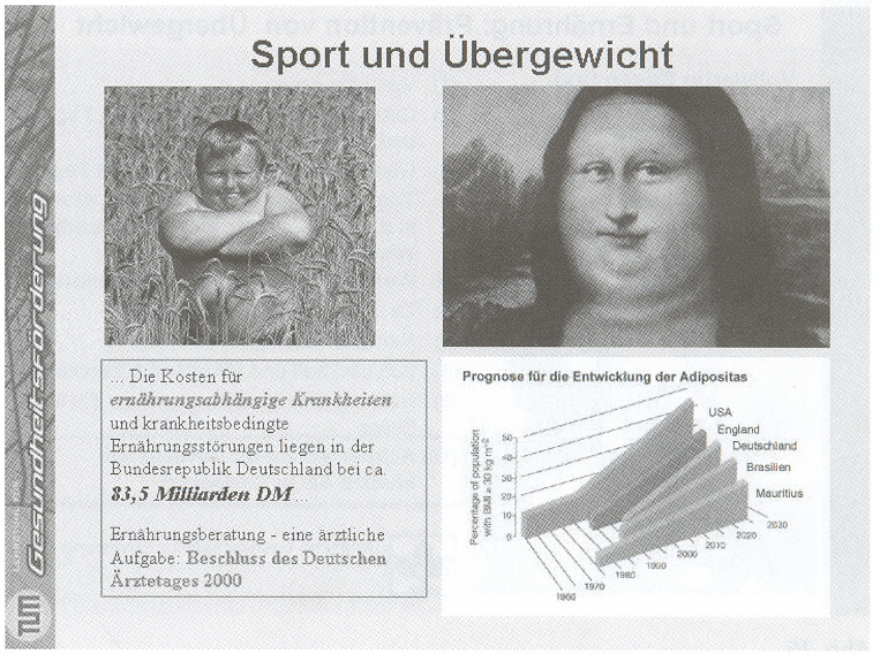

Abb. 25

Interessant ist auch – wenn Sie sich die absolute Zahl von Krebssportgruppen vor Augen halten, immerhin schon mehr als 550 – dass es dagegen aber 6000 Herzgruppen in Deutschland gibt. Der weitere Aufbau solcher Sportgruppen wird sicherlich auch eine Aufgabe sein, der sich unser Lehrstuhl hier in München verschreiben möchte (Abb. 21).

Eine völlig andere Erkrankung:

Volkskrankheit Nr. 1 ist das Rückenleiden. 70% der Bevölkerung werden irgendwann im Laufe des Lebens betroffen. Gigantische Kosten entstehen dadurch und es gibt präzise Kalkulationen, dass sich die Prävention gegen Rückenleiden in der Tat lohnt. Prävention kann also sparen helfen. Häufigkeiten von Rückenschmerzen bei Männern und Frauen in den letzten sieben Tagen: fast jeder Zweite, wie auch im letzten Jahr(Abb. 22).

Bei Frauen stellen sich Rückenleiden noch häufiger ein. In der letzten Woche litt jeder zweite Mann und jede zweite Frau Rückenschmerzen (Abb. 23).

Erfolgversprechende Ansätze: Nun auch hier entsprechende organprotektive Bewegungsprogramme, um auch die Anzahl der Arbeitsunfähig-

keitstage zu reduzieren, das ist das bescheidene Wissen, das bekannt ist (Abb. 24).

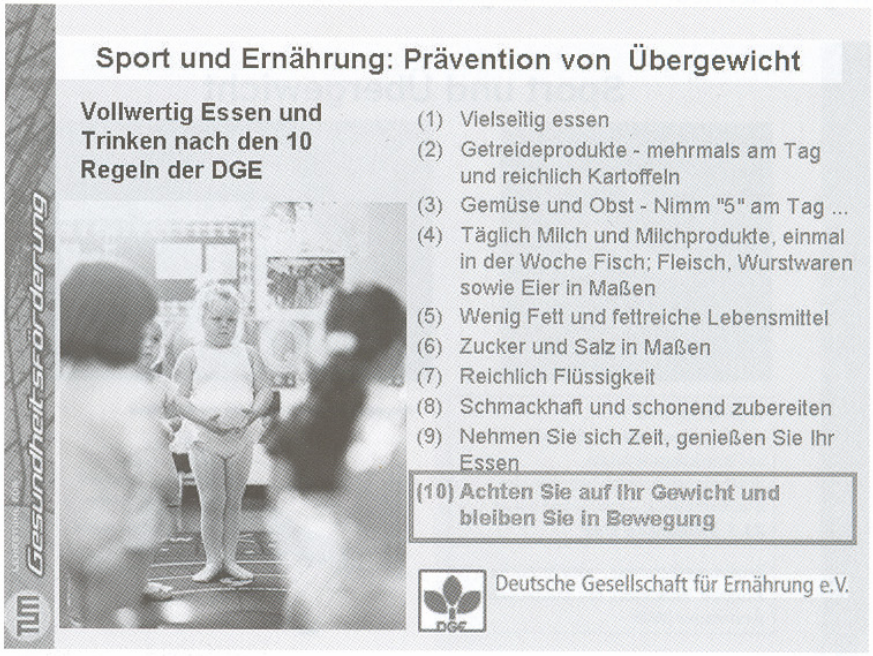

Abb. 26

Ein weiteres Problem im Zusammenhang mit Rückenleiden ist natürlich das Übergewicht (Abb. 25).

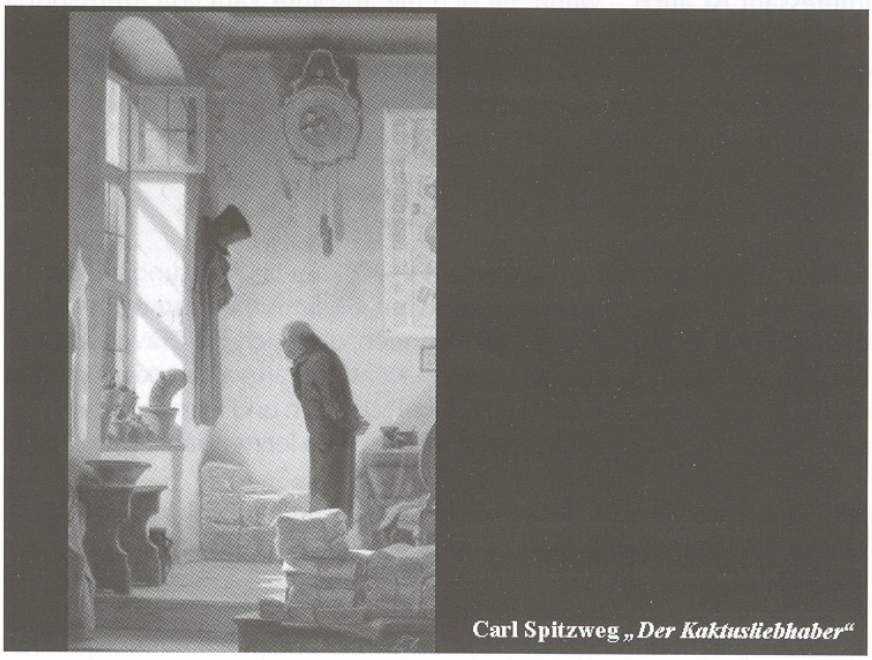

Abb. 27

60

Es gibt Simulationsmodelle, die beschreiben, dass die Adipositas zunehmen wird; Die Kosten für ernährungsbedingte Erkrankungen sind gewaltig und die beiden nachfolgenden Vorträge werden ja darauf auch insbesondere bei den Kindern dann eingehen.

Auch die renommierte Deutsche Gesellschaft für Ernährung, deren Herz ja sicherlich mehr für Ernährung schlägt, empfiehlt mittlerweile in den zehn goldenen Regeln auch die Berücksichtigung von Sport im Alltag (Abb. 26).

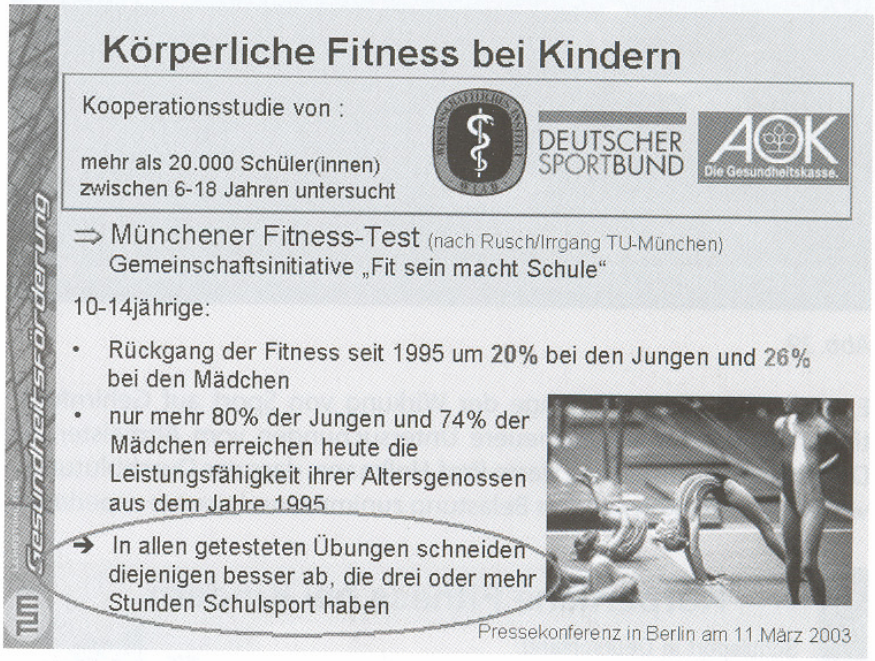

Abb. 28

Kollegen aus der eigenen Fakultät um Herrn Dr. Rusch konnten nachweisen, dass die Fitness von Kindern und Jugendlichen dramatisch reduziert ist. Wir wissen also um die schlechte Fitness der Kinder und es überrascht eigentlich, dass immer noch nicht die tägliche Sportstunde eingeführt wurde, obwohl immer wieder gefordert (Abb. 27).

Nun besteht natürlich die Gefahr, dass insbesondere nach PISA argumentiert wird, jetzt nur noch Mathematik, aber es zeigt sich an neueren Befunden, dass Sport treiben auch schlau machen kann (Abb. 28/29).

Und es zeigte sich an entsprechenden Studien, dass eine tägliche Sportstunde Aggressionen reduzieren kann, die Konzentrationsfähigkeit steigert, auch im Mathematikunterricht, dass eine Körpergewichtsreduktion und dass eine verbesserte soziale Integration der Schüler stattfinden (Abb. 30/31).

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Abb. 29

Es stellt sich damit die Frage der Wirkung von Sport auf Gehirnfunktionen: in der Tat zeigen neuere Untersuchungen vom Altmeister der Deutschen Sportmedizin, Herrn Prof. Hollmann, dass das Durchblutungsverhalten unter körperlicher Belastung zunimmt, und neuere experimen-

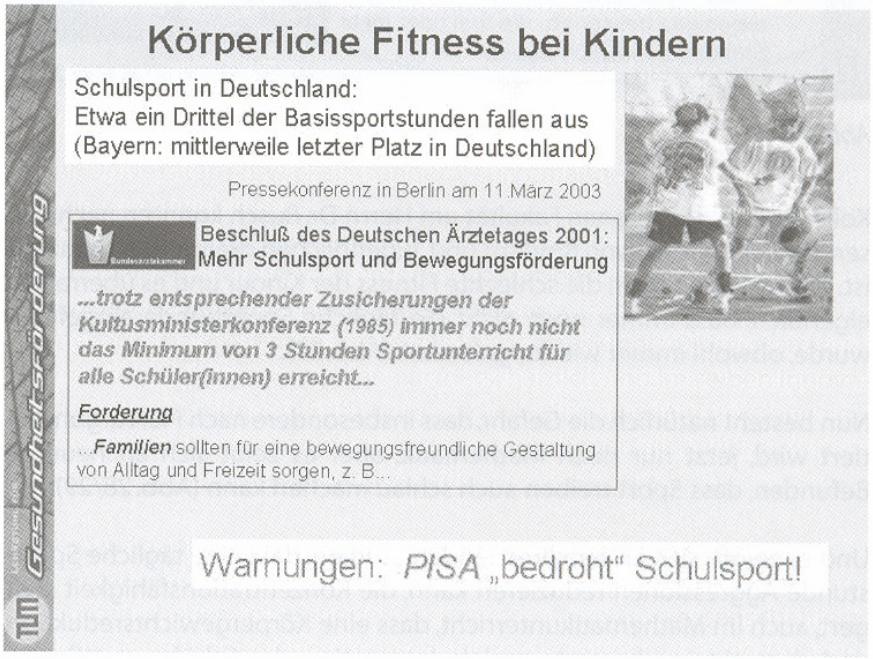

Abb. 30

62

telle Untersuchungen konnten zudem nachweisen, dass die Nervenzelldichte im Gehirn ebenfalls zunimmt und Alterungsphänomene durch aeroben Ausdauersport zumindest verzögert werden können (Abb. 32).

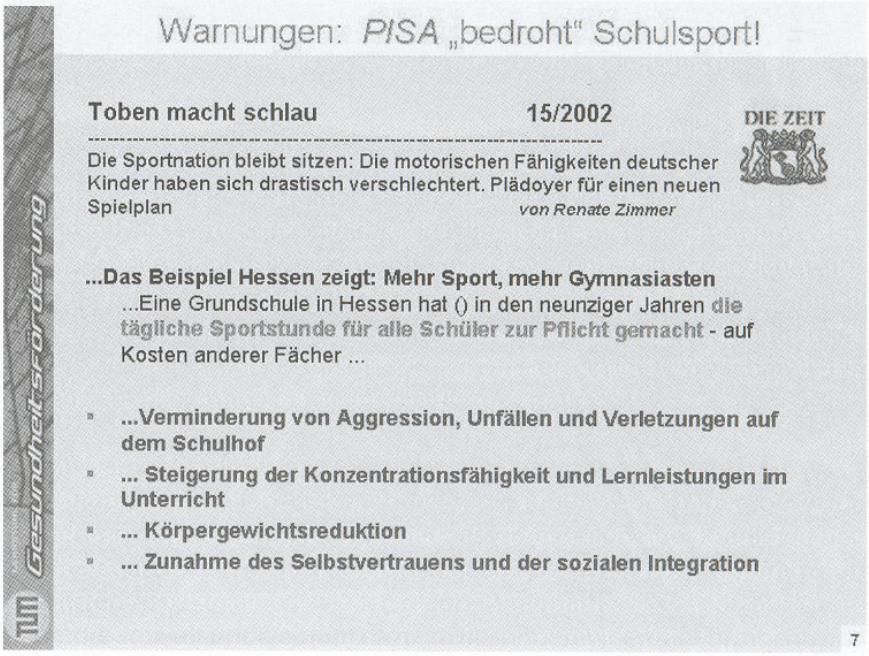

Abb. 31

Abb. 32

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Damit stellt sich die Frage, ob wir durch Sport auch länger leben können, dazu habe ich Ihnen zur Illustration den „Jungbrunnen" von Lucas Kranach. ausgewählt (Abb. 33).

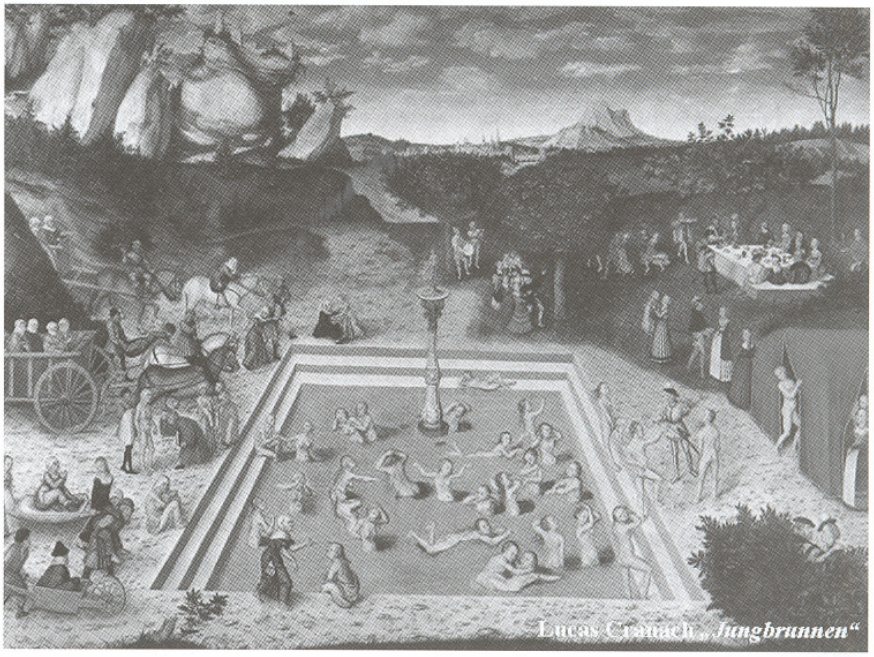

Abb. 33

Zu meinem Bedauern, kann ich Ihnen diese Frage nicht gut beantworten; es gibt hier auf den ersten Blick die Tatsache, dass das gezeigte Mortalitätsrisiko eigentlich ein statistisch längeres Leben bereits implizieren müsste (Abb. 34)

Natürlich muss auch darauf hingewiesen werden, dass auch eine individuelle genetische Prädisposition vorliegen könne. Eine Zwillingsstudie spricht allerdings dagegen; allein die entscheidende offene Frage ist, ob nicht der motorische Antrieb zum Sport bereits bedingt wird durch eine genetische Prädisposition, und damit bereits längeres und gesünderes Leben bewirken könnte; dieses können wir Ihnen anhand der klinischen Studien naturgemäß nicht nachweisen, tierexperimentelle Daten fehlen hier.

Was bewirkt nun alles Sport? Ich denke, ich habe Ihnen ein wenig Einblick geben können, dass Sport sowohl physische Faktoren wie auch psychische Faktoren positiv beeinflussen kann und dass Sport eigentlich ein verschreibungspflichtiges nebenwirkungsarmes Medikament darstellt (R. Rost) (Abb. 35).

Damit stellt sich die Frage, wie beantworte ich nun die Eingangsfrage. Wer von Ihnen hat da eine Idee? Doch vergegenwärtigen wir uns zunächst

einmal, wie viele Menschen überhaupt Sport treiben. Interessanterweise treibt höchstens jeder zweite Mensch ab dem vierten Lebensjahrzehnt Sport! Die bedeutsame Frage ist nun, wie locke ich diese nicht sporttreibenden Menschen zum Sport (Abb. 36) ?

Abb. 34

Die Vision zur Prävention muss sein: Prävention in Deutschland muss sich lohnen! Gestern Abend habe ich von Herrn Henkel gehört, eigentlich lohnt sich Gesunderhaltung in Deutschland nicht, sondern „krankfeiern". Wir müssen Motivation zum Sport schaffen, wir müssen Zugang zum Sport bahnen und wir brauchen mehr Mittel für präventive Maßnahmen der Gesundheitsförderung (Abb. 37).

In diesem Zusammenhang habe ich keinen Kollegen getroffen, der mir überzeugend erklären konnte, warum wir bei der eindeutigen Datenlage immer noch keine tägliche Sportstunde einführen konnten.

Nicht eingehen konnte ich auf die Bedeutung der Dopingprävention in Fitnessstudios für Jugendliche. Gesundheitsförderung muss Eingang finden in die Curricula. Klinisch arbeitende Kollegen, egal ob mit dem Erfahrungshintergrund von Herzsportgruppen oder Adipositaspatientengruppen weisen immer wieder auf die Bedeutung der frühkindlichen Prägung im Elternhaus und die Bedeutung des Vorbildes der Eltern hin. Die segensreiche Wirkung von Sport auf kognitive Fähigkeiten des Heranwachsenden schürt damit die Hoffung, dass Sport auch zur Lösung des sogenannten PISA-Problems beitragen könnte.

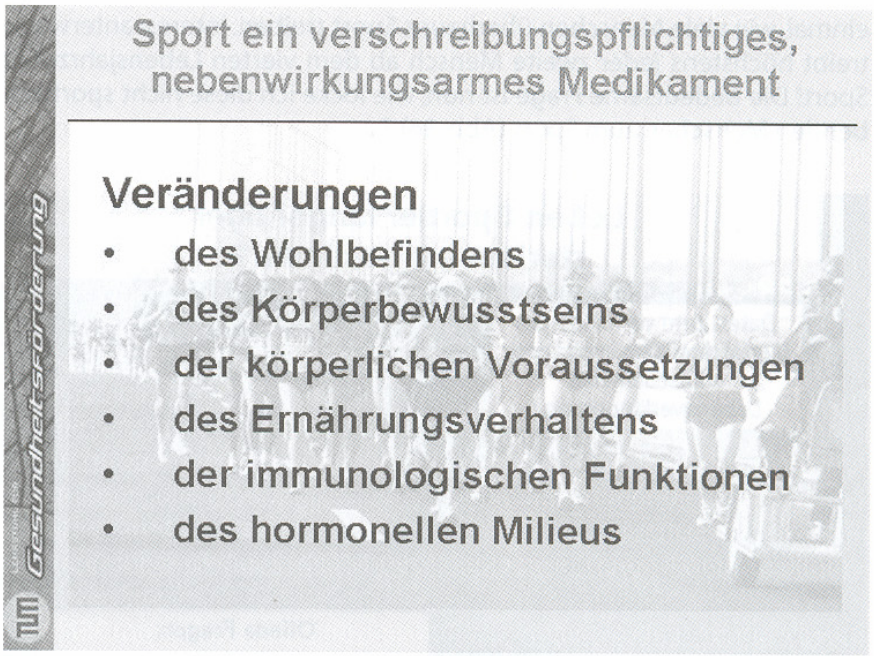

Abb. 35

Somit stellt sich die Frage *„Warum Männer ungesund leben und Frauen gesünder sterben?"* (Abb. 38).

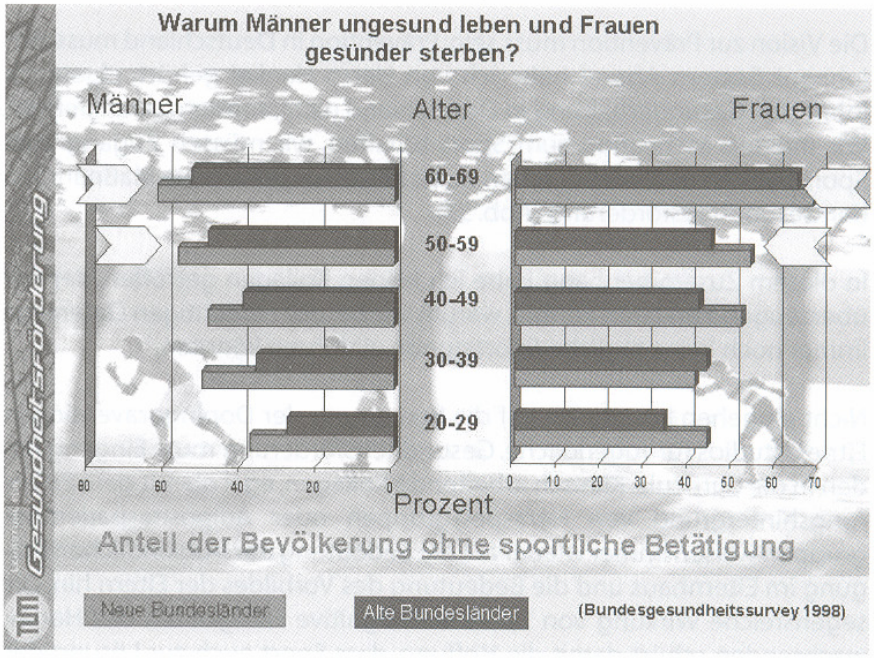

Abb. 36

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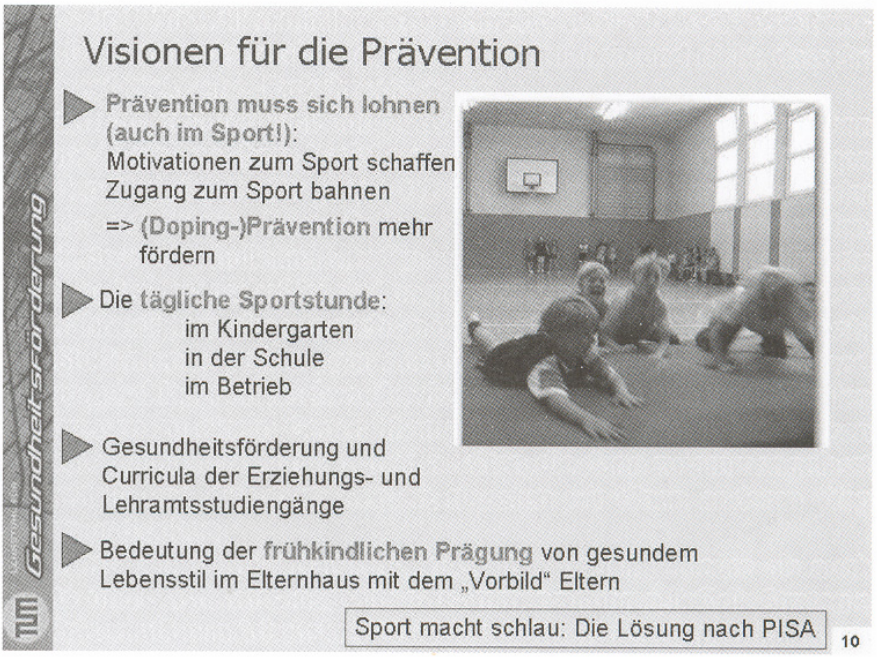

Abb. 37

Das Verhalten der gesunden und an Krebs erkrankten Frauen im Vergleich zu den Männer schürt den Verdacht:
weil Frauen Rat einholen und auch annehmen
und Männer keinen Rat brauchen!

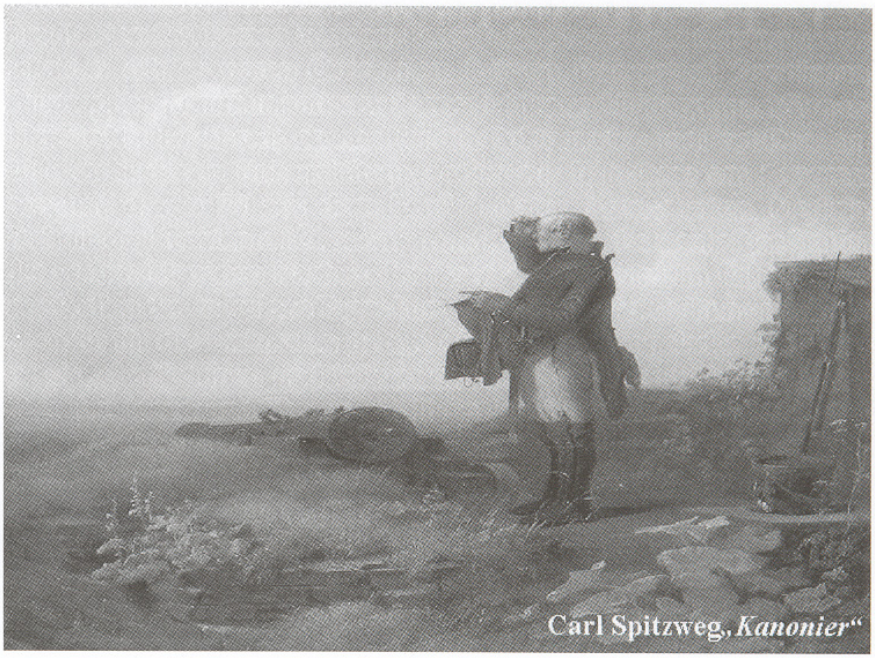

Abb. 38

Appendix V.

Nishino T, Michna H: Zentrale und periphere Wirkungen von natürlichen und synthetischen Glukokortikoiden und deren Relevanz im Doping.

Glukokortikosteroide in der Dopingforschung Bundesinstitut für Sportwissenschaft
Band 06 P 57-74 (2007)

Zentrale und periphere Wirkungen von natürlichen und synthetischen Glukokortikoiden und deren Relevanz im Doping

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Einleitung

In der Nebennierenrinde (NNR) werden aus Cholesterol durch Seitenkettenabbau über Progesteron als Zwischenstufe in der *Zona fasciculata* und *reticularis* insbesondere den Kohlenhydrat-, Fett- und Eiweißstoffwechsel beeinflussende Glukokortikoide. Der Einfluss der Glukokortikoide auf den Glukose Metabolismus brachte ihnen ursprünglich diesen Namen ein.
In der heutigen Nomenklatur definiert man sie als Steroide, welche ihre Wirkungen über Bindungen an spezifische zytosolische Rezeptoren entfalten. Diese Glukokortikoid-Rezeptoren (GR) sind in nahezu allen Geweben anzutreffen [1].
Variationen in der chemischen Struktur der Glukokortikoide führten zu einer Entwicklung von vielen synthetischen Substanzen mit weitaus stärkerer Wirkung im Vergleich zu ihren endogenen Verwandten. Die erhöhte Aktivität dieser Synthetika begründet sich in der deutlich höheren Affinität zum GR und in der signifikant verlangsamten *plasma clearance.* Zusätzlich haben diese neuartigen Verbindungen vernachlässigbar geringe mineralkortikoide Wirkung, so dass bekannte schwere Nebenwirkungen wie Natriumionen-Retention, Hypertension und Hypokaliämie nicht befürchtet werden müssen (Abb.1).

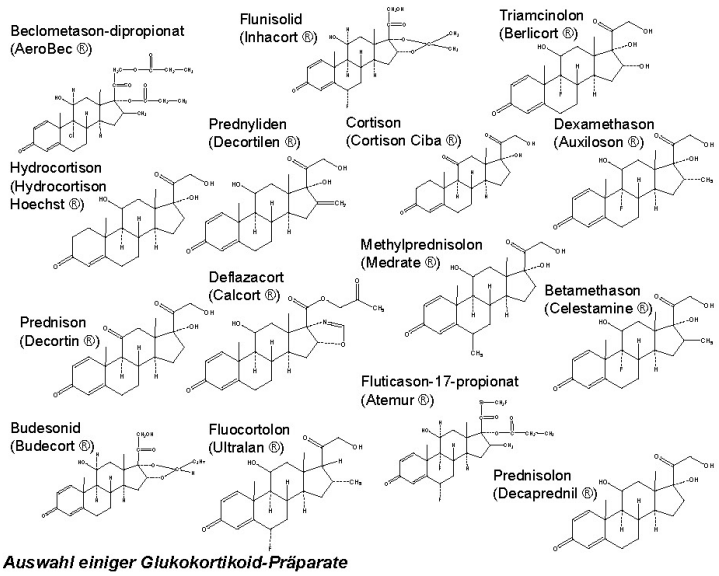

Abb.1 Auswahl einiger Glukokortikoid-Präparate

Chemische Eigenschaften

Steroide sind nahezu unlöslich in Wasser. Ausser in Form von Salzen sind sie derart unlöslich, dass selbst in Konzentrationen von 3% oder niedriger nur Suspensionen entstehen und sie daher für die intravenöse Injektion ungeeignet sind. Sie sind jedoch für topische Cremes und intramuskuläre oder intra-artikuläre Injektionen geeignet. Weiterhin sind sie zur Herstellung von Tabletten zur oralen Gabe prädestiniert [2]. Zwischen der chemischen Struktur und der biologischen Wirksamkeit bestehen feste Beziehungen [3]. So ist für die Glukokortikoidwirkung dieser Steroide die Hydroxylierung an den Positionen C-11 und C-17, die Ketogruppe an C-3, die Ketoseitenkette an C-17 sowie die Doppelbindung zwischen C-4 und C-5 im A-Ring des Steroidgerüsts verantwortlich [3,4,5,6,7,8]. (Abb.2).

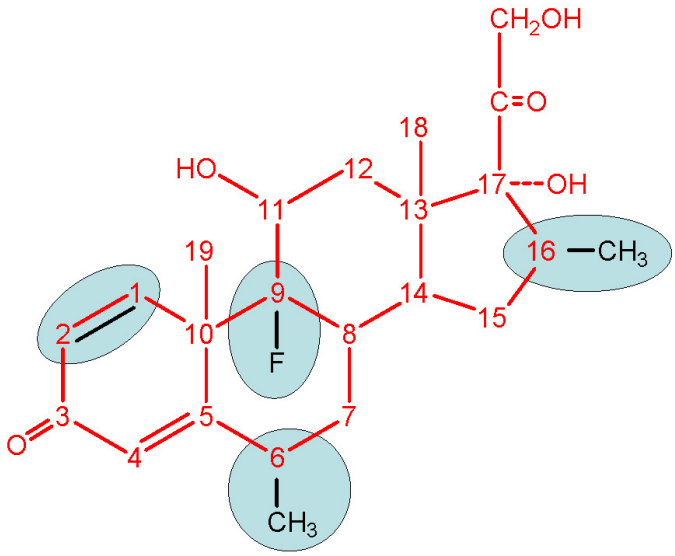

Abb.2 Chemische Modifikationen von Glukokortikoiden

Bereits kleine chemische Modifikationen am Grundgerüst des Cortisols können die Absorptionsrate, den Zeitpunkt des Wirkungsbeginns, die Wirkungsdauer und die Potenz eines Glukokortikoids wesentlich beeinflussen [8]. Eine zusätzliche Doppelbindung im A-Ring des Sterangrundgerüsts (Abb.2: 1-2), und folglich zwei fehlende H-Atome an den entsprechenden C-Atomen, ist der einzige Unterschied zwischen Prednisolon und Hydrocortison. Dies führt zu einer 4 bis 5-fachen Steigerung der glukokortikoiden, und zur Reduktion der mineralkortikoiden Wirkung des Prednisolons. Die Struktur und Seitenketten des Glukokortikoidgrundgerüsts (Abb.2: rot) haben ebenfalls Einfluss auf die Stärke der entzündungshemmenden (glukokortikoiden), sowie der mineralkortikoiden Wirkung und auf die Dauer der Wirkung nach Erreichen des Wirkungsortes [7,9]. Durch die Einführung von Fluoratomen, Methylgruppen sowie durch die Hydroxylierung bzw. Dehydrogenierung mit Bildung neuer Doppelbindungen an verschiedenen Stellen des Sterangrundgerüsts konnte gegenüber den natürlichen Glukokortikoiden eine deutliche glukokortikoide Wirkungssteigerung und zugleich eine Verminderung der unerwünschten mineralkortikoiden Wirkung erreicht werden [3,6].

Die Halbwertszeiten der synthetischen Glukokortikoide sind generell länger als die des Cortisols (80 Minuten). Sie bewegen sich zwischen 1 Stunde für Prednisolon und mehr als 4 Stunden für Dexamethason.

Partialwirkungen von Glukokortikoiden

Durch die strukturelle Ähnlichkeit der Glukokortikoide zu anderen Steroiden wie (Anti-)Androgenen und (Anti-)Gestagenen, entfalten sie neben ihrer Hauptwirkung (z.B. entzündungshemmende Eigenschaften) auch hormonelle Partialwirkung durch Bindung an einem Androgen- und oder Gestagenrezeptor. Bekanntestes Beispiel ist die „Abtreibungspille“ RU486 Mifepriston®. Sie kann neben antigestagener und antiglukokortikoider Wirkung auch antiandrogene Effekte bewirken (Abb.3,4):

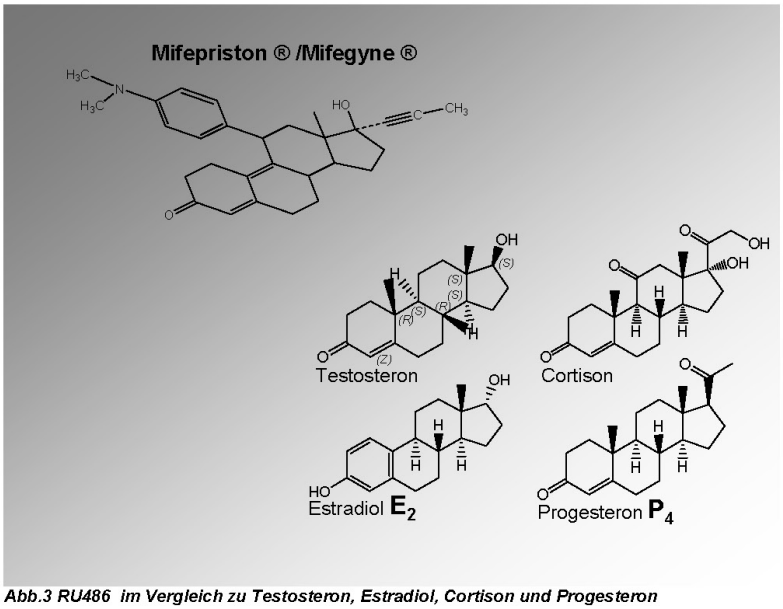

Abb.3 RU486 im Vergleich zu Testosteron, Estradiol, Cortison und Progesteron

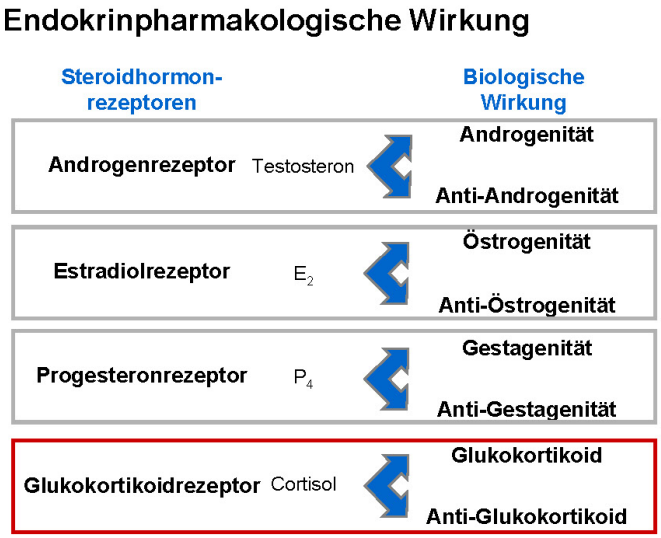

Abb.4 Hormonrezeptoren und endokrinpharmakologische Wirkungen

Der Glukokortikoid-Rezeptor [2]

Glukokortikoide entfalten ihre mannigfaltigen Wirkungen auf Zielzellen über intrazelluläre Rezeptorproteine. Sie passieren die Zellmembran durch passive Diffusion und binden in der Zelle an den GR. Der GR ist ein single-chain Polypeptid mit einem Molekulargewicht von 94kD, welcher die Steroide mit hoher Affinität und Spezifität bindet. Der Rezeptor liegt in der Zelle gebunden an diversen sogenannten heat shock proteinen wie hsp90 und den Immunophilinen hsp56 oder CyP40 und einem 23kD acidic protein vor. Einmal an den Rezeptor gebunden, entsteht ein Hormon-Rezeptor-Komplex, welcher dann befähigt ist, an die DNA zu binden. Die Transkription von Zielgenen wird aktiviert und die mRNA wird in neue Proteine translatiert, welche dann die biologische Aktivität der Glukokortikosteroide entfalten. (Abb.5):

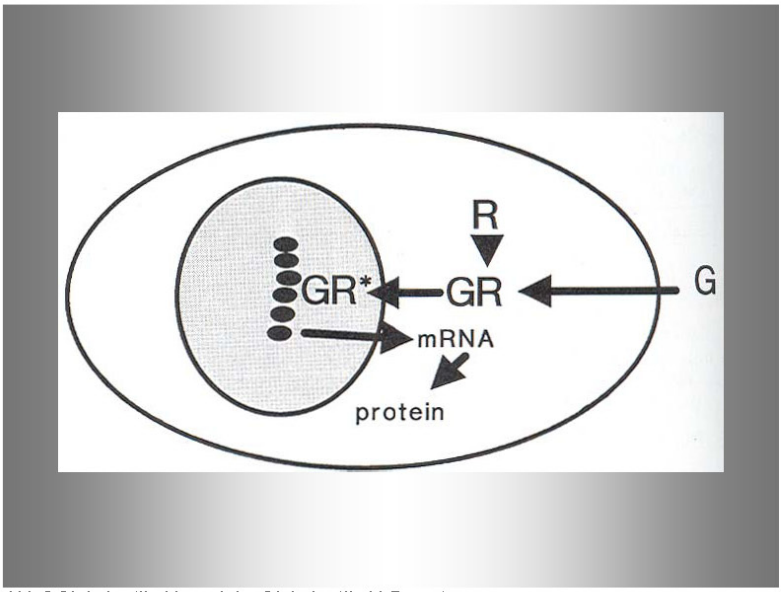

Abb.5 Glukokortikoide und der Glukokortikoid-Rezeptor

Abbildung 6 zeigt die Domänenstruktur des GR Proteins und ihre Homologie (in %) mit anderen Steroidrezeptoren. Insbesondere Ähnlichkeit zeigen ausser den Mineralkortikoid-Rezeptoren die Rezeptoren für **Gestagene** und **Androgene**.

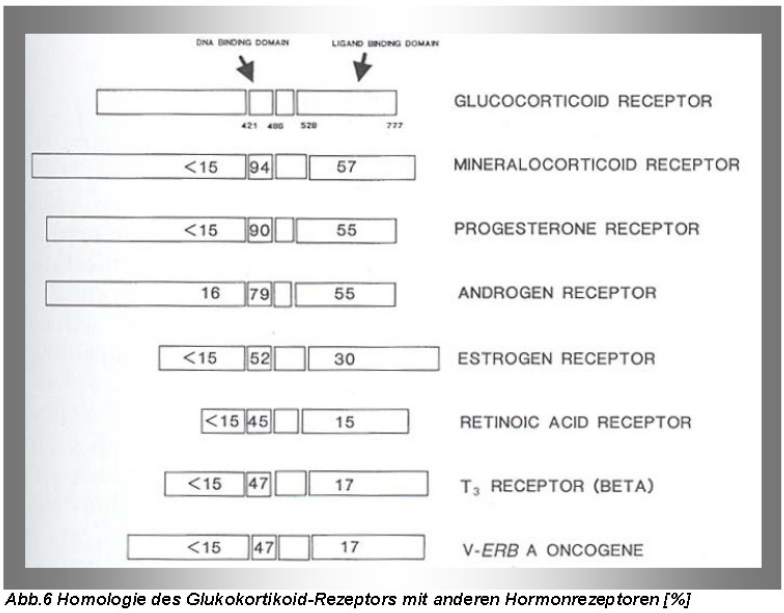

Abb.6 Homologie des Glukokortikoid-Rezeptors mit anderen Hormonrezeptoren [%]

Die Steroid-Bindungsdomäne des GR befindet sich am COOH-Terminus. Die DNA-Bindungsdomäne „Zinkfinger" (Abb.7) des GR ist zwischen den Aminosäuren 421 und 486 lokalisiert.

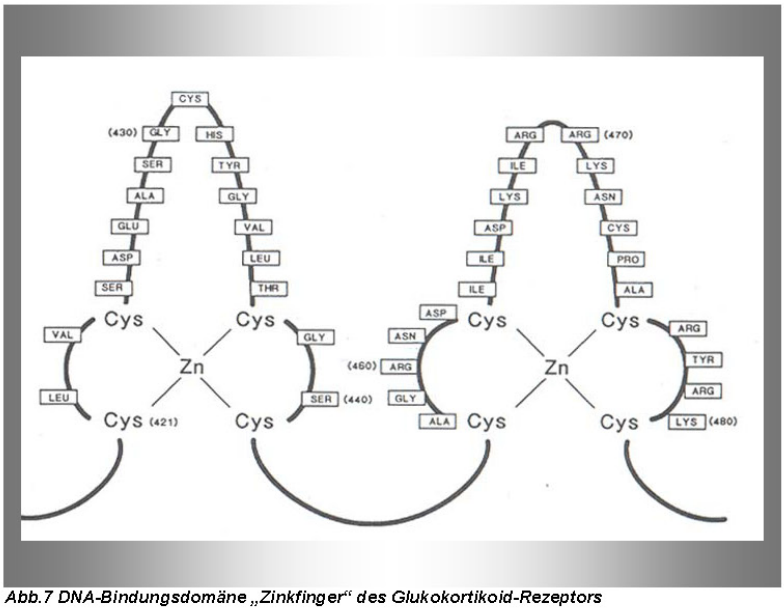

Abb.7 DNA-Bindungsdomäne „Zinkfinger" des Glukokortikoid-Rezeptors

Aus einem GR-Gen entsteht eine sequenzgleiche m-RNA. Was machen nun einzelne Zellen mit der DNA-Bauanleitung des GR? Nach neuesten Untersuchungen von Lu und Cidlowski [10] kann die oben genannte m-RNA zur Synthese von 8 Isoformen eines GR anleiten. Diese Erkenntnis erklärt vielleicht das überaus komplexe Wirkspektrum der Glukokortikoide mit allen bekannten und bei systemischer Anwendung schwerwiegenden Nebenwirkungen.

Glukokortikoide und Androgenität

Glukokortikoide zeigen in Abhängigkeit von ihrer chemischen Struktur in den klassichen Bindungsassays (Scatchard Analysis) Affinität zum Androgen-Rezeptor (AR), wie dies auch in grundlegenden Untersuchungen zur Bindung an radioaktiv markierten R1881 nachgewiesen wurde [11,12,13]. Basierend auf den Nachweis der Bindungsaffinität zum AR konzentrierten wir unsere Untersuchung zur möglichen androgenen Wirkung von Glukokortikoiden auf androgen-abhängigen Parametern in Zellkultur, androgen-abhängigen Tumoren *in vivo* sowie im klassischen Hershberger Assay [14] zur Prüfung von Androgenität *in vivo*.

Im Nachfolgenden seien die Wirkungen von Androgenen auf androgen-abhängige Zellen vorangestellt:

Zur Prüfung der möglichen Androgenität von Glukokortikoiden wurde die menschliche Prostatakarzinomzelllinie aus einem Lymphknoten, die sogenannte LNCaP-Zelllinie verwendet: Die Kulturen wurden in 24-well Platten mit $4x10^4$ Zellen per well ausgesät und über 3 Tage mit verschiedenen Substanzen inkubiert [15]. Als Zeichen der Hormonabhängigkeit stellt sich nach Gabe von Dihydrotestosteron in physiologischen Konzentrationen eine Stimulation des Wachstums ein; interessanterweise zeigt die Behandlung mit 11-Desoxycorticosteron ebenfalls eine den Androgenen in der Höhe vergleichbare Stimulation des Zellkulturwachstums: Die LNCaP Zellen wurden über 3 Tage etwa 20 Stunden mit den Substanzen behandelt (Abb.8):

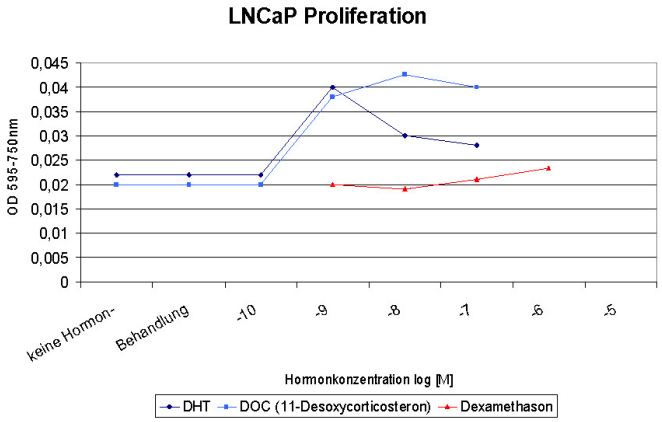

Abb.8 LNCaP Proliferation

Zur Beurteilung eines funktionell androgen-abhängigen Parameters, wurde die Sekretion von ***p***rostata-***s***pezifischem-***a***ntigen (PSA) analysiert (Abb.9):

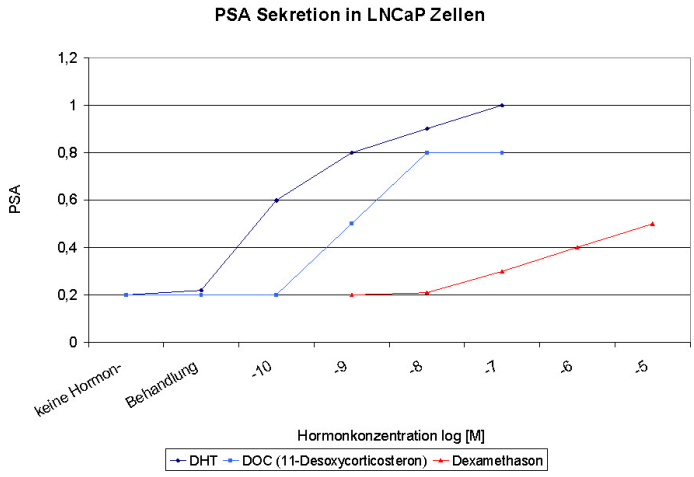

Abb.9 PSA Sekretion in LNCaP Zellen

Hierzu wurde mit einem PSA-Enzym-Immunoassay Testkit (ICN Pharmaceuticals) die Menge des sezernierten PSA im Medium, normalisiert zum Gesamt-DNA Gehalt, gemessen. Hier zeigte sich dann in der Tat, dass die Sekretion von PSA abhängig ist von der Gabe eines Androgens (DHT); besonders interessant ist, dass sowohl 11-Desoxycorticosteron wie auch Dexamethason die Sekretion des androgen-abhängigen Parameters PSA in LNCaP-Zelllinien zu stimulieren vermögen; gleichwohl ist eine ungleich höhere Konzentration von 11-Desoxycorticosteron und Dexamethason notwendig, um androgen-ähnliche Effekte zu erzielen.
In einem weiteren Versuchsansatz konnte der androgenabhängige Effekt auf die Stimulation des Wachstums von LNCaP Zellen (Pregnenolon) in einer Konzentration von (5nM) bewiesen werden und schliesslich konnte dieser Effekt durch Gabe eines reinen AR-Antagonisten (Casodex®) antagonisiert werden; somit kann klar gezeigt werden, dass der Pregnenolon Effekt ein AR mediierter Effekt ist (Abb.10):

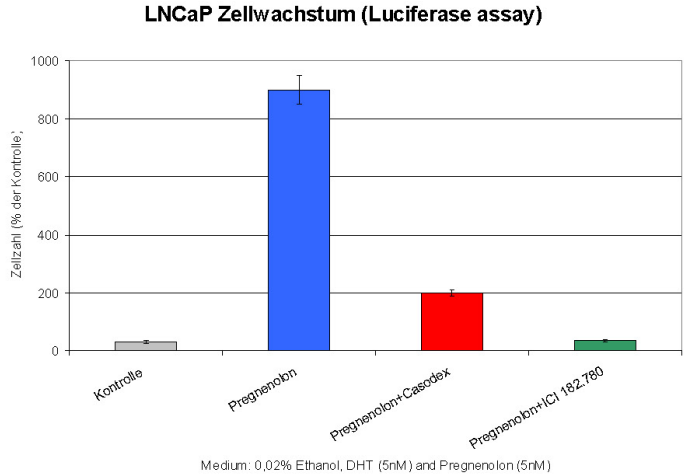

Abb.10 LNCaP Zellwachstum (Luciferase Assay)

Nach Prüfung der androgen-ähnlichen Wirkung von Glukokortikoiden in der Zellkultur wurden nunmehr Experimente angegangen, um den Nachweis eines androgen-ähnlichen Effektes *in vivo* nachzuweisen (Abb.11):

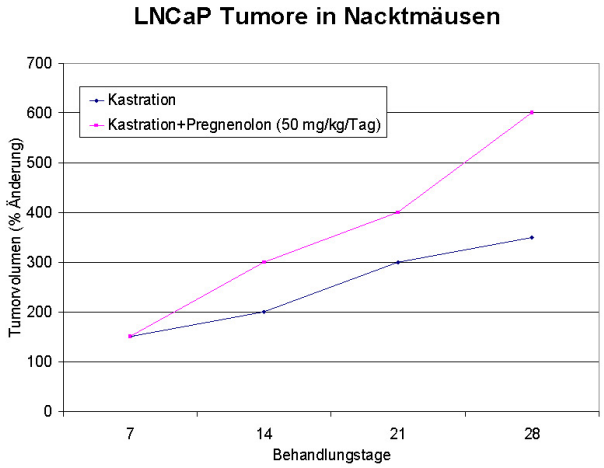

Abb.11 LNCaP Tumore in Nacktmäusen

LNCaP Tumore [16] auf Thymus aplastischen Nacktmäusen können als subkutane oder orthotopische Tumore wachsen. Um ein stetiges Wachstum der Tumore zu gewährleisten, müssen alle mit LNCaP-Zellen inokulierten Tiere mit Testosteron behandelt werden.
In diesem System konnte nachgewiesen werden, dass in orchiektomierten, Thymus aplastischen Nacktmäusen die Gabe von Pregnenolon (50mg/kg/Tag) zu einer

Stimulierung des Wachstums des androgen-abhängigen Tumors führt; ähnlich wie dies in weiteren Experimenten durch die Stimulation mit einem Androgen erfolgt.
Bei der Untersuchung der androgenen Wirkungen von Substanzen, kommt oft auch der industriell validierte Hershberger Assay [14] zum Einsatz (Abb.12). Die pharmazeutische Industrie nutzt diesen seit den 50er Jahren angewandten Test bei der Wirkstofffindung. Unter Verwendung von Positiv- (intakte Tiere) und Negativkontrollen (orchiektomierte Tiere) werden dabei orchiektomierte Tiere mit Glukokortikoiden in unterschiedlichen Dosierungen täglich einmal über 7 Tage behandelt und klassiche akzessorische Geschlechtsorgane der männlichen Ratte gewogen und untersucht. In eigenen Untersuchungen zeigt die Gewichtsanalyse der Prostata im Hershberger Assay eine deutliche Abnahme des Feuchtgewichts nach Orchiektomie. Werden die orchiektomierten Tiere mit 11-Desoxycorticosteron oder Dexamethason behandelt, konnte der Kastrations-Effekt signifikant reduziert werden (Abb.13). Dagegen ist eine deutliche Gewichtszunahme der Prostata bei den Positivkontrollen (Intakt und TP=Testosteronpropionat behandelt) im Vergleich zur Orchiektomie zu erkennen. Die densitometrische Auswertung der immunhistochemischen anti-AR Färbung zeigt ebenfalls den Kastrations-Effekt (14B) im Vergleich zu intakten Tieren (14A) und der mit Testosteronpropionat behandelten Gruppe (14C). Abbildungen 14G-I zeigen Färbungen mit dem Proliferationsantikörper PCNA (***P***roliferating ***C***ell ***N***uclear ***A***ntigen). Auch hier erkennt man die deutlich reduzierte Proliferationsaktivität der orchiektomierten Gruppe (14H) [17,18].

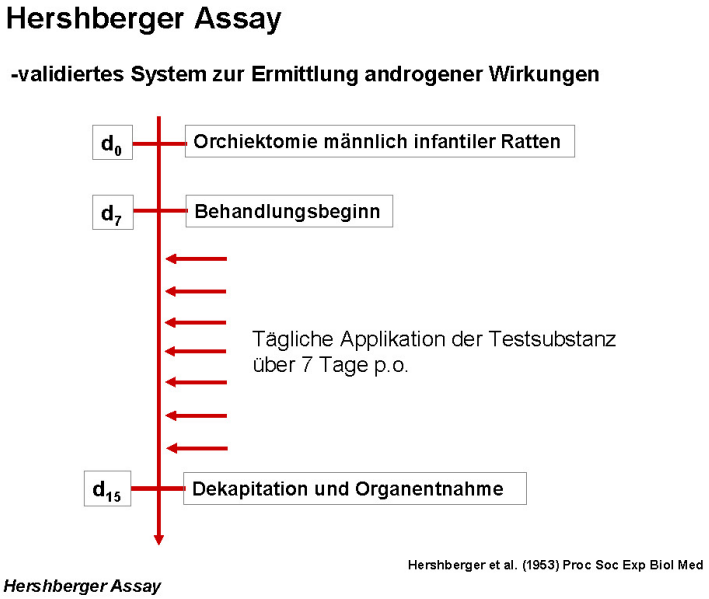

Abb.12 Hershberger Assay

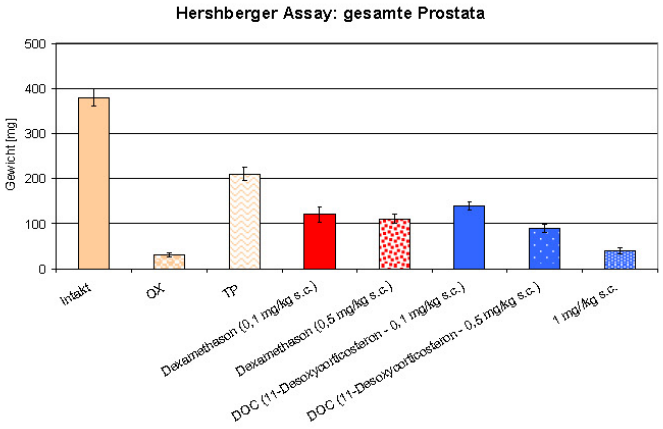

Abb.13 Hershberger Assay: gesamte Prostata

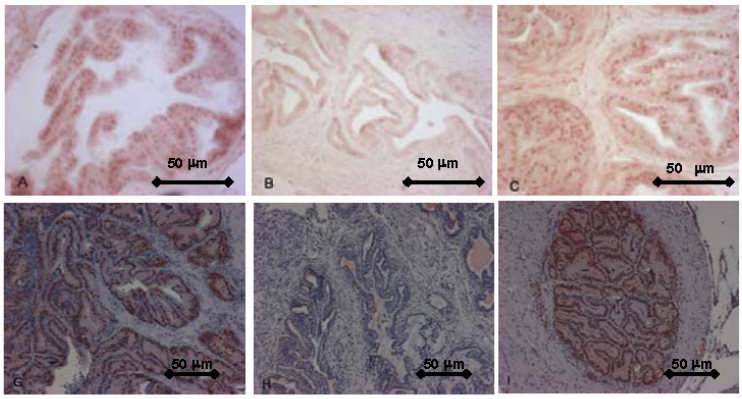

Abb.14 Immunhistochemische anti-AR Färbung der Rattenprostata: obere Reihe von links nach rechts A, B, C Immunhistochemische PCNA Färbung der Rattenprostata: untere Reihe von links nach rechts G, H, I

Neuropsychologische Auswirkungen und Verhaltensstörungen [2]
Einfluss auf die Stimmung:
Glukokortikoide beeinflussen diverse Aspekte des menschlichen Verhaltens, insbesondere Schlafmuster, Stimmung und Sinneswahrnehmung. Die Dauer des REM-Schlafs ist signifikant verkürzt bei Patienten mit Cushing-Syndrom und selbst bei gesunden Menschen, die pharmakologische Dosen von Glukokortikoiden oder ACTH verabreicht bekommen. Durch klinische Observation konnten sehr gegenteilige Beobachtungen die Stimmung betreffend festgestellt werden: Einige Patienten litten unter schwerer Depression wobei andere zu Euphorie neigten. Es gibt jedoch hierzu keine sicheren Daten.
Plihal et al. [19] untersuchten die Auswirkungen von Dexamethason (4mg/Tag) und Cortisol (10mg/h) 7 Tage lang auf das zentrale Nervensystem bei 10 gesunden männlichen Probanden. Dabei stellte sich heraus, dass Dexamethason wie Cortisol akut die Aktivität, Konzentration steigerte und Erregung verursachte. Längere Administration führte zu meist negativen Stimmungsveränderungen wie Depression und Traurigkeit.
Viele Zellen des zentralen Nervensystems besitzen GRs. Die entsprechenden Zellantworten äußern sich in Veränderungen in der elektrischen Aktivität wie zum Beispiel Hyperpolarisation der Zellmembran oder auch Unterdrückung der spontanen elektrischen Aktivität.

Glukokortikoide und Asthma
Glukokortikoide sind aus der heutigen Medizin nicht mehr wegzudenken. Insbesondere ihre entzündungshemmenden Eigenschaften bei Asthma bronchiale sind konkurrenzlos. Die meisten eingereichten ***T***herapeutic ***U***se ***E***xemptions (TUE) im Leistungssport betreffen Glukokortikoide [20].
Die Entzündung der Atemwege ist der Hauptgrund für immer wiederkehrende Asthmaanfälle. Die Glukokortikosteroide bekämpfen die chronische Entzündung der Bronchien und die Überempfindlichkeit der Atemwege. Die gefürchteten Nebenwirkungen dieser Steroide können zumindest das Asthma betreffend vernachlässigt werden, da Kortikosteroide nun auch als Inhalationspräparate zur Verfügung stehen. Inhalierte Kortikosteroide wirken in den Bronchien mit einem zehntel oder hundertstel der Dosis, die benötigt werden würde bei systemischer Gabe. Behandlung von Asthma mit inhalierten $ß_2$-Agonisten, außer Clenbuterol, und Glukokortikoide können bekanntlich im Leistungssport über die ***A***bbreviated ***T***herapeutic ***U***se ***E***xemption (ATUE) angefordert werden (vgl.hierzu: [21]).

Glukokortikoide und sportliche Leistungsfähigkeit
Eine Metaanalyse der durchgeführten Studien zur Frage der leistungssteigernden Wirkung von Glukokortikoiden lässt hingegen keinen signifikant leistungssteigernden Effekt zumindest im Hochleistungssport erkennen [20].

Wirkmechanistisch ist nahe liegend, die in der Literatur klinischer Berichte beschriebene euphorisierende Wirkung von (niedrigdosierten) Glukokortikoiden [19] mit einer möglichen leistungssteigernden Wirkung in Zusammenhang zu bringen.

Gleichwohl dokumentieren die vorgelegten endokrinpharmakologischen Befunde eine schwache androgene Partialwirkung, die sich insbesondere in Sportarten mit hoher Kraftkomponente entfalten könnte; mechanistisch denkbar wäre schliesslich, dass diese schwache Androgenität insbesondere bei Frauen im Leistungssport Wirkung zu erzielen vermag; wie dies auch in einem Diskussionsbeitrag von

Professor Dickhuth basierend auf seinen Erfahrungen in der Betreuung von Leistungssportler(-innen) bestätigt wird.

Fazit

Es wurde aufgezeigt, dass die Gabe von Glukokortikoiden mit erheblichen Nebenwirkungen behaftet sein kann, insbesondere – wenn wie im Leistungssport – suprapharmakologische Dosierungen eingesetzt werden; diese erheblichen Nebenwirkungen sprechen wohl auch weiterhin für die Ächtung dieser Substanzklasse als Dopingmittel.

References:

[1]: Mutschler Arzneimittelwirkungen: *Lehrbuch der Pharmakologie und Toxikologie*
Stuttgart: Wiss. Verl.-Ges., 2001
ISBN 3-8047-1763-2

[2]: Williams *Textbook of Endocrinology*
1998 by W.B. Saunders Company
ISBN: 0-7216-6152-1

[3]: Thun R & Schwartz-Porsche D:
Nebennierenrinde.
In: *Veterinärmedizinische Endokrinolgie* (F Döcke, ed) Gustav Fischer Verlag, Jena (D) 3: pp 309-353, 1992
ISBN: 3-334-60432-2

[4]: MacHarg MA, Bottoms GD, Carter GK & Johnson MA:
Effects of multiple intramuscular injections and doses of dexamethasone on plasma cortisol concentrations and adrenal responses to ACTH in horses.
Am J Vet Res 46(11): 2285-2287, 1985
ISSN: 0002-9645

[5]: Voigt KH & Fehm HL:
Clinical pharmacology of systemic glucocorticoid treatment.
Therapiewoche 31(39): 6230-6240, 1981
ISSN: 0040-5973

[6]: Neumann F, Schenck B, Schleusener H & Schweikert HU:
Endokrinpharmakologie: Pharmakotherapie mit Hormonen.
In: *Allgemeine und spezielle Pharmakologie und Toxikologie* (W Forth), 7 Auflage, Spektrum akademischer Verlag, Heidelberg; pp 581-637, 1998
ISBN: 3-8274-008-0

[7]: Ferguson DC & Hoenig M:
Glucocorticoids, Mineralocorticoids, and Steroid Synthesis Inhibitors.
In: *Veterinary Pharmacology and Therapeutics* (HR Adams, ed.) Iowa State University Press, Ames (USA) 8: pp 649-671, 2001
ISBN: 0-8138-1743-9

[8]: Schimmer BP & Parker KL:
Adrenocorticotropic Hormone; Adrenocortical Steroids and their synthetic Analogs; Inhibitors of the Synthesis and Actions of Adrenocortical Hormones.
In: *Goodman and Gilman's the pharmacological basis of therapeutics* (JG Hardman, LE Limbrid & AG Gilman, eds.), McGraw-Hill, Medical Publishing Division, New York 10(60): pp 1649-1677, 2001
ISBN: 0-07-135469-7

[9]: Ungemach FR:
Pharmaka zur Beeinflussung von Entzündungen.
In: *Pharmakotherapie bei Haus- und Nutztieren* (W Löscher, FR Ungemach & R Kroker, eds.), 6. Auflage, Parey Buchverlag, im Blackwell Verlag GmbH, 10707 Berlin (D) 6: pp 323-357, 2003
ISBN: 3-8263-3450-7

[10]: Lu NZ, Cidlowski JA:
Glucocorticoid receptor isoforms generate transcription specifity. Trends Cell Biol. 2006; 16(6): 301-7

[11]: Maruyama S, Sato S:
Binding of glucocorticoid to the androgen receptor of mouse submandibular glands. J Endocrinol. 1985; 106(3): 329-35.

[12]: Danhaive PA, Rousseau GG:
Binding of glucocorticoid antagonists to androgen and glucocorticoid hormone receptors in rat skeletal muscle. J Steroid Biochem. 1986; 24(2): 481-7.

[13]: Schreiber JR, Hsueh AJ, Baulieu EE:
Binding of the anti-progestin RU-486 to rat ovary steroid receptors. Contraception. 1983; 28(1): 77-85.

[14]: Hershberger L, Shipley E, Meyer R:
Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. Proc Soc Exp Biol Med 1953; 83: 175-180.

[15]: Lee EC, Zhan P, Schallhom R, Packman K, Tenniswood M:
Anti-androgen induced cell-death in LNCaP human prostate cancer cells. Cell Death Differ. 2003; 10(7): 761-71.

[16]: Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP:
LNCaP model of human prostatic carcinoma. Cancer Res. 1983; 43: 1809-1818.

[17]: Nishino T, Wedel T, Schmitt O, Bühlmeyer K, Schönfelder M, Hirtreiter C, Schulz T, Kühnel W, Michna H:
Androgen-dependent morphology of prostates and seminal vesicles in the Hershberger Assay: Evaluation of immunohistochemical and morphometric parameters. Ann Anat 2004; 186: 247-253.

[18]: Nishino T, Wedel T, Schmitt O, Schönfelder M, Hirtreiter C, Schulz T, Kühnel W, Michna H:
The xenoestrogen bisphenol A in the Hershberger assay: Androgen receptor regulation and morphometrical reactions indicate no major effects. Journal of Steroid Biochemistry & Molecular Biology 2006; 98: 155-163.

[19]: Plihal W, Krug R, Pietrowsky R, Fehm HL, Born J:
Corticoid receptor mediated effects on mood in humans. Psychoneuroendocrinology. 1996; 21(6): 515-23.

[20]: Dvorak J, Fedderman N, Grimm K:
Glucocorticosteroids in football: use and misuse. Br J Sports Med. 2006; 40 Suppl. 1: 48-54.

[21]: Kindermann W:
Dopingproblematik und aktuelle Dopingliste. Deutsche Zeitschrift für Sportmedizin 2004; 55(4): 90-95.

Appendix VI.

西野津之[1]、トーステン シュルツ[1]、西野幸重[2]、ホルスト ミヒナ[1]: ラットの前立腺及び精嚢の形態及び免疫化学反応のアンドロゲン感受性について：殊にアンドロゲン受容体に及ぼすビスノール-A の影響.

(*in progress*)

ラットの前立腺及び精嚢の形態及び免疫化学反応の アンドロゲン感受性について：殊にアンドロゲン受容体に 及ぼすビスフェノール-A の影響

On the Androgen-Sensitivity of Morphology and Immunohistochemical Reaction of the Prostate and Seminal Vesicle in Rats: Special Reference to the Effect of Bisphenol-A on the Androgen Receptor

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ABSTRACT

In the Hershberger assay, densitometric analysis of androgen receptor (AR), cell proliferation marker (Ki-67, PCNA) and morphometric analysis of epithelial height/glandular luminal area, all parameters responded to orchiectomy and the replacement with testosterone, indicating to be suitable and reliable for an assessment of the androgenicity of substances. Using these androgen-sensitive parameters, we evaluated effects of bisphenol-A (BPA) in orchiectomized Wistar rats. Animals were treated p.o. either with vehicle or with 3, 50, 200, 500 mg/kg bw/day BPA (n = 13) for 7 days. One group was treated s.c. with 1 mg/kg bw/day testosterone propionate (TP). Flutamide (FL) (3 mg/kg bw/day, p.o.) was used to antagonize androgen effects of the supraphamacological dose (500 mg/kg bw/day) of BPA. In contrast to TP, BPA exerted no effect on absolute weights of prostates and seminal vesicles, whereas the relative weights were increased at higher doses of BPA, most likely due to a decrease in body weight. FL did not show any antagonistic effects. Staining intensity for AR immunoreactivity was increased at low but not at higher doses of BPA in comparison to the orchiectomized rats. Although BPA at all doses tested did not cause an increase in the cell proliferation-index, the epithelial height and glandular luminal area of prostates and seminal vesicles were increased by low doses of BPA. The increase in AR, the

epithelial height and luminal area without affecting organ weights and proliferation-index might be due to a weak androgen-like property of BPA. The present results indicate that the combined application of these parameters may provide an additional tool to test the broad spectrum of endocrine active substances such as endocrine disruptors.

Key Words: Hershberger test, Bisphenol-A, Androgen receptor

1. はじめに

ビスフェノール-A（以下 BPA）は主としてエポキシ樹脂、Polycarbonate（PC）のプラスチック製品や耐火性テトラ臭化ビスフェノール-A 等の素材として用いられる(1)。近年 BPA のみならず Estradiol に似たフェノール環を有する DDT や PCB 等一連の所謂 Xenobiotics にも性ホルモン又は抗ホルモン作用が認められ、これらの化学物質の人体並びに自然界に及ぼす毒性や内分泌機能撹乱作用が大きな問題となっている(2、3)。

例えば、*in vitro* の実験では上記の化合物がエストロゲンの受容体に結合し、その活性化により遺伝子に影響を与えることが報告されている(4-6)。BPA の場合も 0.1 μM から最高 10 μM のレベルで MCF-7 細胞の増殖を僅かに促進し(6)、*in vivo* でも BPA を 100 mg/kg 以下の用量で 3 日間投与した場合子宮の増殖が見られたという(12-14)。他方、1 ml あたり BPA 50 μg（14 mg/kg/day）を含む飲料水の 8 週間投与後に血漿中の遊離型 Testosterone が著しく低下するが、5 μg/ml（0.14 mg/kg/day）の 4 乃至 8 週間投与では変化を認めなかったという Takao 等の報告がある(7)。 vom Saal 等は妊娠マウスにごく微量の BPA や Xenobiotics を与えただけで母性行動や新生雌性動物の発育程度の変化並びに精子減少、前立腺生増大等、性殖機能にも異常が認められたと報告している(8-11)。Gupta(15)もまた妊娠 CD-1 マウスに 1 日つき体重 kg 当りミクログラム範囲の BPA 投与で胎児の開陰間隔、前立腺サイズの増大することを認めている。BPA は前立腺腺癌細胞(LNCaP)のアンドロゲン受容体(以下 AR)を活性化し、細胞分裂を促進をすることも知られている。これに対し Kim 等は Hershberger 試験で BPA（10-100 mg/kg/day）にアンドロゲン/抗アンドロゲン作用認めることは出来なかった(16)。

今回我々はこれらの報告に鑑み、広範囲の BPA 用量（超低用量、薬理学的用量、超薬理学的用量）を選びでそのアンドロゲン作用の有無を検討する目的で、Hershberger 試験法に準じ、ラット前立腺及び精嚢の重量測定のみならず免疫組織化学反応や形態変化を densitometry 及び morphometry で解析することによってアンドロゲンに対する感受性を検討した。

2. 実験材料および方法

生後 2 週目、体重 150 g の Wistar 系雄性ラット（ドイツ Borchen の Harlan Winkelmann 社の HdrBrHan 種）を用いて、2-4 匹毎床敷（ssniff bedding、3/4Faser）の入った Makrolon 製の飼育籠（IV 型）に無作為に入れ、水道水と ssniff R10 標準型ラット用固型飼料（ドイツ Soest の ssniff Spezialdiaet GmbH 製）を無制限に与え、室温 22±3℃、湿度 30-70%、24 時間人口照明下で飼育した。環境順応

後、これ等の動物を1群13匹当て8群に分け、1群はintactとし、他の7群の動物については睾丸摘出を行った。去勢手術はKetanet 10mg/kg （ドイツ Berlin 在駐の Parke-Davis 社製)とRompan 2mg/kg （ドイツ Leverkusen の Bayer AG 製)での麻酔下に行った。

以後はFig. 1に図示したように、去勢(d0)後8日目(d7)から7日間に亘り、4群に夫々BPAを3、50、200及び500mg/kg/day経口投与し、他の1群にはBPA 500mg/kg/dayとFlutamide(以下 FL) 3mg/kg/dayを併用経口投与した。残りの3群の内1群にはTestosterone propionate(以下 TP) 1mg/kg/dayを皮下投与し、残りの2群、即ち去勢動物群並びにintact群の動物には溶媒のPropylene glycolを経口投与した。

なおBPA並びにFLはPropylene glycolに、またTPは落花生油に溶解し投与した。

BPA(PtNr. 97001/Prod.Nr. 04111095、CasNr. 80-05)はドイツLeverkusenのバイエル社から、またPropylene glycolはドイツ Darmstadt の Merck 社から購入、 TP と FL はドイツ Berlin の Schering社の好意により提供を受けたものである。

投与終了24時間後、全ての動物を断頭屠殺し(d15)、直ちに精嚢腺及び前立腺を摘出し、重量測定を行い、4% Paraformaldehydeで24時間固定した。

2.1 免疫組織化学的検索法

固定済みの臓器を常法通り濃度の異なるアルコール溶液で段階的に連続脱水し、パラフィン固定後、5μmの切片を1試料当り10枚作成した。ARと増殖マーカーの免疫組織化学的検索は下記の一次抗体を用い、製造元既定の標準法に従って行った。

2.1.1 抗アンドロゲン受容体

sc-815 (米国カリフォルニア Santa Cruz Biotechnology 社の家兎 poyclonal 抗体、1:100)及び554224(ドイツBD PharMingen社のマウスmonoclonal抗体、1:100)を使用。

2.1.2 抗増殖マーカー

anti-PCNA (英国 New Castle Novocastra 社の PC-10 マウス monoclonal 抗体、1：200)及びanti-Ki-67(MIB-5:デンマーク DakoCytomation社のM7248マウスmonoclonal抗体、1：100)を使用。

2.2 染色強度(densitometry)並びに形態変化(morphometry)の測定法

免疫組織化学染色度及び上皮細胞の高さ及び分泌腺腔面積の測定はドイツ Jena Zeiss-Vision 社製のKS100、KS RUNを用い、顕微鏡はZeiss社製のAxiocam(高解析能のscannerカメラ)を着装したAxiophot光学顕微鏡を使用した。

測定にあたり解析画像のサイズをすべて一定(1300 x 1030 画素)にした。

染色強度の測定にはZeiss社のソフトKS400を用いた。使用したレンズは20倍の対物レンズと1.0倍の接眼レンズで、最終解析画像の一画素の縁長を0.32μmとした。各画素のグレー値は0(白色)-255(黒色)の範囲とし、画素毎に吸光度に変換した(17)。測定は一切片当り5回とし、動物一匹当り5切片をとり合計25測定を行った。

形態変化の測定には上記Zeiss社のKS100付属のソフト(version 3.0)を用いた。上皮細胞の高さは40倍の対物レンズと1.0倍の対眼レンズを、分泌腺腔の面積は40倍の対物レンズと1.0倍の対眼レンズを用い、細胞増殖の定量的測定には40倍の対物レンズと1.0倍の対眼レンズを使用し

た。その際一切片当り 1000 個の細胞を数え、細胞数の過剰測定を妨ぐ為無核細胞は除外した。

2.3 統計処理

ANOVA-test(one way analysis of variance)と Bartletts-test (equal variance analysis) で分散分析を行い, two side t-test($p<0.05$)と Bonferroni post-test ($p<0.001$) で平均値の有意差検定を行った。結果は平均値±標準偏差(SD)で表し、p 値が 0.05 以下の場合を有意とした。尚、統計処理には GraphPad Instat software (Intuitive Software for Science, San Diega, CA, USA)を用いた。

2.4 動物実験の為の動物保護法

本実験は「動物実験実施上の基礎、規定、審査に関する法律」: 1998 年 5 月 25 日発令の動物保護法(BGBI. IS. 1105, 1818)に準じて行った。

3. 実験成績

3.1 Hershberger 試験

BPA の低用量(3, 50 mg/kg)では体重、前立腺及び精嚢の絶対重量/比重量に変化は見られなかったが、高容量(200 及び 500mg/kg)の投与で体重の減少及び前立腺及び精嚢の比重量の有意な増加が見られ、絶対重量に変化は見られなかった(Tab. 1)(Fig. 2, Fig. 3)。BPA 500mg/kg 投与の影響は FL の併用により変化しなかった。BPA 200、500mg/kg 及び BPA 500mg/kg+FL 投与群の動物で毒性と思われる飼料摂取の減少並びに下痢やガスの貯留等消化器系機能障害症状が認められた。

3.2 AR 染色度の測定(densitometry)

polyclonal 抗体(sc-815)を用いた場合、各実験群の AR 染色の吸光度は以下の通りであった：前立腺では intact 群＝75±21、去勢群＝43±12、TP 群＝61±24、BPA 3mg 群＝65±27、BPA 50mg 群＝57±19、BPA 200mg 群＝48±18、BPA 500mg 群＝39±9、BPA 500mg+FL 群=33±10 で、精嚢では intact 群＝42±17、去勢群＝11±10、TP 群＝19±11、BPA 3mg 群＝8±5, BPA 50mg 群＝11±15、BPA 200mg 群＝7±3、BPA 500mg 群＝3±1、BPA 500mg+FL 群=15±17 であった。

上記の結果から、前立腺について見た場合 intact 群と TP 群の値は去勢群のそれに比べ高く、有意差が認められた($p<0.05$)。一方、BPA 投与群では 3mg と 50mg で AR 染色度の有意な上昇が見られ、高用量の 200mg では影響は無く、500mg では寧ろ低下した。亦、BPA 500mg の作用は FL 併用によって強まる傾向が見られた。

精嚢に関しては去勢による AR 反応の低下は TP 投与により回復の傾向を示したが、去勢群との間に有意差は認められなかった。去勢動物に BPA を投与した場合有意な用量依存性変化は見られなかった。

上記の結果を確認する目的で monoclonal 抗体(554224)を用い前立腺につき調べた場合の AR 染色の吸光度は次の通りである：intact 群＝104±20、去勢群＝56±17、TP 群＝77±22、BPA 3mg 群＝99±7、BPA 50mg 群＝103±23、BPA 200mg 群＝78±16、BPA 500mg 群＝49±23、BPA 500mg+FL 群=36±6 であった(Fig. 5)。

統計処理の結果、polyclonal 抗体の場合と同様に、intact 群と TP 群の染色強度は去勢群の比して有意に高く、去勢により低下した AR 値が TP 投与により上昇することが再確認された。BPA を

3mg、50mg 及び 200mg 投与した場合では去勢対照群に比べ AR 値は有意な上昇を示したが、500mg 投与では変化はなく、500mg+FL 投与では低下した。

3.3 細胞増殖マーカー

Ki-67 を指標とした前立腺上皮細胞増殖率は intact 群で 85±9% 、去勢群で 9±1%、TP 群で 90±2%であった。BPA 投与群では 3mg で 10±1%、50mg で 8±1%、200mg で 2%、500mg で 1%、500mg+FL で 1%であった(Fig. 6a)。

PCNA を指標とした場合も概ね同様の結果が得られた(Fig. 6b)：intact 群で 90±9% 、去勢群で 10±2%、TP 群で 88±9%であった。BPA 投与群 3mg で 2%、50mg で 4%、200mg で 2%、500mg で 5±1%、500mg+FL で 6±1%であった。

以上二種類のマーカーを用いた実験で、前立腺上皮細胞の増殖は去勢により著明に減少し、TP の投与によって intact 群のレベルまで回復するが、BPA は増殖率にはほとんど影響しないことが認められた。

3.4 前立腺上皮の高さと分泌腺腔の面積

Fig. 7a に示す如く、前立腺上皮の高さは intact 群と TP 投与群共に 17±2μm で、去勢群では 11±1μm で有意な減少が見られた。BPA 投与後の値は 3mg 群では 14±2μm、50mg 群では 14±2μm で去勢による減少の一部回復が見られたが、200mg 群では 10±1μm で、500mg 群(9±1μm)と 500mg+FL 群(8±1μm)の値は去勢群の値に比べより低かった。

一方精嚢腺上皮の高さに関してもほぼ同様の結果が得られた(Fig. 7b)：intact 群で 18±3μm、TP 投与群で 15±2μm で、去勢群の値 9±2μm との間に有意差が認められた。BPA 投与後の値は 3mg 群で 11±1μm、50mg 群で 11±1μm、200mg 群で 8±1μm、500mg 群で 8±1μm、500mg+FL 群で 8±1μm であった。

分泌腺腔の面積測定の結果は第 8a、b 図の通りで、前立腺の場合 intact 群で 131000±40000μm^2、去勢群で 7000±5000μm^2、TP 投与群で 87000±30000μm^2であった。BPA 投与群では 3mg で 23000±16000μm^2、50mg 群で 25000±12000μm^2、200mg 群で 15000±10000μm^2、500mg 群で 4000±500μm^2、500mg+FL 群で 4000±3000μm^2であった。

精嚢腺では intact 群で 113000±80000μm^2、去勢群で 7000±5000μm^2、TP 投与群で 151000±860000μm^2であった。BPA 投与群では 3mg で 35000±43000μm^2、50mg 群で 27000±20000μm^2、200mg 群で 8000±3000μm^2、500mg 群で 4000±1000μm^2、500mg+FL 群で 3000±2000μm^2であった。

以上前立腺並びに精嚢腺で上皮の高さ及び分泌腺腔を面積測定した結果、去勢により値が有意に低下し、これが TP 投与によって回復することが確認され、BPA 低用量投与群でも一定の回復が見られた。これに対し高用量の BPA を投与した場合、前立腺上皮の高さの有意な低下が認められた。

4. 考察

4.1 Parameter について

諸種生体内物質の濃度が免疫反応シグナルの吸光度から測定されることが既に知られている(26,27)。この方法の長所は組織切片の広範囲にわたり迅速に測定することが出来、しかも再現性の高いことにある。しかしこの半定量的測定では場合によってシグナルを見逃す可能性も若干あ

り得るので出来る限り反応性の高いシグナルを選ぶべきであると云われている(28)。これらのことを考慮して、本実験ではHershberger法を用い、前立腺や精嚢の重量測定のみならずAR量並びに生殖器官機能並びに組織形態に関連した parameter の免疫組織化学反応による染色強度をcomputerを介したdensitometry及びmorphometryにより半定量的に測定した。

4.1.1 Hershberger 試験

今回用いた Hershberger 法はアンドロゲン/抗アンドロゲン作用の検定法として今日でも広く用いられている。本実験でも前立腺と精嚢の重量が去勢により著しく減少し、TPの投与で回復することが認められた。これは両生殖臓器の増殖がアンドロゲンに依存していることを示すが、測定の end point が重量のみであるため、得られたデータの評価に限界がある。例えば、エストロゲンの paradox effect として知られている精嚢重量増加作用が挙げられる(18-20)。従ってアンドロゲン作用をより正しく評価する為には特異的且つより鋭敏なパラメーターを測定することが肝要である。

4.1.2 アンドロゲン受容体

前立腺の機能がアンドロゲンにより維持されることは周知の通りであるが、我々はアンドロゲンの低下によりラット前立腺中にテナシンが誘発されることを認めている(21)。とりわけ前立腺細胞内のAR量がアンドロゲンの影響を受けることが知られており(22,23)、前立腺や精嚢細胞中のARを免疫組織化学的方法で染色し、その強度を光学的に測定することが出来る(24,25)。
本実験でも前立腺及び精嚢のAR陽性細胞の免疫化学反応度は去勢により著明に低下し、この低下はTPの薬理学的用量の投与でほぼ正常に回復することが確認された。

4.1.3 細胞上皮と分泌腺腔

雄性ラットの生殖器官、特に副睾丸、前立腺、精嚢の上皮細胞の増殖がアンドロゲンに大きく依存しており(36)、今回の実験でも前立腺上皮の高さ及び分泌腺腔の面積が去勢により減少し、TP補充によりほぼ正常値まで回復することが認められた。

4.1.4 細胞増殖マーカー

細胞増殖にともない発現する増殖細胞核抗原(PCNA)及びKi-67抗原は特に悪性腫瘍の診断マーカーとして広く用いられる(37,38)。PCNAはcell cycleの細胞増殖期や核修復期に発現する36kDaの酸性核蛋白で(39,40)、DNA-polymerase delta及びRF-C蛋白がDNA primer-template junctionと結合する際に作用すると云われる。免疫染色によればDNA合成期にあたるS期の核に認められる。亦、Ki-67抗原はG_0期には見られないが、G_1、S、G_2、M期に発現し、その半減期は短く、増殖旺盛な細胞のマーカーとみなされる(41,42)。
本実験でも前立腺と精嚢の両マーカーのアンドロゲン依存性が確認された。

4.1.5 BPAの作用

ラットでの3世代に亘る飼育試験で胎児サイズの減少度を指標とした場合、経口投与によるBPAの最大無有害作用量(NOAEL)は50mg/kg/dayで、これに基づいて低作用量は5mg/kg/dayと定められている(31)。他方、喫歯類での開発試験の結果によれば、BPAが有毒物質であるとは考えられてない(32)。これらの記載を参考にして、本実験ではBPAの3mg/kg/dayを超低用量, 50-200mg/kg/dayを薬理学的用量、500mg/kg/dayを超薬理学的用量とした。その結果Kimら(16)の報告

と同様、BPA 投与群(3, 50 mg/kg/day)で前立腺重量に変化は認められなかった。ただし高用量の BPA 投与群(200mg, 500mg/kg/day)で体重当りの臓器重量増加が見られた。これは実験中に認められた食欲減少、下痢といった毒性又は副作用による体重減少に起因するものでと考えられる。剖検では全消化管内にガスの貯留もみとめられた。同様の副作用が Morissey ら(29)、Yamasaki ら(30)、や Tyl ら(31)によって既に報告されている。

低用量の BPA(3mg, 50mg/kg /day)を去勢動物に投与した場合、上皮の高さと分泌腺腔面積の増加が見られ、加えて BPA 投与群で、TP 投与群と同様に、前立腺の AR 染色度の増加が認められたことは一定のアンドロゲン様作用が BPA に潜在することを示唆するものと考えられる。しかし前立腺及び精嚢の臓器重量に変化は認められず、増殖マーカーの増加も見られなかったことから、BPA のアンドロゲン様作用は極微弱なものと推測される。

一方において BPA がエストロゲン様の性質を有することが多くの研究者によって報告されているが、大抵の場合 100mg/kg/day 以上の投与量でこの作用が見られる(12-14)。BPA の低用量で認められるアンドロゲン様作用の発現機構は現在のところ未詳であるが、本実験で見られた BPA の影響が既知のエストロゲン様作用によるものとは推測しがたい。何故なら、エストロゲンは前立腺及び精嚢の繊維組織や筋肉壁の増殖を促すが上皮や分泌腺には影響しないとされている(20,33)。従って、エストロゲンが精嚢に影響すること無く前立腺細胞中の AR の免疫反応性を選択的に高めるとは考えがたい。更に、エストロゲンは副腎皮質機能を促進し、副腎性アンドロゲンの分泌調整に関与する要素の一つのとしても知られているが(34)、BPA が副腎機能を亢進することにより前立腺だけに選択的作用を及ぼすという考えも肯定しがたい。

以上、ラットの前立腺及び精嚢の AR の免疫組織化学反応性、細胞形態変化、細胞増殖度等の測定データの解析により BPA に微弱なアンドロゲン様作用の潜在することを認めた。これらの結果から本実験に用いられた方法が今後多くの物質、特に環境や人体に悪影響を及ぼすと思われる所謂内分泌撹乱物質のアンドロゲン作用を定性的且つ定量的に評価するうえで役立つものと考えられる。

5. 結論

5.1. ラット前立腺及び精嚢の臓器重量、AR、上皮細胞の高さ/分泌腺腔の面積及び細胞増殖が去勢により減少し、アンドロゲン投与で回復する。

5.2. 去勢ラットの前立腺及び精嚢重量を指標とした場合、BPA の低用量(30, 50mg/kg/day)では影響が見られない。高用量(200, 500mg/kg/day)で見られた生殖臓器の比重量の増加は BPA の副作用による体重の減少に起因すると思われる。

5.3. BPA は 3,50,200mg/kg/day の低用量で、TP と同様に、去勢により減少した前立腺細胞 AR の免疫化学的染色度を正常の範囲まで増加回復させる。

5.4. BPA は低用量で去勢後の前立腺及び精嚢の上皮細胞の高さ及び分泌腺の面積の減少を軽度に回復させる。

5.5. 増殖マーカーKi-67 及び PCMA を指標とした場合、BPA は雄生殖腺細胞の増殖に影響を与えない。

以上、雄性生殖腺の免疫組織化学的変化並びに形態学的変化を densitometry/morphometry により半定量的に測定した結果、BPA に微弱なアンドロゲン様作用の潜在することを認めたが、今後この方法が内分泌撹乱物質等のアンドロゲン作用の評価検定に役立つものと考える。

参考文献

1) T. Zincke (1905) Mittheilungen aus dem chemischen Laboratorium der Universitat Marburg, *Justus Liebigs Annalen Chemie*. 343, 75-99.

2) S. Krimski (2000) Hormonal Chaos, The Johns Hopkins University Press, Baltimore-London, 1-54.

3) J. Ashby, J. Odum, D. Paton, P.A. Lefevre, N. Beresford, J.P. Sumpter (2000) Re-evaluation of the first synthetic estrogen, 1-keto-1,2,3,4-tetrahydrophenantrene, and bisphenol A, using both the ovariectomized rat model used in 1933 and additional assays, *Toxicol. Lett.* 115 231-238.

4) F. Paris, P. Balaguer, B. Terouanne, N. Servant, C. Lacoste, J.P. Cravedi, J.C. Nicolas, C. Sultan (2002) Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines, *Mol. Cell Endocrinol.* 193 43-9.

5) S.C. Nagel, F.S. vom Saal, K.A. Thayer, M.G. Dhar, M. Boechler, W.V. Welshons (1997) Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol, *Environ. Health Perspect.* 105 10-6.

6) H.S. Kim, S.Y. Han, S.D. Yoo, B.M. Lee, K.L. Park (2001) Potential estrogenic effects of bisphenol-A estimated by in vitro and in vivo combination assays, *J. Toxicol. Sci.* 26 111-8.

7) T. Takao, W. Nanamiya, I. Nagano, K. Asaba, K. Kawabata, K. Hashimoto (1999) Exposure with the environmental estrogen bisphenol A disrupts the male reproductive tract in young mice, *Life Sciences* 65 2351-2357.

8) W.V. Welshons, S.C. Nagel, K.A. Thayer, B.M. Judy, F.S. vom Saal (1999) Low-dose bioactivity of xenoestrogens in animals: fetal exposure to low doses of methoxychlor and other xenoestrogens increases adult prostate size in mice, *Toxicol. Ind. Health* 15 12-25.

9) F.S. vom Saal, P.S. Cooke, D.L. Buchanan, P. Palanza, K.A. Thayer, S.C. Nagel, S. Parmigiani, W.V. Welshons (1998) A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production and behaviour, *Toxicol. Ind. Health* 14 239-60.

10) P.L. Palanza, K.L. Howdeshell, S. Parmigiani, F.S. vom Saal (2002) Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice, *Environ. Health perspect.* 110 415-422.

11) K.L. Howdeshell, A.K. Hotchkiss, K.A. Thayer, J.G. Vandenbergh, F.S. vom Saal (1999) Environmental toxins: Exposure to bisphenol A advances puberty, *Nature* 401 763-764.

12) P. Diel, T. Schulz, K. Smolnikar, E. Strunck, G. Vollmer, H. Michna (2000) Ability of Xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotrophic activity, *J. Steroid Biochem. Mol. Biol.* 73 1-10.

13) C.M. Markey, C.L. Michaelson, E.C. Veson, C. Sonnenschein, A.M. Soto (2001) The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A, *Environ. Health Perspect.* 109 55-60.

14) H. Tinwell, R. Joiner, I. Pate, A. Soames, J. Foster, J. Ashby (2000) Uterotrophic activity of bisphenol A in the immature mouse, *Regul. Toxicol. Pharmacol.* 32 118-126.

15) C. Gupta (2000) Reproductive Malformation of the Male Offspring Following Maternal Exposure to Estrogenic Chemicals (44516), *Proc. Soc. Exp. Biol. Med.* 224 61-68.

16) H. Kim, S. Han, T. Kim, S. Kwack, R. Lee, I. Kim, J. Seok, B. Lee, S. Yoo, K. Park (2002) No androgenic/anti-androgenic effects of bisphenol-A in Hershberger assay using immature castrated rats, *Toxicol. Lett.* 5; 135 (1-2) 111.

17) M. Oberholzer, M. Oestreicher, H. Christen, M. Bruehlmann (1996) Methods in quantitative image analysis, *Histochem. Cell Biol.* 105 333-355.

18) S.E. De Jongh (1935) Paradoxe Wirkungen von Follikelhormon (Menformon) bei männlichen Tieren; ihre Beeinflussbarkeit durch männliches Hormon, *Arch. Int. Pharmacodyn.* 50 348-355.

19) S.E. De Jongh (1937) De beinvloeding van het mannelijk genitaal apparaat door hormonen, *Hormoon,* VII: 42-51.

20) J. Freud (1933) Conditions of hypertrophy of the seminal vesicles in rats, *Biochem. J.* 27 1438-1445.

21) G. Vollmer, H. Michna, K. Ebert, R. Knuppen (1994) Androgen ablation induces tenascin expression in the rat prostate, *Prostate* 25 81-90.

22) R.J. Moore, J.M. Gazak, J.D. Wilson (1979) Regulation of cytoplasmic dihydrotestosterone binding in dog prostate by 17β-estradiol, *J. Clin. Invest.* 63 351-357.

23) J.P. Blondeau, E.E. Baulieu, P. Robel (1982) Androgen-dependent regulation of androgen nuclear receptor in the rat ventral prostate, *Endocrinology* 110 1926-1932.

24) C. Huggins, C.V. Hodges (2002) Studies on prostatic cancer. I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941, *J. Urol.* 167 948-951.

25) C. Rolf, E. Nieschlag (1998) Potential adverse effects of long-term testosterone therapy, *Baillieres Clin. Endocrinol. Metab.* 12 521-534.

26) L.B. Nabors, E. Songu-Mize, R.R. Mize (1988) Quantitative immunocytochemistry using an image analyzer. II. Concentration standards for transmitter immunocytochemistry, *J. Neurosci. Methods.* 26(1) 25-34.

27) R.R. Mize, R.N. Holdefer, L.B. Nabors (1988) Quantitative immunocytochemistry using an image analyzer. I. Hardware evaluation, image processing and data analysis, *J. Neurosci. Methods.* 26(1) 1-23.

28) W.I. De Boer, P.S. Hiemstra, J.K. Sont, E. De Heer, K.F. Rabe, J.H.J.M. Van Krieken, P.J. Sterk (2001)

Image analysis and quantification in lung tissue, *Clin. Exp. Allergy* 31 504-508.

29) R.E. Morissey, J.D. George, C.J. Price, R.W. Tyl, M.C. Marr, C.A. Kimmel (1987) The developmental toxicity of bisphenol A in rats and mice, *Fundam. Appl. Toxicol.* 8 571-82.

30) K. Yamasaki, M. Sawaki, S. Noda, N. Imatanaka, M. Takatsuki (2002) Subacute oral toxicity study of ethynylestradiol and bisphenol A, based on the draft protocol for the "Enhanced OECD Test Guideline no. 407", *Arch. Toxicol.* 76 65-74.

31) R.W. Tyl, C.B. Myers, M.C. Marr, B.F. Thomas, A.R. Keimowitz, D.R. Brine, M.M. Veselica, P.A. Fail, T.Y. Chang, J.C. Seely, R.L. Joiner, J.H. Butala, S.S. Dimond, S.Z. Cagen, R.N. Shiotsuka, G.D. Stropp, J.M. Waechter (2002) Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats, *Toxicol. Sci.* 68 121-146.

32) European Chemicals Bureau: EU-Risk-Assessment Summary Vol.37 (2003) on 4,4'-isopropylidenephenol (bisphenol-A), CAS#: 80-05-7, EINECS#: 201-245-8.

33) K.David, J. Freud, S.E. de Jongh (1934) Conditions of hypertrophy of seminal vesicle in rats II, *Biochem. J.* 28 1360-1367.

34) W.D. Odell, L.N. Parker (1984) Control of adrenal androgen production, *Endocr. Res.* 10 617-630.

35) J.E. McNeal (1988) Normal histology of the prostate, *Am. J. Surg. Pathol.* 12 619-633.

36) I. F. Lissbrant, E. Lissbrant, A. Persson, J.E. Damber, A. Bergh (2003) Endothelial cell proliferation in male reproductive organs of adult rat is high and regulated by testicular factors, *Biol. Reprod.* 68 1107-1111.

37) P. Corkolopoulou, J. Oates, J. Clocker, C. Edwards (1993) p53 expression in oat and non-oat small cell lungcarcinomas: correlation with proliferating cell nuclear antigen, *J. Clin. Pathol.* 46 1093-1096.

38) T. Kawai, M. Suzuki, S. Kono, N. Shinoyama, M. Rokutanda, K. Takagi, T. Ogata, S. Tamai (1194) Proliferating cell nuclear antigen and Ki-67 in lung carcinoma, Correlation with DNA flow cytometric analysis, *Cancer* 74 2468-2475.

39) M.K.K Shivji, M.K. Kenny, R.D Wood (1992) Proliferating cell nuclear antigen is required for DNA excision repair, *Cell* 69 367-374.

40) P.A. Hall,D.A. Levison, A.L. Woods, C.C.W. Yu, D.B. Kellock, J.A. Watkins, D.M. Barnes, C.E. Gillett, R. Camplejohn, R. Dover, N.H. Waseem, D.P. Lane (1990) Proliferating cell nuclear antigen (PCNA) immnolocalization in paraffin sections: An index of cell proliferation with evidence of deregulated expression in some neoplasms, *J. Pathol.* 162 285-294.

41) D. McCormick, H. Chong, C. Hobbs, C. Datta, P.A. Hall (1993) Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MBI-1, *Pathol.* 22 355-360.

42) S.E. Pinder, P. Wencyk, D.M. Sibbering, J.A. Bell, C.W. Elston, R. Nicholson, J.F.R. Robertson, R.W.

Blamey, I.O. Ellis (1995) Assessment of the new proliferation marker MBI-1 in breast carcinoma using image analysis: Associations with other prognostic factors and survival, Brit. J. Cancer 71 146-149.

Figure Legends

Tab. 1

Absolute and relative wet weights of prostates and seminal vesicles in intact and castrated (OX) rats. Castrated animals were treated with either TP or BPA (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group..

Fig. 1.

Experimental design of the Hershberger assay.

Fig. 2.

Comparison of body weights in [g] between the intact group, orchiectomized group (OX) and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Fig. 3.

Comparison of absolute wet weights of the whole prostate in [mg] between the intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Fig. 4.

All panels show photographs of the prostate. Zones are described according to McNeal (35).

A-H: Immunohistochemical staining of androgen receptor (s. red coloured cells) using monoclonal antibody shows the transition zone of the intact group (A), OX group (B) and TP group (C) in relation to the BPA-treated groups (D-H) (3, 50, 200, 500 and 500+FL). Original magnification 20x.

I-P: Immunohistochemical staining of MIB-5 (s. red coloured cells) showing the peripheral zone of the intact group (I), OX group (J) and TP group (K) in relation to the BPA-treated groups (L-P) (3, 50, 200, 500 and 500+FL). Original magnification 10x.

Q-X: Immunohistochemical staining of PCNA (s. red coloured cells) showing the transition zone of the intact group (Q), OX group (R) and TP group (S) in relation to the BPA-treated groups (T-X) (3, 50, 200, 500 and 500+FL). Original magnification 10x.

Fig. 5.

Densitometric values of the intact group, orchiectomized group (OX), TP group and the BPA-treated groups (3, 50, 200, 500 and 500+FL) after immunohistochemical staining (monoclonal antibody) of the androgen receptor in the prostate. Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Fig. 6a.

Quantitative comparison of MIB-5-immunoreactive epithelial prostatic cells between the intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Fig. 6b.

Quantitative comparison of PCNA-immunoreactive epithelial prostatic cells between the intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Fig. 7a.

Comparison of epithelial height of prostate between the intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Fig. 7b.

Comparison of epithelial height of seminal vesicle between the intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Fig. 8a.

Comparison of luminal glandular area of prostate between the intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Fig. 8b.

Comparison of luminal glandular area of seminal vesicle between the intact group, OX group and TP group in relation to the BPA-treated groups (3, 50, 200, 500 and 500+FL). Asterisks indicate statistically significant differences ($p<0.05$) which refer to the castrated control group.

Tab. 1:

	Prostate		Seminal vesicle	
	mg	mg/kg body weight	mg	mg/kg body weight
Intact	372 ± 61*	1453 ± 191*	236 ± 61*	928 ± 243*
OX	27 ± 13	116 ± 56	18 ± 5	77 ± 23
TP	213 ± 31*	864 ± 116*	141 ± 41*	573 ± 168*
BPA 3mg	35 ± 17	156 ± 79	16 ± 5	73 ± 25
BPA 50mg	30 ± 14	142 ± 49	16 ± 2	69 ± 13
BPA 200mg	29 ± 7	176 ± 52*	17 ± 3	100 ± 30*
BPA 500mg	26 ± 5	166 ± 37*	17 ± 5	94 ± 24*
BPA 500mg + FL	28 ± 8	185 ± 69*	17 ± 3	115 ± 35*

Fig. 1:

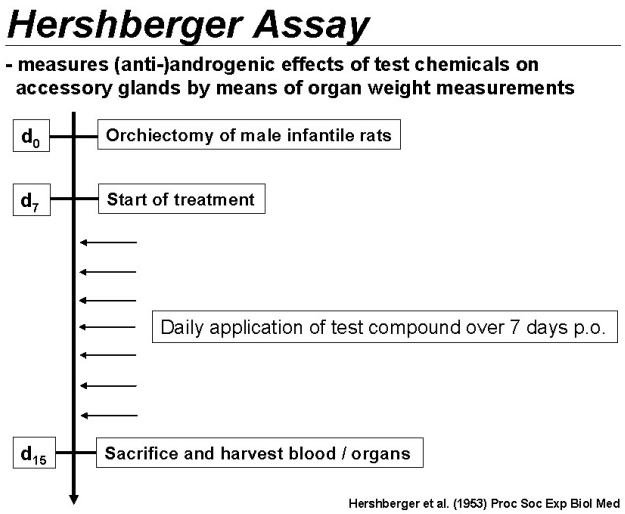

Hershberger Assay
- measures (anti-)androgenic effects of test chemicals on accessory glands by means of organ weight measurements
d0
Orchiectomy of male infantile rats
d7
Start of treatment
Daily application of test compound over 7 days p.o.
d15
Sacrifice and harvest blood / organs
Hershberger et al. (1953) Proc Soc Exp Biol Med

Fig. 2:

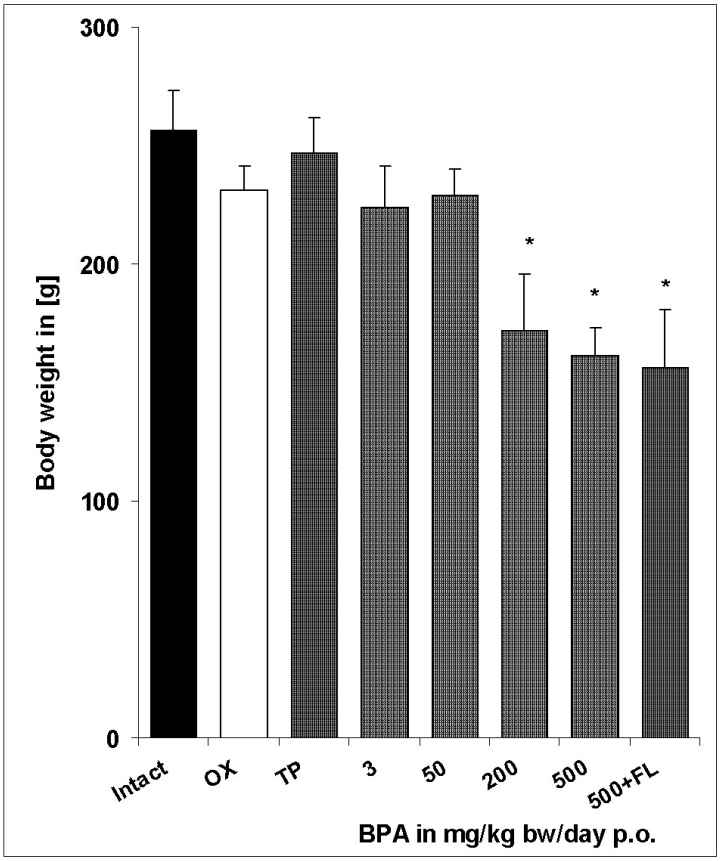

300
200
100
0
Body weight in [g]
*
*
*
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.

Fig. 3:

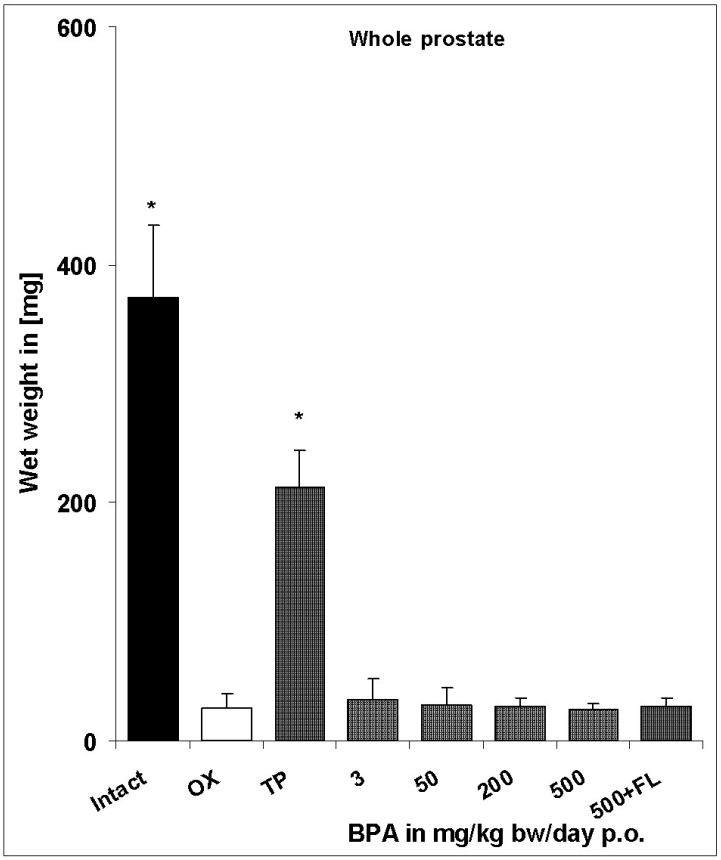

Whole prostate
600
400
200
0
Wet weight in [mg]
*
*
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.

Fig. 5:

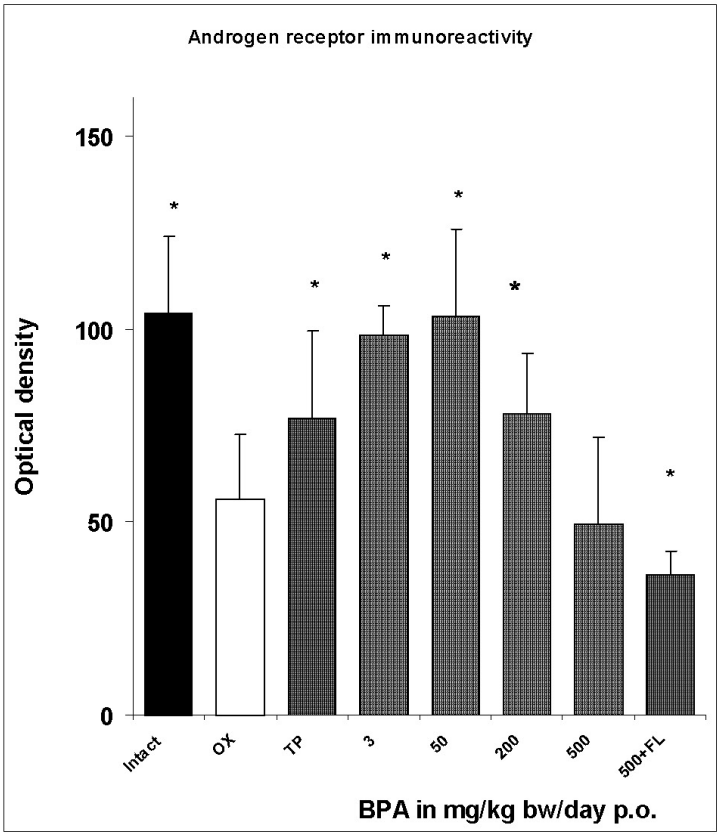
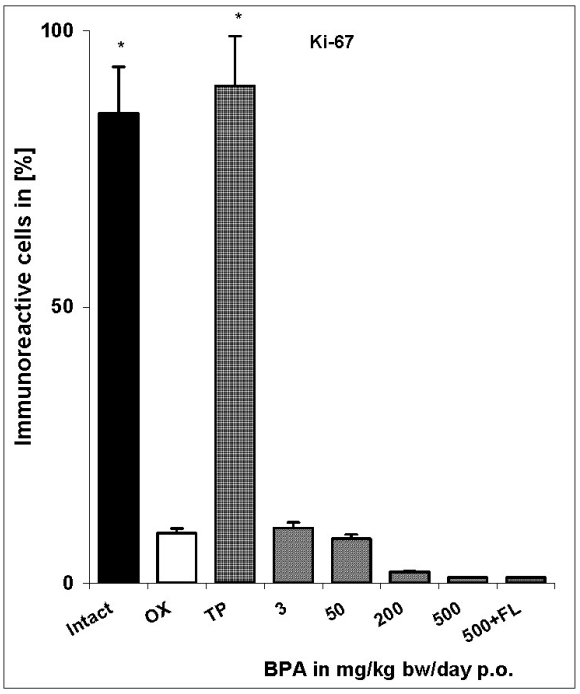

Androgen receptor immunoreactivity
Optical density
150
100
50
0
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.

Fig. 6a, 6b:


Ki-67
Immunoreactive cells in [%]
100
50
0
*
*
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.

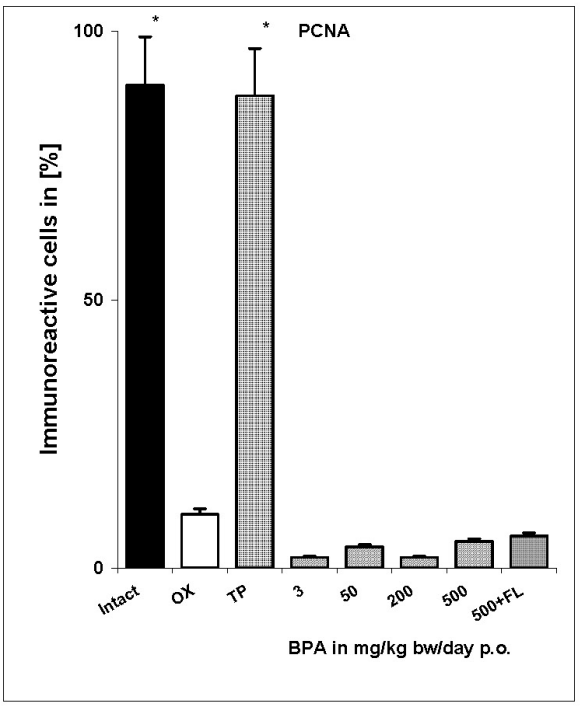
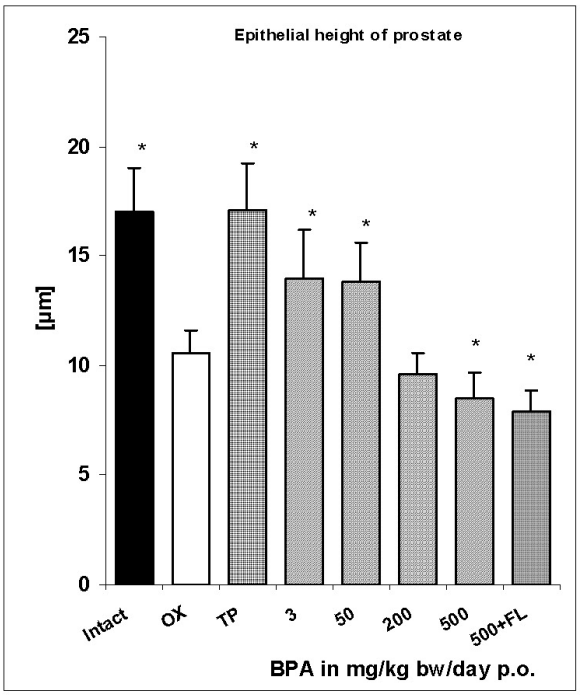

PCNA
Immunoreactive cells in [%]
100
50
0
*
*
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.

Fig. 7a, 7b:


Epithelial height of prostate
25
20
15
10
5
0
[µm]
*
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.

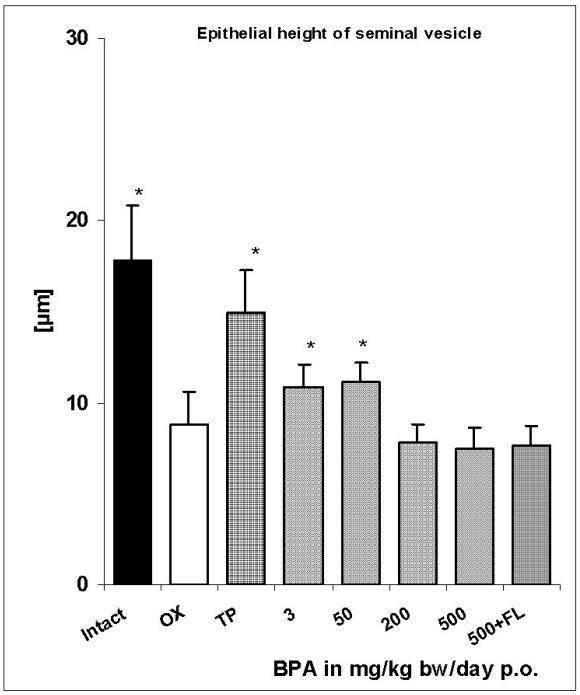

Epithelial height of seminal vesicle
30
20
10
0
[µm]
*
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.

Fig.8a, 8b

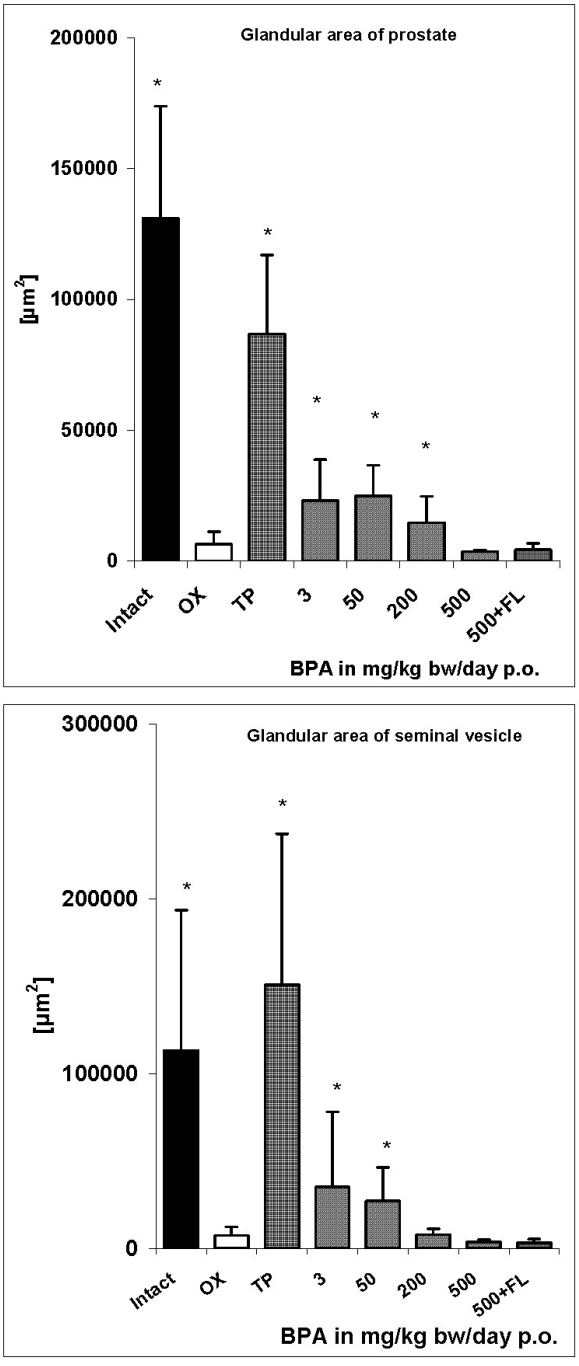
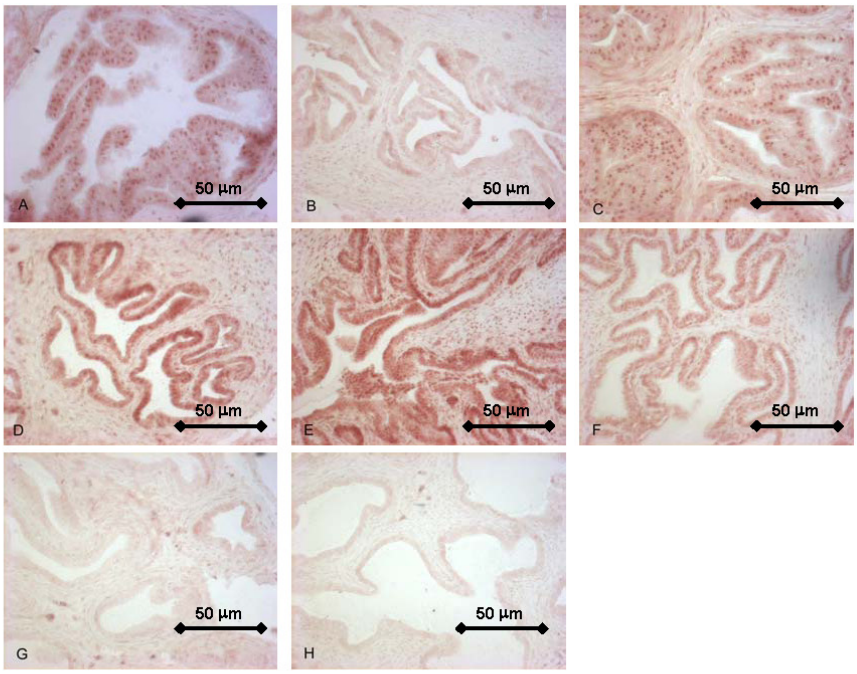
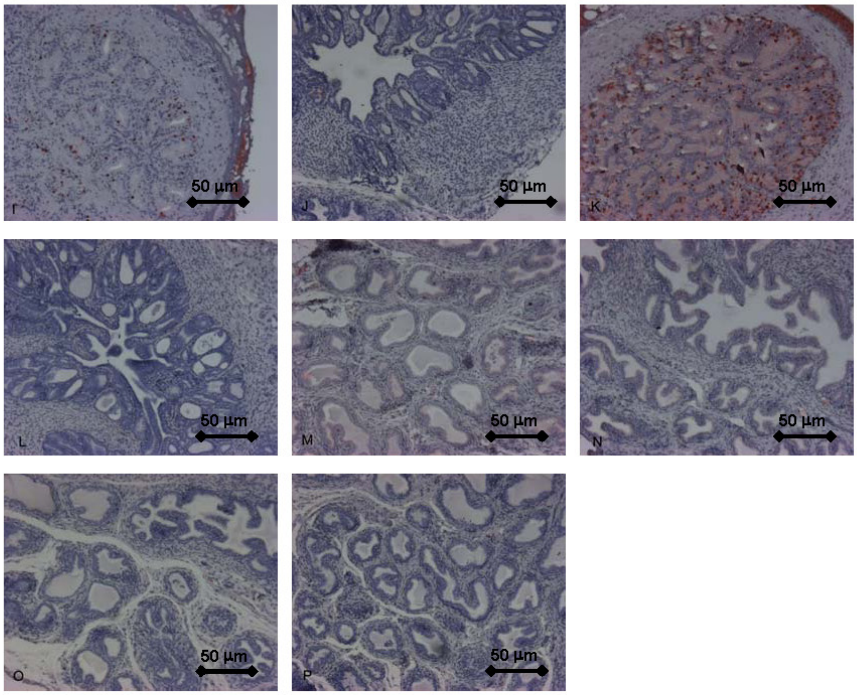
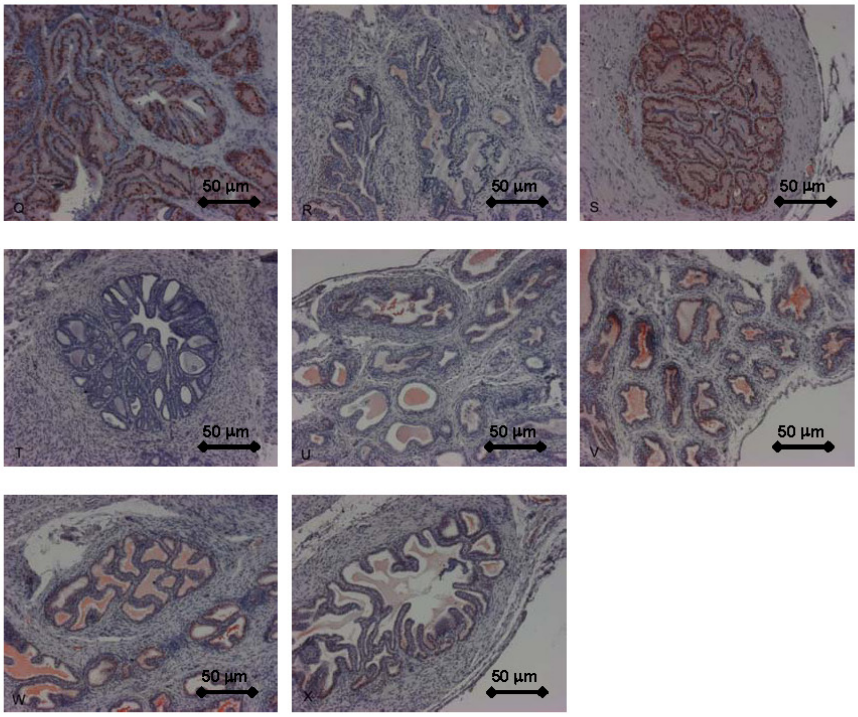

Glandular area of prostate
200000
150000
100000
50000
0
[µm²]
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.
Glandular area of seminal vesicle
300000
200000
100000
0
[µm²]
Intact
OX
TP
3
50
200
500
500+FL
BPA in mg/kg bw/day p.o.

Fig.4A-4X





23


Q
50 µm
R
50 µm
S
50 µm
T
50 µm
U
50 µm
V
50 µm
W
50 µm
X
50 µm

Appendix VII.

Kaufmann K, Pojarová M, Vogel S, Liebl R, Gastpar R, Gross D, Nishino T, Pfaller T, von Angerer E: Antimitotic activities of 2-phenylindole-3-carbaldehydes in human breast cancer cells.

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Antimitotic activities of 2-phenylindole-3-carbaldehydes in human breast cancer cells

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Abstract—Small molecules such as indoles are attractive as inhibitors of tubulin polymerization. Thus a number of 2-phenylindole-3-carbaldehydes with lipophilic substituents in both aromatic rings was synthesized and evaluated for antitumor activity in MDA-MB 231 and MCF-7 breast cancer cells. Some 5-alkylindole derivatives with a 4-methoxy group in the 2-phenyl ring strongly inhibit the growth of breast cancer cells with IC_{50} values of 5–20 nM. Their action can be rationalized by the cell cycle arrest in G_2/M phase due to the inhibition of tubulin polymerization.

1. Introduction

The microtubule system is essential for a number of cellular functions including mitosis and cell replication, maintenance of cell shape, cellular transport, and motility. Microtubules are hollow fibers formed by the polymerization of α- and β-tubulin heterodimers. The formation of microtubules and their depolymerization is a dynamic process which can be interrupted by both stabilization of microtubules and inhibition of polymerization. A large number of natural products are known to shift the dynamic equilibrium of the microtubule system to one or the other side and abrogate the biological functions of microtubules thereby. The taxanes and some other natural products such as epothilones stabilize the microtubule structures, whereas other agents such as colchicine, combretastatin A-4, and the vinca alkaloids inhibit the polymerization of α-/β-tubulin dimers.[1] Some of the natural products are characterized by complex chemical structures which makes synthesis and chemical modifications difficult. Others such as combretastatin A-4 are based on rather simple scaffolds which can easily be modified.[2]

The biological importance of microtubules makes them an interesting target for the development of anticancer drugs. For systematic studies on the use of antimitotic agents in cancer therapy molecules with simple structures are very attractive because they allow extensive chemical modifications. Examples are stilbenes,[2] aryl-substituted heterocycles,[3] anthracenones,[4] benzophenones,[5], and analogues[6] to name only a few. A variety of synthetic antimitotic compounds are based on the indole structure.[7–10] In Figure 1, some aryl-substituted indoles such as **1**,[11–13] **2**,[14] **3**,[15] and **4**[16] are presented. Three of these examples carry a trimethoxyphenyl ring which is considered as important for binding to the colchicine site on tubulin.

An interesting aspect in the application of combretastatin A-4 and related agents in cancer chemotherapy is their antivascular effect.[17] Since microtubules of the cytoskeleton play a major role in maintaining cell shape, the elongated endothelial cells of the tumor neovasculature are particularly sensitive to drugs that depolymerize microtubules and degrade the cytoskeleton. In contrast to drugs that target tumor angiogenesis compounds such as combretastatin A-4 phosphate disrupt the already formed tumor vasculature.[18]

In a previous study we discovered that some methoxy-substituted 2-phenylindole-3-carbaldehydes strongly inhibit the growth of human breast cancer cells.[19] Investigations on the mode of action revealed that the microtubules are the primary target of these agents. In an

Keywords: Phenylindoles; Breast cancer; Tubulin polymerization; Cell cycle arrest.
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doi:10.1016/j.bmc.2007.05.030

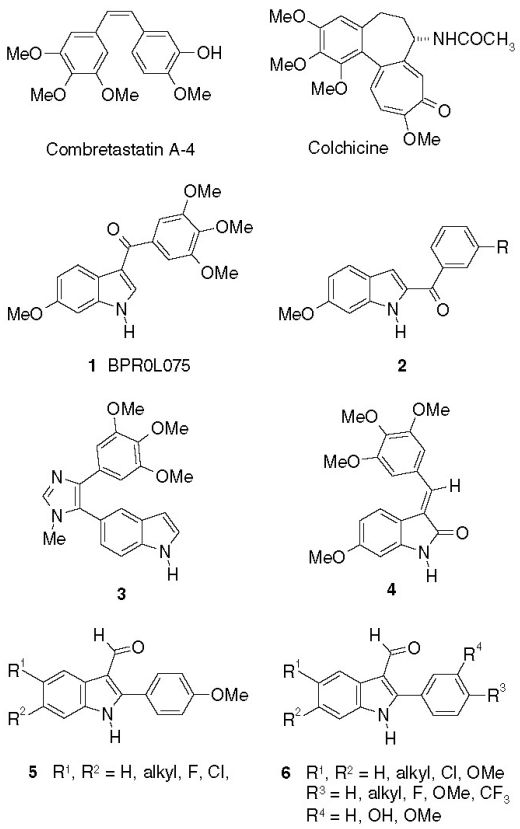


Figure 1. Chemical structures of combretastatin A-4, colchicine, and of some indole-based synthetic inhibitors of tubulin polymerization.

assay with bovine tubulin a marked inhibition on tubulin polymerization was observed. These indole derivatives were also shown to inhibit the binding of colchicine to tubulin. These findings prompted us to study this class of compounds in more detail. Since these derivatives of 2-phenylindole lack structural features considered typical for inhibitors of tubulin polymerization, for example, the 3,4,5-trimethoxyphenyl group which is characteristic for colchicine, combretastatin A-4, and podophyllotoxin, the structural requirements for activity had to be elaborated by a systematic variation of the substituents in both aromatic rings. Since the first investigations showed that these substituents should be lipophilic in nature, alkyl groups of variable length were introduced. Halogen atoms and the trifluoromethyl group completed the spectrum of substituents. The 1-position of the five-membered ring was kept unsubstituted because the N–H element of the indole is essential for the anti-proliferative activity.[19] All derivatives were tested for cytostatic activity. Since the interaction with the tubulin system usually results in an arrest of the cell cycle in the G_2/M-phase, the most potent compounds were submitted to a FACS analysis to record the cell cycle distribution. A small number of derivatives were studied for their inhibitory effects on tubulin polymerization to confirm previous findings. A representative example was used to investigate morphological changes of the cells by confocal laser microscopy.

2. Results and discussion

2.1. Chemistry

For the systematic variation of the substitution pattern in both aromatic rings a versatile route of synthesis was required. An appropriate procedure for the synthesis of the substituted 2-phenylindoles **11** and **12** was the Bischler method which involved the condensation of a substituted aniline **9** with an α-bromophenacyl derivative **10** (Fig. 2). Subsequently, the formyl group was introduced in position 3 of the indole by a Vilsmeier reaction with DMF and $POCl_3$ to give the 2-phenylindole-3-carbaldehydes **5** and **6**. The reaction of the aldehydes with methylamine and hydroxylamine, respectively, afforded the corresponding imines **7** and **8**.

2.2. Anti-proliferative activity

All of the compounds synthesized were first evaluated for cytostatic activity using hormone-independent human MDA-MB 231 breast cancer cells in a microplate assay. Based on previous investigations with methoxy-substituted 2-phenylindole-3-carbaldehydes, first, a series of compounds with a methoxy group in the *para*-position of the phenyl ring and a variety of lipophilic substituents in positions 5 and/or 6 of the indole including halogens and alkyl groups of variable length were tested. Vincristine was used as reference drug in most of the assays. All 2-(4-methoxyphenyl)indole derivatives strongly inhibited the growth of MDA-MB 231 cells with IC_{50} values below 1 μM (Table 1). The presence of fluorine in the indole moiety gave only rise to a positive effect when located in 6-position (**5c**). The cytostatic potency is strongly influenced by the length and the structure of the alkyl substituent in 5-position. It increases with the length up to 5 carbon atoms and decreases with ramification as demonstrated for the butyl derivatives **5j–l**. The lowest IC_{50} values (5.5–7.4 nM) were found for the *n*-butyl (**5j**), *n*-pentyl (**5m**), and *n*-hexyl (**5n**) derivatives. These values are close to the one of vincristine (4.5 nM).

Subsequently the substituent in the phenyl ring was modified (Table 2). Shifting of the methoxy group to the *meta*-position (**6a**) or introduction of an additional methoxy (**6b**) or hydroxyl (**6c**) group into this position strongly reduced the activity. A similar decrease in activity was observed when methoxy was replaced by a *n*-butyl (**6i**) or a fluoro (**6j**) substituent. Replacement of the 4-methoxy group by smaller groups such as methyl, ethyl or trifluoromethyl had only a minor effect on the potency. The lowest IC_{50} value (7.8 nM) was recorded for the 4-methyl derivative **6e**. The comparison of derivatives with *n*-butyl and ethyl as substituents (**6h** and **6i**) revealed that the indole moiety tolerates larger lipophilic substituents than the phenyl ring.

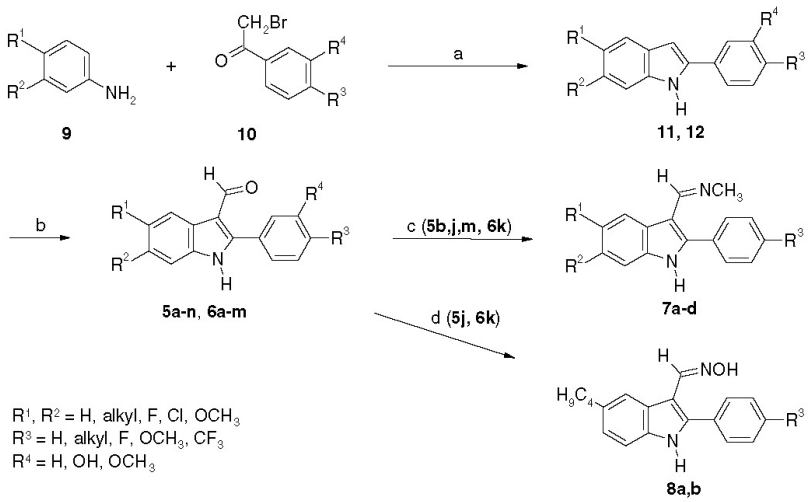
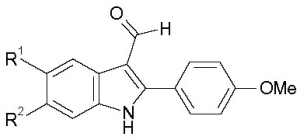


Figure 2. Syntheses of 2-phenylindole-3-carbaldehydes **5** and **6**, and of derivatives **7** and **8**. Reagents and conditions: (a) xylene, *N*,*N*-dimethylaniline, 170 °C; (b) $POCl_3$, DMF, 15 → 70 °C, 3 h; (c) $MeNH_2$, EtOH, 40 °C, 16 h; (d) $H_3NOH^+Cl^-$, H_2O/EtOH, NaOAc, reflux, 3 h.

Table 1. Anti-proliferative activities of 2-(4-methoxyphenyl)indole-3-carbaldehydes **5a n** on human MDA-MB 231 and MCF-7 breast cancer cells



Compound	R^1	R^2	MDA-MB 231[a] IC_{50} (nM)	MCF-7[b] IC_{50} (nM)
5a	OMe	H	260	180
5b	H	OMe	35	160
5c	H	F	59	43
5d	F	H	540	240
5e	H	Cl	27	65
5f	Me	Cl	26	62
5g	Me	H	86	140
5h	Pr	H	20	54
5i	*i*-Pr	H	29	97
5j	*n*-Bu	H	6.7	22
5k	*sec*-Bu	H	72	180
5l	*t*-Bu	H	280	580
5m	*n*-Pent	H	5.5	20
5n	*n*-Hex	H	7.4	6.0
Vincristine			4.5	n.d.[c]

[a] Inhibition of cell growth determined after incubation for 4 days and subsequent crystal violet staining of viable cells. Mean values of two-independent experiments with 16 24 replicates, SD are less than 20%.
[b] Analogous experiment as described for MDA-MB 231 cells with one exception: the incubation period was 5 days.
[c] Not determined.

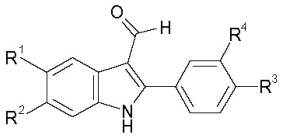
Additional modifications concerned the aldehyde function. Conversion to the methylamines (**7a–d**) did not affect the activity in comparison to the free aldehyde, whereas the formation of oximes (**8a** and **8b**) reduced the anti-proliferative activity by one order of magnitude (Table 3). This difference in bioactivity can be rationalized by the higher rate of hydrolysis of the methyl imines in comparison to the oximes. Hydrolysis experiments with **7b** and **8a** followed by HPLC analysis revealed that **7b** undergoes significant hydrolysis to the aldehyde **5j** at 37 °C, whereas **8a** remains stable under these conditions.

All 2-phenylindole derivatives were also tested for anti-proliferative activity in MCF-7 breast cancer cells (Tables 1–3). Though similar activities were observed in both cell lines a tendency to higher IC_{50} values in MCF-7 cells was noticed especially for the two oximes **8a** and **8b** (Table 3).

2.3. Cell cycle arrest in G_2/M-phase

Previous studies with 2-phenylindole-3-carbaldehydes suggested that these aldehydes exert their cytotoxic effects via tubulin as the intracellular target. Both the inhibition of tubulin polymerization and stabilization of microtubules can lead to an arrest of the cell cycle in the G_2/M-phase. Thus, a selection of representative derivatives including aldehydes, methyl imines, and oximes was examined for their effects on the cell cycle progression with MDA-MB 231 cells. The cell cycle-dependent DNA content was determined by flow cytometry using propidium iodide in permeabilized cells. All compounds were tested in different concentrations and compared with vincristine as reference drug. Figure 3 shows the typical decrease of the peak for cells in the G_1/G_0 phase and the parallel increase of the number of cells in G_2/M phase for compound **5n**. This derivative and vincristine are rather similar in potency. Only at the lowest concentration (10 nM) the vinca alkaloid proved to be somewhat more active (Fig. 4a).

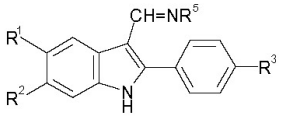
Table 2. Anti-proliferative activities of 2-phenylindole-3-carbaldehydes **6a m** on human MDA-MB 231 and MCF-7 breast cancer cells



Compound	R^1	R^2	R^3	R^4	MDA-MB 231[a] IC_{50} (nM)	MCF-7[b] IC_{50} (nM)
5b	H	OMe	OMe	H	35	160
6a	H	OMe	H	OMe	1030	390
6b	H	OMe	OMe	OMe	270	20
6c	H	OMe	OMe	OH	800	1650
6d	H	OMe	Me	H	31	100
6e	H	Cl	Me	H	7.8	37
6f	Me	H	Me	H	48	165
6g	*n*-Bu	H	Me	H	34	54
6h	*n*-Bu	H	Et	H	27	58
6i	Et	H	*n*-Bu	H	300	200
6j	*n*-Bu	H	F	H	350	200
6k	*n*-Bu	H	CF_3	H	33	66
6l	*n*-Pent	H	CF_3	H	42	67
6m	*n*-Hex	H	CF_3	H	43	22
Vincristine					4.5	n.d.[c]

[a] Inhibition of cell growth determined after incubation for 4 days and subsequent crystal violet staining of viable cells. Mean values of two-independent experiments with 16 24 replicates, SD are less than 20%.
[b] Analogous experiment as described for MDA-MB 231 cells with one exception: the incubation period was 5 days.
[c] Not determined.

Table 3. Anti-proliferative activities of 3-iminomethyl-2-phenylindoles **7** and **8** on human MDA-MB 231 and MCF-7 breast cancer cells



Compound	R^1	R^2	R^3	R^5	MDA-MB 231[a] IC_{50} (nM)	MCF-7[b] IC_{50} (nM)
7a	H	OMe	OMe	Me	34	220
7b	*n*-Bu	H	OMe	Me	6	27
7c	*n*-Pent	H	OMe	Me	6	21
7d	*n*-Bu	H	CF_3	Me	32	140
8a	*n*-Bu	H	OMe	OH	40	212
8b	*n*-Bu	H	CF_3	OH	497	1660

[a] Inhibition of cell growth determined after incubation for 4 days and subsequent crystal violet staining of viable cells. Mean values of two-independent experiments with 16 24 replicates, SD are less than 20%.
[b] Analogous experiment as described for MDA-MB 231 cells with one exception: the incubation period was 5 days.

All indole derivatives tested were able to block the cell cycle in G_2/M phase (Figs. 4 and 5). Generally, this blockade was accompanied by the appearance of a significant sub-G_1 peak ('debris') probably due to apoptotic processes. The cell cycle arrest occurs at concentrations which were similar to those observed for the inhibition of cell growth except for the oxime **8a** which required a 10-fold higher concentration (Fig. 5b).

2.4. Inhibition of tubulin polymerization

A rational explanation for the cell cycle arrest is the inhibition of tubulin polymerization as it is also observed for colchicine, combretastatin A-4, and many other antimitotic agents. In order to prove this assumption two aldehydes **5j** and **6k**, the imine **7b**, and the oxime **8a** were tested in a microplate assay to obtain preliminary data on their interaction with tubulin. The progression of tubulin polymerization was measured turbidimetrically at 350 nm over 28 min after the temperature had been raised from 2 to 37 °C. At a concentration of 5 μM, the aldehyde **6k** and the oxime were inactive, whereas **5j** exerted a minor inhibition (23%) of tubulin polymerization. However, the imine **7b**, derived from the aldehyde **5j**, strongly inhibited tubulin polymerization. From the curves recorded for various concentrations (Fig. 6) an IC_{50} value of 1.2 μM was calculated which was lower than that of colchicine (5 μM) in this assay.

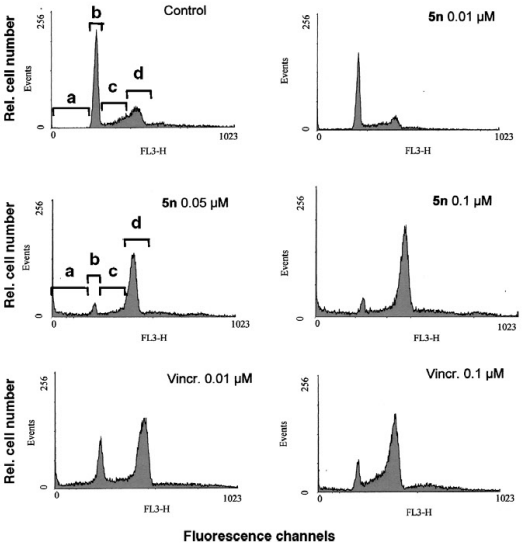


Figure 3. Flow cytometry analysis of cell cycle. MDA-MB 231 breast cancer cells were exposed to compound **5n** and vincristine in various concentrations for 24 h. The DNA content was quantified by the standard propidium iodide procedure, as described in Section 4. Cells are assigned to those in G_0/G_1- (label b), S- (label c), and G_2/M-phase (label d), and to sub-G_1 cells (label a), respectively, according to their DNA content.

A similar assay was performed with a tubulin preparation from calf brains using temperature-controlled cuvettes instead of microplates. This assay is more suitable for the determination of IC_{50} values than the microplate assay because it works with larger volumes and with a better temperature control. However, it requires the multi-step isolation of tubulin from fresh calf brains prior to the experiment. In this assay, the aldehydes **5a–c** and the imine **7a** were tested for inhibitory activity on tubulin polymerization in various concentrations. All four compounds showed a dose-dependent inhibition of polymerization with IC_{50} values of 4.0 (**5a**), 1.5 (**5b**), and 1.8 μM (**5c** and **7a**) (Fig. 7). The figures for the latter three compounds are similar to that of colchicine (1.9 μM). These concentrations are nearly two orders of magnitude higher than those observed for the anti-proliferative effect and the blockade of cell cycle progression. However, this discrepancy in the effective concentrations is generally noticed for antimitotic agents such as combretastatin A-4 and related structures.[12,13,16,20]

2.5. Change of cell morphology

Another method to identify the intracellular target of the 2-phenylindole-3-carbaldehydes was the histological examination of cells by fluorescence microscopy. Confocal laser microscopy allows the simultaneous visualization of chromatin and microtubules and the analysis of their spatial arrangement. Untreated U-87 MG human glioma cells display the normal distribution of microtubules (Fig. 8, panel A). In dividing cells their functional role can be demonstrated (Fig. 8A, panels a–c). Treatment of cells with 10 nM vincristine gave rise to a disruption of the microtubule network and a loss of

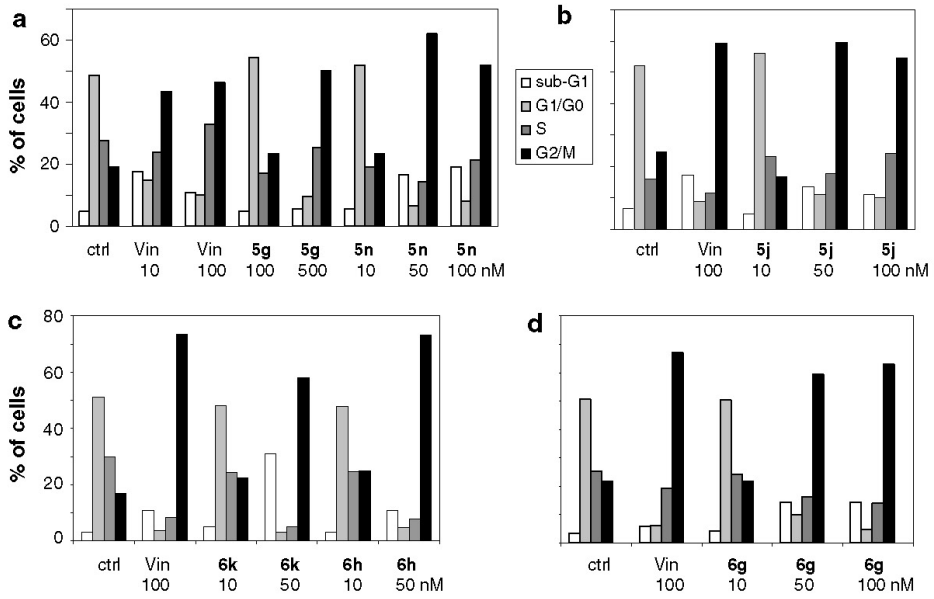


Figure 4. Cell cycle distribution of MDA-MB 231 cells treated for 24 h with 2-phenylindole-3-carbaldehydes **5 g**, **5n** (a), **5j** (b), **6k**, **6h** (c), and **6g** (d) in various concentrations. Vincristine (Vin) was used as reference drug. Percentages of sub-G_1 cells and cells in G_1/G_0-phase, S-phase, and G_2/M-phase are shown. Data refer to a representative experiment out of two.

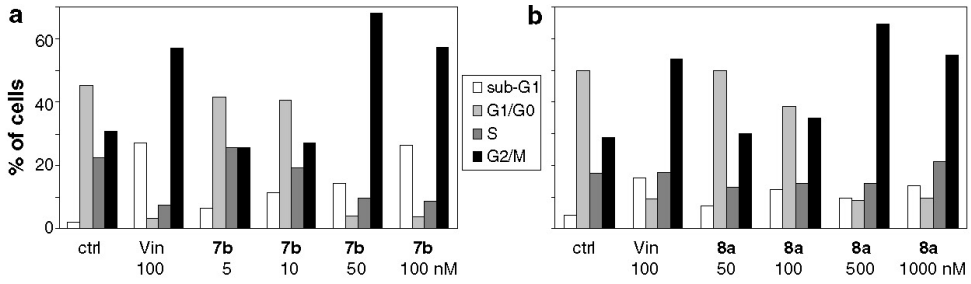


Figure 5. Cell cycle distribution of MDA-MB 231 cells treated for 24 h with the methylimine **7b** (a) and the oxime **8a** (b) in various concentrations. Vincristine (Vin) was used as reference drug. Percentages of sub-G_1 cells and cells in G_1/G_0-phase, S-phase, and G_2/M-phase are shown. Data refer to a representative experiment out of two.

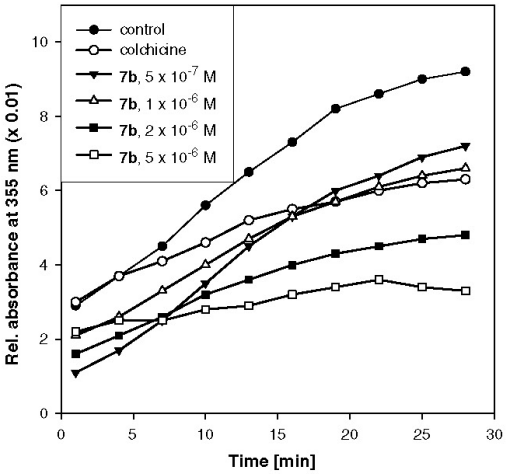


Figure 6. Effect of methylimine **7b** on tubulin assembly. Optical density at 355 nm was measured every minute over a period of 28 min simultaneously for all compounds and concentrations after temperature had been switched from 2 to 37 °C. Control wells contained tubulin and the solvent (0.1% DMSO). The concentration of colchicine was 5 μM.

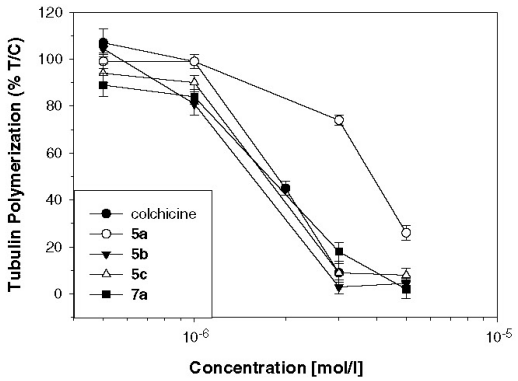


Figure 7. Inhibitory effects of various 2-phenylindole derivatives and of colchicine on the polymerization of calf brain tubulin. Assembly of microtubules was assessed turbidimetrically at 350 nm 20 min after the temperature had been switched from 2 to 37 °C. Polymerization in the absence of inhibitor gave the control readings. Values are means of three-independent experiments ±SEM.

its function in the mitotic process (Fig. 8, panel B). When the cells were treated with the aldehyde **5j** (50 nM) the microtubules condensed around the nucleus and were no longer capable of separating the chromosomes for mitosis (Fig. 8, panel C). The nucleus showed chromatin condensation and disintegration of the nuclear matrix probably due to an apoptotic cell death (Fig. 8C, panel b).[21]

2.6. Discussion

The aim of this study was to identify the most favorable substitution pattern of the 2-phenylindole-3-carbaldehydes for antimitotic activity. Since hydrophilic substituents such as hydroxy functions have been shown to reduce the antitumor effect dramatically[19] only lipophilic groups were considered. Preliminary investigations have shown that substituents in positions 1, 4, and 7 of the indole and 3 in the phenyl ring had a detrimental effect on the anti-proliferative activity. Thus, only substituents in 5- and 6-position of the indole and in the *para*-position of the phenyl ring were modified. The most favorable substitution pattern comprises an alkyl chain of 4 to 6 carbon atoms and a methoxy or a methyl group in the phenyl ring. Though a number of synthetic indole-based inhibitors of tubulin polymerization are known (Fig. 1)[7] the structure of the 2-phenylindole-3-carbaldehydes is unique because it possesses an aldehyde function and lacks the 3,4,5-trimethoxyphenyl ring which is typical for the majority of indole-based inhibitors of tubulin polymerization.[11–13,15,16,22,23] Other indoles with a carbonyl function are the 2-aroylindoles.[14]

Though the 2-phenylindole-3-carbaldehydes can be considered as stilbene analogues they differ from combretastatin A-4 by the trans arrangement of the phenyl rings. This may explain the lack of cytotoxicity of the 4,5,6-trimethoxy-2-(4-methoxyphenyl)indole-3-carbal-

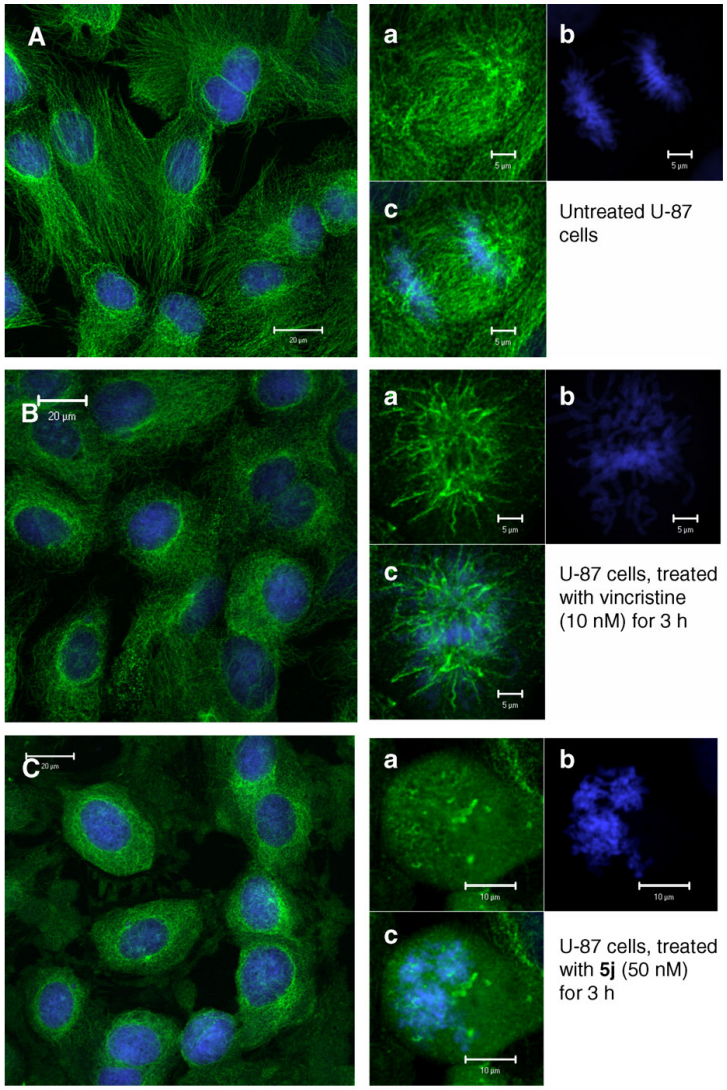


Figure 8. Effects of vincristine and 2-phenylindole-3-carbaldehyde **5j** on the organization of cellular microtubule network and nuclear structure of U-87 MG human glioblastoma cells, visualized by confocal laser fluorescence microscopy. (Panel A) Untreated control cells which undergo normal mitoses as shown in panels a, b, and c (a, stained tubulin; b, stained nucleus; c, merged images). (Panel B) Vincristine (10 nM) leads to a disturbed and partially degraded tubulin network that has lost its function (a, b, c). (Panel C) Indole **5j** (50 nM) causes a condensation of microtubules around the nucleus and a loss of function (a, b, c). Some of the nuclei desintegrate upon treatment (b).

dehyde which is furnished with the 3,4,5-trimethoxyphenyl group (data not shown). Also the low activity found with **6c** which possesses the same substitution pattern as that of the second phenyl ring of combretastatin A-4 is in agreement with these considerations. The results of this and other studies suggest that the colchicine binding site tolerates a large variety of different chemical structures as long as they contain two aromatic moieties with appropriate substituents.[2,3]

From the data presented the primary site of action appears to be the α/β-tubulin heterodimer whose polymerization is strongly inhibited by the aldehydes tested. This action results in the destruction of the microtubule network and abrogates its function. This action can be visualized by fluorescence microscopy as demonstrated for the aldehyde **5j**. Already at a concentration of 50 nM the microtubules condense around the nucleus and the filament structure is lost. At higher concentra-

tions most of the microtubules have disappeared.[19] This change is accompanied by a structural alteration of the nucleus which desintegrates probably due to apoptotic processes.

Interesting results were obtained from the comparison of the aldehydes with the derivatives that possess an imine structure. In the cytotoxicity assays all of the methyl imines showed activities identical to those of the corresponding aldehydes which can be rationalized by the hydrolysis of the imines. The hydrolytic conversion of the imine to the parent aldehyde was confirmed by HPLC analysis. A significant difference was observed when experiments such as the inhibition of tubulin polymerization last only for a short period of time. The imine **7b** proved to be much more potent as inhibitor of tubulin polymerization than the corresponding aldehyde **5j**. This difference may be due to a higher binding affinity of the imine for tubulin compared to the aldehyde, but this assumption has not yet been proved. The oximes which are shown to be resistant to hydrolysis at 37 °C are less active than the corresponding aldehydes by one order of magnitude. This difference is reflected by their inactivity in the tubulin polymerization assay (data not shown).

3. Conclusion

This study revealed that the 2-phenylindole-3-carbaldehydes are an interesting class of compounds with high anti-proliferative activity in two breast cancer cell lines. We were able to show that tubulin is the primary target of these agents which inhibit the polymerization of tubulin to functional microtubules by binding to the colchicine binding site. This interaction with tubulin leads to cell cycle arrest in the G_2/M phase and probably leads to an apoptotic cell death. The in vitro potencies of some of the aldehydes are in same range as those of vincristine and combretastatin A-4. Preliminary investigations on the in vivo activity, however, showed that these aldehydes do not inhibit the growth of transplanted murine tumors. One of the possible reasons might be the instability of the aldehyde function toward metabolic reactions. Insufficient bioavailability could be another reason. In order to overcome this problem we are going to modify the carbonyl function to improve the metabolic stability of this essential structural element. Two of these modifications, conversion of the aldehydes to methyl imines and oximes, respectively, are included in this study. Though both types of imines are active, their mode of action seems to be different. Results from other modifications will be reported in due time.

4. Experimental

4.1. General methods

Melting points were determined on a Büchi 510 apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AC-250 spectrometer with TMS as internal standard and were in accord with the assigned structures. Mass spectra (PI-EI MS) were measured on a Varian MAT-311A spectrometer at 70 eV. Purity of all compounds was checked by TLC. Elemental analyses of crystalline compounds were performed by the Mikroanalytisches Laboratorium, University of Regensburg. The syntheses of 5-methoxy-2-(4-methoxyphenyl)indole-3-carbaldehyde (**5a**), 6-methoxy-2-(4-methoxyphenyl)indole-3-carbaldehyde (**5b**), and 6-fluoro-2-(4-methoxyphenyl)-indole-3-carbaldehyde (**5c**) have been described previously.[19]

4.2. Preparation of 2-phenylindoles 11 and 12

A solution of 4′(3′)-substituted 2-bromoacetophenone (26 mmol) in xylene (150 mL) was added dropwise to a solution of the substituted aniline (66 mmol) in *N*,*N*-dimethylaniline (15 mL) at 170 °C over a period of 1 h. The reaction mixture was stirred at this temperature for 3–24 h. After cooling, the dark brown solution was poured into 400 mL of a 2 N HCl/EtOAc mixture (1:1). The aqueous phase was extracted twice with EtOAc (200 mL). The organic phase was washed with sat. NaCl solution and dried over Na_2SO_4. The solvent was removed under vacuum and the product was purified by column chromatography (SiO_2) with different mobile phases on the basis of dichloromethane and subsequent crystallization.

4.2.1. 5-Fluoro-2-(4-methoxyphenyl)indole (11d). Colorless crystals (21% yield), mp 215 °C. ^{1}H NMR (DMSO-d_6) δ 3.83 (s, 3H, –OCH_3); 6.81 (d, ^4J = 2.0 Hz, 1H, indole-H^3); 6.88 (dd, ^4J = 2.2 Hz, ^4J = 2.0 Hz, 1H, indole-H^4); 7.03, 7.77 (AA′BB′, ^3J = 8.8 Hz, 4H, phenyl-H); 7.23 (d, ^3J = 8.0 Hz, 1H, indole-H^7); 7.33 (dd, ^3J = 8.0 Hz, ^4J = 2.2 Hz, 1H, indole-H^6); 11.50 (s, 1H, N–H). Anal. for $C_{15}H_{12}FNO$; calcd C, 74.68; H, 5.01; N, 5.81; found C, 74.39; H, 5.08; N, 5.77.

4.2.2. 6-Chloro-2-(4-methoxyphenyl)indole (11e). Greenish solid (71% yield), mp 198 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, –OCH_3); 6.64 (s, 1H, indole-H^3); 7.07, 7.49 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.25 (dd, ^3J = 8 Hz, ^4J = 2 Hz, 1H, indole-H^5); 7.49 (d, ^4J = 2 Hz, 1H, indole-H^7); 7.59 (d, ^3J = 8 Hz, 1H, indole-H^4); 8.32 (s, br, 1H, N–H). Anal. for $C_{15}H_{12}ClNO$; calcd C, 69.91; H, 4.69; N, 5.46; found C, 69.12; H, 4.48; N, 5.54.

4.2.3. 6-Chloro-2-(4-methoxyphenyl)-5-methylindole (11f). The preparation afforded two products, **11f** (22% yield) and isomeric 4-chloro-2-(4-methoxyphenyl)-5-methylindole (14% yield), which were separated by column chromatography. The main product (**11f**) was obtained as colorless solid, mp 198 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 2.37 (s, 3H, –CH_3); 3.81 (s, 3H, –OCH_3); 6.70 (s, 1H, indole-H^3); 7.03, 7.76 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.36 (s, 1H, indole-H^4); 7.43 (s, 1H, indole-H^7); 11.43 (s, 1H, N–H). Anal. for $C_{16}H_{14}ClNO$; calcd C, 70.72; H, 5.19; N, 5.15; found C, 70.49; H, 5.21; N, 5.10.

4.2.4. 2-(4-Methoxyphenyl)-5-methylindole (11g). Colorless crystals (30% yield), mp 237 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.46 (s, 3H, –CH_3); 3.88 (s, 3H, –OCH_3); 6.66 (s, 1H, indole-H^3); 7.00–7.61 (m, 3H, indole-

$H^{4,6,7}$); 7.00, 7.61 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 8.19 (s, br, 1H, N–H). Anal. for $C_{16}H_{15}NO$; calcd C, 80.98; H, 6.37; N, 5.90; found C, 80.46; H, 6.11; N, 5.80.

4.2.5. 2-(4-Methoxyphenyl)-5-*n*-propylindole (11h). Colorless crystals (20% yield), mp 195–196 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 0.96 (t, ^3J = 7 Hz, 3H, –CH_2–CH_3); 1.68 (sext, ^3J = 7 Hz, 3H, –CH_2–CH_2–CH_3); 2.67 (t, ^3J = 7 Hz, 3H, –CH_2–CH_2–); 3.84 (s, 3H, –OCH_3); 6.64 (d, ^4J = 1 Hz, 1H, indole-H^3); 6.94–7.01 (m, 1H, indole-H^6); 6.96, 7.26 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.26 (d, ^3J = 7 Hz, 1H, indole-H^7); 7.39 (s, 1H, indole-H^4); 8.13 (s, br, 1H, N–H). Anal. for $C_{18}H_{19}NO$; calcd C, 81.48; H, 7.22, N, 5.28; found C, 81.23; H, 7.32; N, 5.18.

4.2.6. 5-Isopropyl-2-(4-methoxyphenyl)indole (11i). Colorless crystals (14% yield), mp 203 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 1.24 (d, ^3J = 7 Hz, 6H, –CH–(CH_3)$_2$); 2.93 (sept, ^3J = 7 Hz, 1H, –CH–(CH_3)$_2$); 3.80 (s, 3H, –OCH_3); 6.68 (d, 1H, ^4J = 2 Hz, indole-H^3); 6.96 (dd, ^4J = 2 Hz, ^3J = 8 Hz, 1H, indole-H^6); 7.01, 7.76 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.26 (d, ^4J = 2 Hz, 1H, indole-H^4); 7.30 (d, ^3J = 8 Hz, 1H, indole-H^7); 11.26 (s, br, 1H, N–H). Anal. for $C_{19}H_{21}NO$; calcd C, 81.48; H, 7.22; N, 5.28; found C, 80.34; H, 7.07; N, 5.18.

4.2.7. 5-*n*-Butyl-2-(4-methoxyphenyl)indole (11j). Colorless crystals (76% yield), mp 228 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 0.94 (t, ^3J = 7 Hz, 3H, –CH_2–CH_3); 1.38 (sext, ^3J = 7 Hz, 2H, –CH_2–CH_2–CH_3); 1.65 (quin, ^3J = 7 Hz, 2H, –CH_2–CH_2–CH_2–); 2.70 (t, ^3J = 7 Hz, 2H, –CH_2–CH_2–); 3.85 (s, 3H, –OCH_3); 6.63 (d, 1H, ^4J = 2 Hz, indole-H^3), 6.94–7.01 (m, 1H, indole-H^6); 6.96, 7.57 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.26 (d, ^3J = 8 Hz, 1H, indole-H^7); 7.38 (s, 1H, indole-H^4); 8.14 (s, br, 1H, –NH). Anal. for $C_{19}H_{21}NO$; calcd C, 81.68; H, 7.58; N, 5.01; found C, 81.51; H, 7.61; N, 4.97.

4.2.8. 5-*sec*-Butyl-2-(4-methoxyphenyl)indole (11k). Colorless crystals (38% yield), mp 191 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 0.85 (t, ^3J = 7 Hz, 3H, –CH_2–CH_3); 1.29 (d, ^3J = 7 Hz, 3H, –CH–CH_3); 1.65 (quin, ^3J = 7 Hz, 2H, –CH_2–CH_3); 2.67 (t, ^3J = 7 Hz, 1H, –CH–CH_3); 3.85 (s, 3H, –OCH_3); 6.65 (s, 1H, indole-H^3); 6.95-7.02 (m, 1H, indole-H^6); 6.97, 7.57 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.30 (d, ^3J = 8 Hz, 1H, indole-H^7); 7.39 (s, 1H, indole-H^4); 8.15 (s, br, 1H, –NH). Anal. for $C_{19}H_{21}NO$; calcd C, 81.68; H, 7.58; N, 5.01; found C, 81.51; H, 7.60; N, 4.94.

4.2.9. 5-*tert*-Butyl-2-(4-methoxyphenyl)indole (11l). White crystals (36% yield), mp 224 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 1.24 (s, 9H, –$(CH_3)_3$); 3.80 (s, 3H, –OCH_3); 6.70 (d, ^4J = 2 Hz, 1H, indole-H^3); 7.02, 7.76 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.14 (dd, ^5J = 2 Hz, ^3J = 8 Hz, 1H, indole-H^6); 7.29 (d, ^3J = 8 Hz, 1H, indole-H^7); 7.45 (d, ^4J = 2 Hz, 1H, indole-H^4); 11.25 (s, 1H, –NH). Anal. for $C_{19}H_{21}NO$; calcd C, 81.68; H, 7.58; N, 5.01; found C, 81.31; H, 7.57; N, 4.91.

4.2.10. 2-(4-Methoxyphenyl)-5-*n*-pentylindole (11m). White solid (21% yield), mp 197–198 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 0.89 (t, ^3J = 7 Hz, 3H, –CH_2–CH_3); 1.34 (m, 2H, –CH_2–CH_2–CH_3); 1.66 (m, 2H, –CH_2–CH_2–CH_2–); 2.69 (t, ^3J = 7 Hz, 2H, –CH_2–CH_2–); 3.85 (s, 3H, –OCH_3); 6.64 (d, ^4J = 1 Hz, 1H, indole-H^3); 6.94–7.01 (m, 1H, indole-H^6); 6.96, 7.57 (AA′BB′, ^3J = 9 Hz, 4H, Phenyl-H); 7.26 (d, ^3J = 8 Hz, 1H, indole-H^7); 7.39 (s, 1H, indole-H^4); 8.13 (s, br, 1H, –NH). Anal. for $C_{20}H_{23}NO$; calcd C, 81.87; H, 7.90; N, 4.77; found C, 82.05; H, 7.79, N, 5.33.

4.2.11. 5-*n*-Hexyl-2-(4-methoxyphenyl)indole (11n). White solid (14% yield), mp 197–198 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 0.88 (t, ^3J = 7 Hz, 3H, –CH_2–CH_3); 1.32 (m, 2H, –CH_2–CH_2–CH_3); 1.65 (m, 2H, –CH_2–CH_2–CH_2–); 2.17 (m, 4H, –CH_2–(CH_2)$_2$–CH_2–); 2.71 (t, ^3J = 7 Hz, 2H, –CH_2–CH_2–); 3.85 (s, 3H, –OCH_3), 6.64 (d, ^5J = 1 Hz, 1H, indole-H^3); 6.94–7.01 (m, 1H, indole-H^6); 6.97, 7.57 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.27 (d, ^3J = 8 Hz, 1H, indole-H^7); 7.39 (s, 1H, indole-H^4); 8.20 (s, br, 1H, –NH). Anal. for $C_{21}H_{25}NO$; calcd C, 82.05; H, 8.20; N, 4.56; found C, 82.04; H, 7.85; N, 4.42.

4.2.12. 2-(3,4-Dimethoxyphenyl)-6-methoxyindole (12b). Colorless crystals (15% yield), mp 161 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H, –OCH_3); 3.92 (s, 3H, –OCH_3); 3.96 (s, 3H, –OCH_3); 6.65 (s, 1H, indole-H^3); 6.79 (dd, ^3J = 7 Hz, ^5J = 2 Hz, 1H, ArH); 6.88–7.15 (m, 4H, indole-$H^{4,5,7}$, ArH); 7.47 (d, ^3J = 7 Hz, 1H, ArH); 8.20 (s, br, 1H, N–H). Anal. for $C_{17}H_{17}NO_3$; calcd C, 72.07; H, 6.05; N, 4.94; found C, 71.84; H, 5.99; N, 4.90.

4.2.13. 2-(3-Hydroxy-4-methoxyphenyl)-6-methoxyindole (12c). The title compound was not isolated, but used immediately for the next step of synthesis.

4.2.14. 6-Methoxy-2-(4-methylphenyl)indole (12d). White solid (42% yield), mp 162 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H, –CH_3); 3.88 (s, 3H, –OCH_3); 6.72 (s, br, 1H, indole-H^3); 6.81 (dd, ^3J = 8 Hz, ^4J = 2 Hz, 1H, indole-H^5); 6.92 (s, 1H, indole-H^7); 7.26, 7.50 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.24–7.32 (m, 1H, indol-H^4), 8.24 (s, br, 1H, N–H); MS *m/z* (%) 238 (16, $[MH]^{+\cdot}$), 237 (90, $M^{+\cdot}$), 222 (100, $[M-{}^{\cdot}CH_3]^+$), 194 (12, $[M-{}^{\cdot+}CO-CH_3]^+$).

4.2.15. 6-Chloro-2-(4-methylphenyl)indole (12e). Orange solid (40% yield), mp 145 °C (EtOH). ^{1}H NMR (DMSO-d_6) δ 2.34 (s, 3H, –CH_3); 6.87 (d, ^4J = 1 Hz, 1H, indole-H^3); 7.00 (dd, ^3J = 8 Hz, ^4J = 2 Hz, 1H, indole-H^5); 7.28, 7.52 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.38 (d, ^4J = 2 Hz, 1H, indole-H^7); 7.51 (d, ^3J = 8 Hz, 1H, indole-H^4); 11.64 (s, 1H, N–H). Anal. for $C_{15}H_{12}ClN$; calcd C, 74.53; H, 5.00; N, 7.79; found C, 74.46; H, 5.02; N, 5.61.

4.2.16. 5-Methyl-2-(4-methylphenyl)indole (12f). White solid (36% yield), mp 227 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.38 (s, 3H, –CH_3); 2.44 (s, 3H, –CH_3); 6.69 (s, 1H, indole-H^3); 6.99 (dd, ^3J = 8 Hz, ^4J = 1 Hz, 1H, indole-H^6); 7.24, 7.53 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.20–7.27 (m, 2H, indole-$H^{4,7}$); 8.19 (s, br, 1H, N–H). Anal. for $C_{16}H_{15}N$; calcd C, 86.82; H, 6.83; N, 6.35; C, 85.94; H, 6.68; N, 6.21.

4.2.17. 5-Butyl-2-(4-methylphenyl)indole (12g). Orange powder (15% yield), mp 181–182 °C. ^{1}H NMR ($CDCl_3$) δ 0.94 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.39 (m, 2H, –CH_2–CH_2–CH_3); 1.65 (m, 2H, –CH_2–CH_2–CH_2–); 2.37 (s, 3H, –CH_3); 2.70 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 6.70 (dd, 1H, ^4J = 2 Hz, ^5J = 1 Hz, indole-H^4); 7.01 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.22 (m, 3H, indole-H^7 and phenyl-H); 7.40 (s, 1H, indole-H^3); 7.51 (d, 2H, ^3J = 8 Hz, phenyl-H); 8.16 (s, br, 1H, N–H). Anal. for $C_{19}H_{21}N$; calcd C, 86.64; H, 8.04; N, 5.32; found C, 86.31; H, 7.65; N, 5.34.

4.2.18. 5-Butyl-2-(4-ethylphenyl)indole (12h). Yellow powder (18% yield), mp 167–168 °C. ^{1}H NMR ($CDCl_3$) δ 0.93 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.26 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.38 (m, 2H, –CH_2–CH_2–CH_3); 1.65 (m, 2H, –CH_2–CH_2–CH_2–); 2.68 (m, 4H, –CH_2–CH_3 and –CH_2–CH_2–); 6.71 (d, 1H, ^4J = 2 Hz, indole-H^4); 7.01 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.27 (m, 3H, phenyl-H and indole-H^7); 7.40 (s, 1H, indole-H^3); 7.57 (d, 2H, ^3J = 8 Hz, phenyl-H); 8.21 (s, br, 1H, N–H). Anal. for $C_{20}H_{23}N$; calcd C, 86.59; H, 8.36; N, 5.05; found C, 86.55; H, 8.07; N, 5.03.

4.2.19. 2-(4-Butylphenyl)-5-ethylindole (12i). Orange powder (20% yield), mp 183–184 °C. ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.21 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.32 (m, 2H, –CH_2–CH_2–CH_3); 1.58 (m, 2H, –CH_2–CH_2–CH_2); 2.63 (m, 4H, –CH_2–CH_3 and –CH_2–CH_2–); 6.74 (d, 1H, ^4J = 2 Hz, indole-H^4); 7.04 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.31 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.43 (s, 1H, indole-H^3); 7.56, 7.24 (AA′BB′, 4H, ^3J = 8 Hz, phenyl-H); 8.22 (s, br, 1H, N–H). Anal. For $C_{20}H_{23}N$; calcd C, 86.59; H, 8.36; N, 5.05; found C, 86.10; H, 8.33; N, 4.93.

4.2.20. 5-Butyl-2-(4-fluorophenyl)indole (12j). Orange powder (48% yield), mp 140–142 °C. ^{1}H NMR ($CDCl_3$) δ 0.94 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.45 (m, 2H, –CH_2–CH_2–CH_3); 1.65 (m, 2H, –CH_2–CH_2–CH_2); 2.70 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 6.69 (1, 1H, indole-H^4); 7.03 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.29 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.41 (s, 1H, indole-H^3); 7.65, 7.13 (m, 4H, phenyl-H); 8.16 (s, br, 1H, –NH–). Anal. for $C_{18}H_{18}FN$; calcd C, 80.96; H, 6.78; N, 5.24; found C, 80.12; H, 6.65; N, 5.11.

4.2.21. 5-Butyl-2-[4-(trifluoromethyl)phenyl]indole (12k). Yellow powder (31% yield), mp 120–121 °C. ^{1}H NMR ($CDCl_3$) δ 0.94 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.38 (m, 2H, –CH_2–CH_2–CH_3); 1.70 (m, 2H, –CH_2–CH_2–CH_2–); 2.71 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 6.85 (d, 1H, ^4J = 2 Hz, indole-H^4); 7.07 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.33 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.43 (s, 1H, indole-H^3); 7.74, 7.67 (AA′BB′, 4H, ^3J = 8 Hz, phenyl-H); 8.29 (s, br, 1H, N–H). Anal. for $C_{19}H_{18}F_3N$; calcd C, 71.91; H, 5.72; N, 4.41; found C, 71.56; H, 5.85; N, 4.15.

4.2.22. 5-Pentyl-2-[4-(trifluoromethyl)phenyl]indole (12l). Orange powder (32% yield). The compound was directly used in the following reaction step. ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.34 (m, 4H, –CH_2–(CH_2)$_2$–CH_3); 1.66 (m, 2H, –CH_2–CH_2–CH_2–); 2.70 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 6.68 (d, 1H, ^4J = 2 Hz, indole-H^4); 7.07 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.32 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.43 (s, 1H, indole-H^3); 7.74, 7.68 (AA′BB′, 4H, ^3J = 8 Hz, phenyl-H); 8.29 (s, br, 1H, N–H).

4.2.23. 5-Hexyl-2-[4-(trifluoromethyl)phenyl]indole (12m). Orange powder (31% yield). The compound was directly used in the following reaction step. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.36 (m, 4H, –CH_2–(CH_2)$_2$–CH_3); 1.65 (m, 4H, –CH_2–(CH_2)$_2$–CH_2–); 2.70 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 6.87 (d, 1H, ^4J = 2 Hz, indole-H^4); 7.07 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.33 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.43 (s, 1H, indole-H^3); 7.74, 7.67 (AA′BB′, 4H, ^3J = 8 Hz, phenyl-H); 8.30 (s, br, 1H, N–H).

4.3. Preparation of 2-phenylindole-3-carbaldehydes 5 and 6

Under nitrogen, $POCl_3$ (10 mmol) was added dropwise to DMF (10 mmol) at 15 °C. The mixture was stirred for 15 min, then the solution of the respective 2-phenylindole (1 mmol) in DMF (50 mL) was added dropwise to the mixture, which was then heated to 70 °C. After 3 h of heating, the reaction mixture was diluted with ice water (200 mL), neutralized with 40% NaOH, and extracted with chloroform. The chloroform extract was washed with water and dried over $NaSO_4$. The solvent was removed under vacuum. Crystallization of the residue from methanol gave the pure product.

4.3.1. 5-Fluoro-2-(4-methoxyphenyl)indole-3-carbaldehyde (5d). Beige crystals (85% yield), mp 250 °C. ^{1}H NMR (DMSO-d_6) δ 3.86 (s, 3H, –OCH_3); 7.05–7.13 (m, 1H, indole-H); 7.15, 7.72 (AA′BB′, ^3J = 8.7 Hz, 4H, phenyl-H); 7.44–7.89 (m, 1H, indole-H); 7.81–7.89 (m, 1H, indole-H); 9.92 (s, 1H, –CHO); 12.39 (s, 1H, N–H). Anal. for $C_{16}H_{12}FNO$; calcd C, 71.37; H, 4.49; N, 5.20; found C, 71.05; H, 4.39; N, 5.08.

4.3.2. 6-Chloro-2-(4-methoxyphenyl)indole-3-carbaldehyde (5e). Gray solid (28% yield), mp 260 °C. ^{1}H NMR (DMSO-d_6) δ 3.86 (s, 3H, –OCH_3); 7.16, 7.73 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.25 (dd, ^3J = 7 Hz, ^4J = 2 Hz, 1H, indole-H^5); 7.49 (d, ^4J = 2 Hz, 1H, indole-H^7); 8.16 (d, ^3J = 7 Hz, 1H, indole-H^4); 9.95 (s, 1H, –CHO); 12.40 (s, 1H, N–H). Anal. for $C_{16}H_{12}ClNO_2$: calcd C, 67.26; H, 4.23; N, 4.90; found C, 67.48; H, 4.44; N, 4.76.

4.3.3. 6-Chloro-2-(4-methoxyphenyl)-5-methylindole-3-carbaldehyde (5f). Gray solid (28% yield), mp 287 °C. ^{1}H NMR (DMSO-d_6) δ 2.43 (s, 3H, –CH_3); 3.33 (s, 3H, –OCH_3); 7.16, 7.73 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.49 (s, 1H, indole-H^4); 8.14 (s, 1H, indole-H^7); 9.92 (s, 1H, –CHO); 12.31 (s, br, 1H, N–H). Anal. for $C_{17}H_{14}ClNO_2$; calcd C, 68.12; H, 4.71; N, 4.67; found C, 67.82; H, 4.85; N, 4.68.

4.3.4. 2-(4-Methoxyphenyl)-5-methylindole-3-carbaldehyde (5g). Gray solid (79% yield), mp 242 °C. ^{1}H NMR (DMSO-d_6) δ 2.42 (s, 3H, –CH_3); 3.86 (s, 3H, –OCH_3);

7.09 (dd, ^3J = 9 Hz, ^4J = 1 Hz, 1H, indole-H^6); 7.15, 7.71 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.36 (d, ^3J = 8 Hz, 1H, indole-H^7); 8.01 (s, 1H, indole-H^4); 9.92 (s, 1H, –CHO); 12.17 (s, br, 1H, N–H). Anal. for $C_{17}H_{15}NO_2$; calcd C, 76.96; H, 5.70; N, 5.28; found C, 76.57; H, 5.65; N, 5.17.

4.3.5. 2-(4-Methoxyphenyl)-5-*n*-propylindole-3-carbaldehyde (5h). Colorless solid (35% yield), mp 209 °C. ^{1}H NMR (DMSO-d_6) δ 0.91 (t, ^3J = 7 Hz, 3H, –CH$_2$–C*H*$_3$); 1.63 (sext, ^3J = 7 Hz, 2 H, –CH$_2$–C*H*$_2$–CH$_3$); 2.67 (t, ^3J = 7 Hz, 3H, –C*H*$_2$–CH$_2$–); 3.86 (s, 3H, –OCH$_3$), 7.10 (dd, ^4J = 2 Hz, ^3J = 8 Hz, 1H, indole-H^6), 7.15 und 7.70 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H), 7.38 (d, ^3J = 8 Hz, 1H, indole-H^7), 8.01 (d, ^4J = 2 Hz, 1H, indole-H^4), 9.93 (s, 1H, –CHO), 12.17 (s, 1H, N–H). Anal. for $C_{19}H_{19}NO_2$; calcd C, 77.96; H, 6.53; N, 4.77; found C, 77.55; H, 6.47; N, 4.80.

4.3.6. 5-Isopropyl-2-(4-methoxyphenyl)indole-3-carbaldehyde (5i). Colorless solid (54% yield), mp 217 °C. ^{1}H NMR (DMSO-d_6) δ 1.25 (d, ^3J = 7 Hz, 6H, –CH–(C*H*$_3$)$_2$); 2.99 (sept, ^3J = 7 Hz, 1H, –C*H*–(CH$_3$)$_2$); 3.85 (s, 3H, –OCH$_3$); 7.15, 7.69 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.16 (d, ^3J = 8 Hz, 1H, indole-H^6); 7.38 (d, ^3J = 8 Hz, 1H, indole-H^7); 8.05 (s, 1H, indole-H^4); 9.92 (s, 1H, –CHO); 12.16 (s, 1H, N–H). Anal. for $C_{19}H_{19}NO_2$; calcd C, 77.96; H, 6.53; N, 4.77; found C, 77.57; H, 6.45; N, 4.81.

4.3.7. 5-*n*-Butyl-2-(4-methoxyphenyl)indole-3-carbaldehyde (5j). Orange crystals (44% yield), mp 210 °C. ^{1}H NMR (DMSO-d_6) δ 0.92 (t, ^3J = 7 Hz, 3H, –CH$_2$–C*H*$_3$); 1.34 (sext, ^3J = 7 Hz, 2H, –CH$_2$–C*H*$_2$–CH$_3$); 1.63 (quin, ^3J = 7 Hz, 2H, –CH$_2$–C*H*$_2$–CH$_2$); 2.73 (t, ^3J = 7 Hz, 2H, –C*H*$_2$–CH$_2$–); 3.89 (s, 3H, –OCH$_3$); 7.22-7.24 (m, 1H, indole-H^6); 7.21, 7.67 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.22–7.24 (m, 1H, indole-H^6); 7.52 (d, ^3J = 8 Hz, 1H, indole-H^7); 7.57 (s, 1H, indole-H^4), 8.71 (s, 1H, –CHO); 13.19 (s, 1H, N–H). Anal. for $C_{20}H_{21}NO_2$; calcd C, 78.15; H, 6.89; N, 4.56; found C, 78.08; H, 6.83; N, 4.66.

4.3.8. 5-*sec*-Butyl-2-(4-methoxyphenyl)indole-3-carbaldehyde (5k). Slightly violet solid (68% yield), mp 179 °C. ^{1}H NMR (DMSO-d_6) δ 0.80 (t, ^3J = 7 Hz, 3H, –CH$_2$–C*H*$_3$); 1.25 (d, ^3J = 7 Hz, 3H, –CH–C*H*$_3$); 1.61 (quin, ^3J = 7 Hz, 2H, –C*H*$_2$–CH$_3$); 2.69 (t, ^3J = 7 Hz, 1H, –C*H*–CH$_3$); 3.86 (s, 3H, –OCH$_3$); 7.10–7.17 (m, 1H, indole-H^6), 7.15, 7.70 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.39 (d, ^3J = 8 Hz, 1H, indole-H^7); 8.02 (s, 1H, indole-H^4); 9.92 (s, 1H, –CHO); 12.14 (s, 1H, N–H). Anal. for $C_{20}H_{21}NO_2$; calcd C, 78.15; H, 6.89; N, 4.56; found C, 77.81; H, 6.86; N, 4.51.

4.3.9. 5-*tert*-Butyl-2-(4-methoxyphenyl)indole-3-carbaldehyde (5l). Colorless solid (67% yield), mp 223 °C. ^{1}H NMR (DMSO-d_6) δ 1.36 (s, 9H, –CH$_3$); 3.86 (s, 3H, –OCH$_3$); 7.15, 7.69 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.36–7.42 (m, 2H, indole-H^{6,7}); 8.23 (s, 1H, indole-H^4); 9.93 (s, 1H, –CHO); 12.13 (s, 1H, N–H); MS: *m*/*z* (%) = 307 (61, M $^{\cdot+}$), 292 (100, [M−$^{\cdot}$CH$_3$]$^+$).

4.3.10. 2-(4-Methoxyphenyl)-5-*n*-pentylindole-3-carbaldehyde (5m). Orange crystals (60% yield), mp 182 °C. ^{1}H NMR (DMSO-d_6) δ 0.87 (t, ^3J = 7 Hz, 3H, –CH$_2$–C*H*$_3$); 1.30 (m, 4H, –CH$_2$–(C*H*$_2$)$_2$–CH$_3$); 1.62 (m, 2H, –CH$_2$–C*H*$_2$–CH$_2$); 2.68 (t, ^3J = 7 Hz, 2H, –C*H*$_2$–CH$_2$–); 3.86 (s, 3H, –OCH$_3$); 7.10 (dd, ^3J = 8 Hz, ^4J = 2 Hz, 1H, indole-H^6); 7.16, 7.70 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.38 (d, ^3J = 8 Hz, 1H, indole-H^7); 8.00 (s, 1H, indole-H^4); 9.92 (s, 1H, –CHO); 12.18 (s, 1H, N–H); Anal. for $C_{21}H_{23}NO_2$; calcd C, 78.47; H, 7.21; N, 4.36; found C, 77.97; H, 6.70; N, 4.25.

4.3.11. 5-*n*-Hexyl-2-(4-methoxyphenyl)indole-3-carbaldehyde (5n). Orange crystals (60% yield), mp 176 °C. ^{1}H NMR (DMSO-d_6) δ 0.88 (t, ^3J = 7 Hz, 3H, –CH$_2$–C*H*$_3$); 1.29 (m, 6H, –CH$_2$–(C*H*$_2$)$_3$–CH$_3$); 1.61 (m, 2H, –CH$_2$–C*H*$_2$–CH$_2$–); 2.68 (t, ^3J = 7 Hz, 2H, –C*H*$_2$–CH$_2$–); 3.86 (s, 3H, –OCH$_3$); 7.10 (d, ^3J = 8 Hz, 1H, indole-H^6); 7.15, 7.70 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.37 (d, ^3J = 8 Hz, 1H, indole-H^7); 8.00 (s, 1H, indole-H^4); 9.92 (s, 1H, –CHO); 12.17 (s, 1H, N–H). Anal. for $C_{22}H_{25}NO_2$; calcd C, 78.77; H, 7.51; N, 4.18; found C, 77.95; H, 7.57; N, 4.25.

4.3.12. 6-Methoxy-2-(3-methoxyphenyl)indole-3-carbaldehyde (6a). White crystals (80% yield), mp 190 °C. ^{1}H NMR (DMSO-d_6) δ 3.80 (s, 3H, –OCH$_3$); 3.85 (s, 3H, –OCH$_3$); 6.80-8.10 (m, 7H, ArH); 9.94 (s, 1H, –CHO); 12.19 (s, 1H, N–H). Anal. for $C_{17}H_{15}NO_3$; calcd C, 72.58; H, 5.37; N, 4.98; found C, 71.97; H, 5.60; N, 4.61.

4.3.13. 2-(3,4-Dimethoxyphenyl)-6-methoxyindole-3-carbaldehyde (6b). Yellow crystals (55% yield), mp 210 °C. ^{1}H NMR (DMSO-d_6) δ 3.81 (s, 3H, –OCH$_3$); 3.85 (s, 3H, –OCH$_3$); 3.87 (s, 3H, –OCH$_3$); 6.87 (dd, ^3J = 9 Hz, ^4J = 2 Hz, 1H, phenyl-H^6); 6.95 (d, ^4J = 2 Hz, 1H, indole-H^7); 7.15 (d, ^3J = 8 Hz, 1H, indole-H^5); 7.28 (d, ^4J = 2 Hz, 1H, phenyl-H^2); 7.27-7.32 (m, 1H, phenyl-H^5); 8.05 (d, ^3J = 8 Hz, 1H, indole-H^4); 9.95 (s, 1H, –CHO); 12.06 (s, br, 1H, N–H). Anal. for $C_{18}H_{17}NO_4$; calcd C, 69.44; H, 5.50; N, 4.50; found C, 69.23; H, 5.58; N, 4.22.

4.3.14. 2-(3-Hydroxy-4-methoxyphenyl)-6-methoxyindole-3-carbaldehyde (6c). Colorless crystals (57% yield), mp 253 °C. ^{1}H NMR (DMSO-d_6) δ 3.81 (s, 3H, –OCH$_3$); 3.88 (s, 3H, –OCH$_3$); 6.85 (dd, ^3J = 9 Hz, ^4J = 2 Hz, 1H, indole-H^5); 6.96–6.97 (m, 2H, phenyl-H^{2,5}); 7.16 (dd, ^3J = 8 Hz, ^4J = 2 Hz, 1H, phenyl-H^6); 7.28 (dd, ^4J = 2 Hz, 1H, indole-H^7); 8.03 (d, ^3J = 9 Hz, 1H, indole-H^4); 9.54 (s, 1H, –OH); 9.93 (s, 1H, –CHO); 11.99 (s, 1H, N–H). Anal. for $C_{17}H_{15}NO_4$; calcd C, 68.68; H, 5.08, N, 4.71; found C, 68.53; H, 5.02; N, 4.65.

4.3.15. 6-Methoxy-2-(4-methylphenyl)indole-3-carbaldehyde (6d). Colorless crystals (60% yield), mp 250 °C. ^{1}H NMR (DMSO-d_6) δ 2.42 (s, 3H, –CH$_3$); 3.81 (s, 3H, –OCH$_3$); 6.87 (dd, ^3J = 8 Hz, ^4J = 2 Hz, 1H, indole-H^5); 6.95 (d, ^4J = 2 Hz, 1H, indole-H^7); 7.40, 7.63 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 8.05 (d, ^3J = 8 Hz, 1H, indole-H^4); 9.91 (s, 1H, –CHO); 12.17 (s, 1H, N–H). Anal. for $C_{17}H_{15}NO_2$; calcd C, 76.96; H, 5.70; N, 5.28; found C, 76.24; H, 5.89; N, 4.91.

4.3.16. 6-Chloro-2-(4-methylphenyl)indole-3-carbaldehyde (6e). Colorless crystals (57% yield), mp 268 °C.

^{1}H NMR (DMSO-d_6) δ 2.42 (s, 3H, –CH_3); 7.26 (dd, ^3J = 8 Hz, ^4J = 2 Hz, 1H, indole-H^5); 7.42, 7.68 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.50 (d, ^4J = 2 Hz, 1H, indole-H^7); 8.18 (d, ^3J = 9 Hz, 1H, indole-H^4); 9.96 (s, 1H, –CHO); 12.46 (s, 1H, N–H). Anal. for $C_{16}H_{12}ClNO$; calcd C, 71.25; H, 4.48; N, 5.19; found C, 71.45; H, 4.63; N, 4.86.

4.3.17. 5-Methyl-2-(4-methylphenyl)indole-3-carbaldehyde (6f). Colorless crystals (48% yield), mp 226 °C. ^{1}H NMR (DMSO-d_6) δ 2.41 (s, 3H, –CH_3); 2.43 (s, 3H, –CH_3); 7.10 (dd, ^3J = 8 Hz, ^4J = 1 Hz, 1H, indole-H^6); 7.40, 7.65 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 7.39 (d, ^3J = 8 Hz, 1H, indole-H^7); 8.02 (d, ^4J = 1 Hz, 1H, indole-H^4); 9.94 (s, 1H, –CHO); 12.20 (s, 1H, N–H). Anal. for $C_{17}H_{15}NO$; calcd C, 81.90; H, 6.06; N, 5.62; found C, 81.64; H, 5.95; N, 5.38.

4.3.18. 5-Butyl-2-(4-methylphenyl)indole-3-carbaldehyde (6g). Orange solid (68% yield), mp 175–178 °C. ^{1}H NMR (DMSO-d_6) δ 0.92 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.34 (m, 2H, –CH_2–CH_2–CH_3); 1.61 (m, 2H, –CH_2–CH_2–CH_2–); 2.41 (s, 3H, –CH_3); 2.68 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 7.13 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.41 (m, 3H, indole-H^7, phenyl-H); 7.66 (d, 2H, ^3J = 8 Hz, phenyl-H); 8.03 (s, 1H, indole-H^4); 9.94 (s, 1H, –CHO); 12.24 (s, br, 1H, N–H); MS: *m/z* (%) = 291 (67, $[M]^+O$); 248 (100, $[M]^+O$–OC_3H_7). Anal. for $C_{20}H_{21}NO$; calcd C, 82.44; H, 7.26; N, 4.81; found C, 81.22; H, 7.07; N, 4.12.

4.3.19. 5-Butyl-2-(4-ethylphenyl)indole-3-carbaldehyde (6h). Yellow solid (46% yield), mp 178–180 °C. ^{1}H NMR (DMSO-d_6) δ 0.91 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.30 (m, 5H, –CH_2–CH_3, –CH_2–CH_2–CH_3); 1.59 (m, 2H, –CH_2–CH_2–CH_2–); 2.70 (m, 4H, –CH_2–CH_3, –CH_2–CH_2–); 7.11 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.39 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.67, 7.43 (AA′BB′, 4H, ^3J = 8 Hz, phenyl-H); 8.01 (s, 1H, indole-H^4); 9.93 (s, 1H, –CHO); 12.22 (s, br, 1H, N–H); MS: *m/z* (%) = 305 (90, $[M]^+O$); 262 (100, $[M]^+O$–OC_3H_7). Anal. for $C_{21}H_{23}NO$; calcd C, 82.59; H, 7.59; N, 4.59; found C, 81.38; H, 7.43; N, 4.21.

4.3.20. 2-(4-Butylphenyl)-5-ethylindole-3-carbaldehyde (6i). Light brown solid (30% yield), mp 175–176 °C. ^{1}H NMR (DMSO-d_6) δ 0.91 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.23 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.35 (m, 2H, –CH_2–CH_2–CH_3); 1.61 (m, 2H, –CH_2–CH_2–CH_2–); 2.70 (m, 4H, –CH_2–CH_3, –CH_2–CH_2–); 7.17 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.40 (m, 3H, indole-H^7, phenyl-H); 7.66 (d, 2H, ^3J = 8 Hz, phenyl-H); 8.03 (s, 1H, indole-H^4); 9.94 (s, 1H, –CHO); 12.22 (s, br, 1H, N–H). Anal. for $C_{21}H_{23}NO$; calcd C, 82.59; H, 7.59; N, 4.59; found C, 81.55; 7.25; N, 4.16.

4.3.21. 5-Butyl-2-(4-fluorophenyl)indole-3-carbaldehyde (6j). Orange solid (40% yield), mp 192–194 °C. ^{1}H NMR (DMSO-d_6) δ 0.91 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.32 (m, 2H, –CH_2–CH_2–CH_3); 1.60 (m, 2H, –CH_2–CH_2–CH_2); 2.69 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 7.13 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.43 (m, 3H, indole-H7, phenyl-H); 7.82 (d, 2H, phenyl-H); 8.02 (s, 1H, indole-H^4); 9.87 (s, 1H, –CHO); 12.30 (s, br, 1H, N–H); MS: *m/z* (%) = 295 (64, $[M]^+O$); 252 (100, $[M]^+O$–OC_3H_7). Anal. for $C_{19}H_{18}FNO$; calcd C, 77.27; H, 6.14; N, 4.74; found C, 76.40; H, 6.10; N, 4.60.

4.3.22. 5-*n*-Butyl-2-[4-(trifluoromethyl)phenyl]indole-3-carbaldehyde (6k). Orange powder (45% yield), mp 230–231 °C. ^{1}H NMR (DMSO-d_6) δ 0.91 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.33 (m, 2H, –CH_2–CH_2–CH_3); 1.60 (m, 2H, –CH_2–CH_2–CH_2); 2.70 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 7.17 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.44 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.95, 8.00 (AA′BB′, 4H, ^3J = 8 Hz, phenyl-H); 8.05 (s, 1H, indole-H^4); 9.97 (s, 1H, –CHO); 12.48 (s, br, 1H, N–H). Anal. for $C_{20}H_{18}F_3NO$; calcd C, 69.55; H, 5.25; N, 4.05; found C, 69.14; H, 5.18; N, 4.09.

4.3.23. 5-Pentyl-2-[4-(trifluoromethyl)phenyl]indole-3-carbaldehyde (6l). Yellow powder (32% yield), mp 226–227 °C. ^{1}H NMR (DMSO-d_6) δ 0.88 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.31 (m, 4H, –CH_2–(CH_2)$_2$–CH_3); 1.64 (m, 2H, –CH_2–CH_2–CH_2–); 2.71 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 7.19 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.46 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.97, 8.02 (AA′BB′, 4H, ^3J = 8 Hz, phenyl-H); 8.07 (s, 1H, indole-H^4); 9.97 (s, 1H, –CHO); 12.51 (s, br, 1H, N–H). Anal. for $C_{21}H_{20}F_3NO$; calcd C, 70.18; H, 5.61; N, 3.89; found C, 70.00; H, 5.49; N, 3.62.

4.3.24. 5-Hexyl-2-[4-(trifluoromethyl)phenyl]indole-3-carbaldehyde (6m). Yellow solid (34% yield), mp 224–225 °C. ^{1}H NMR (DMSO-d_6) δ 0.85 (t, 3H, ^3J = 7 Hz, –CH_2–CH_3); 1.28 (m, 6H, –CH_2–(CH_2)$_3$–CH_3); 1.61 (m, 2H, –CH_2–CH_2–CH_2–); 2.69 (t, 2H, ^3J = 7 Hz, –CH_2–CH_2–); 7.16 (dd, 1H, ^3J = 8 Hz, ^4J = 2 Hz, indole-H^6); 7.44 (d, 1H, ^3J = 8 Hz, indole-H^7); 7.95, 8.00 (AA′BB′, 4H, ^3J = 8 Hz, phenyl-H); 8.04 (s, 1H, indole-H^4); 9.97 (s, 1H, –CHO); 12.48 (s, br, 1H, N–H). Anal. for $C_{22}H_{22}F_3NO$; calcd C, 70.76; H, 5.94; N, 3.75; found C, 70.98; H, 6.01; N, 3.51.

4.4. Preparation of the carbaldehyde methyl imines 7

To a solution of the 2-phenylindole-3-carbaldehyde **5** or **6** (2 mmol) in CH_2Cl_2 (100 mL) was added under nitrogen methylamine (0.01 mol) as 33% solution in EtOH. The mixture was stirred at 40 °C overnight. Then, H_2O (100 mL) was added and the aqueous mixture was extracted several times with 50-mL portions of $CHCl_3$. The combined organic layers were washed with H_2O and brine, and dried over Na_2SO_4. After evaporation of the solvent in vacuo, the residue was purified by crystallization from EtOH.

4.4.1. 6-Methoxy-2-(4-methoxyphenyl)-3-[(methylimino)-methyl]indole (7a). Yellow resin (85% yield). ^{1}H NMR (d_6-acetone) δ 3.35 (d, ^4J = 1 Hz, 3H, =N–CH_3); 3.73 (s, 3H, –OCH_3); 3.82 (s, 3H, –OCH_3); 6.70 (dd, ^3J = 9 Hz, ^4J = 2 Hz, 1H, indole-H^5); 6.86 (d, ^4J = 2 Hz, 1H, indole-H^7); 6.99, 7.49 (AA′BB′, ^3J = 9 Hz, 4H, phenyl-H); 8.24 (d, ^3J = 9 Hz, 1H, indole-H^4); 8.42 (q, ^4J = 1 Hz, 1H, –CH =N–CH_3).

4.4.2. 5-*n*-Butyl-2-(4-methoxyphenyl)-3-[(methylimino)-methyl]indole (7b). Colorless crystals (67% yield), mp 207–209 °C. ^{1}H NMR (DMSO-d_6) δ 0.91 (t, ^3J = 7.3 Hz, 3H, –CH_2 –CH_3); 1.33 (sext, ^3J = 7.4 Hz, 2H, –CH_2–CH_2–CH_3); 1.58 (quin, ^3J = 7.5 Hz, 2H, –CH_2–CH_2–CH_2–); 2.65 (t, ^3J = 7.6 Hz, 2H, –CH_2–CH_2–CH_2–); 3.40 (d, ^4J = 1.3 Hz, 3H, =N–CH_3); 3.84 (s, 3H, –OCH_3); 7.01 (dd, ^3J = 8.3 Hz, ^4J = 1.6 Hz, 1H, indole-H^6); 7.13, 7.55 (AA′BB′, ^3J = 8.8 Hz, 4H, phenyl-H); 7.29 (d, ^3J = 8.2 Hz, 1H, indole-H^7); 8.09 (d, ^4J = 0.9 Hz, 1H, indole-H^4); 8.43 (d, ^4J = 1.4 Hz, 1H, –CH =NCH_3); 11.56 (s, 1H, N–H). Anal. for $C_{21}H_{24}N_2O$; calcd C, 78.71; H, 7.55; N, 8.74; found C, 78.72; H, 7.55; N, 8.53.

4.4.3. 5-*n*-Pentyl-2-(4-methoxyphenyl)-indol-3-[(methylimino)methyl]indole (7c). Colorless crystals (77% yield), mp 203 °C. ^{1}H NMR (DMSO-d_6) δ 0.86 (t, ^3J = 6.8 Hz, 3H, –CH_2–CH_3); 1.29 (m, 4H, –CH_2–(CH_2)$_2$–CH_3); 1.59 (m, 2H, –CH_2–CH_2–CH_2); 2.64 (t, ^3J = 7.5 Hz, 2H, –CH_2–CH_2–); 3.40 (d, ^4J = 1.1 Hz, 3H, =N–CH_3); 3.84 (s, 3H, –OMe); 7.01 (dd, ^3J = 8.3 Hz, ^4J = 1.6 Hz, 1H, indole-H^6); 7.13, 7.55 (AA′BB′, ^3J = 8.7 Hz, 4H, phenyl-H); 7.29 (d, ^3J = 8.2 Hz, 1H, indole-H^7); 8.09 (s, 1H, indole-H^4); 8.43 (s, 1H, –CH =NCH_3); 11.56 (s, 1H, N–H). Anal. for $C_{22}H_{26}N_2O$; calcd C, 79.01; H, 7.84; N, 8.38; found C, 78.65; H, 7.51; N, 8.27.

4.4.4. 5-*n*-Butyl-2-[4-(trifluoromethyl)phenyl]-3-[(methylimino)methyl]indole (7d). Colorless solid (63% yield), mp 186 °C. ^{1}H NMR (DMSO-d_6) δ 0.91 (t, ^3J = 7.3 Hz, 3H, –CH_2–CH_3); 1.33 (sext, ^3J = 7.4 Hz, 2H, –CH_2–CH_2–CH_3);1.59 (quin, ^3J = 7.5 Hz, 2H, –CH_2–CH_2–CH_2–); 2.67 (t, ^3J = 7.6 Hz, 2H, –CH_2–CH_2–), 3.43 (d, ^4J = 1.2 Hz, 3H, =N–CH_3); 7.08 (dd, ^3J = 8.3 Hz, ^4J = 1.6 Hz, 1H, indole-H^6); 7.36 (d, ^3J = 8.3 Hz, 1H, indole-H^7); 7.85, 7.93 (AA′BB′, ^3J = 8.3 Hz, 4H, phenyl-H); 8.14 (s, 1H, indole-H^4); 8.49 (s, 1H, –CH =NCH_3); 11.85 (s, 1H, N–H). Anal. for $C_{21}H_{21}F_3N_2$; calcd C, 70.37; H, 5.91; N, 7.82; found C, 70.17; H, 5.69; N, 7.04.

4.5. Preparation of the oximes 8

To a solution of hydroxylammonium chloride (1.2 mmol) in H_2O (8 mL), a solution of the 2-phenylindole-3-carbaldehyde (1 mmol) in EtOH (8 mL) and NaOAc (1.6 mmol) were added. The mixture was heated under reflux for 3 h and stirred at room temperature overnight. After repeated extraction with CH_2Cl_2 the combined organic layers were washed with water and dried (Na_2SO_4). The solvent was removed in vacuo and the residue recrystallized from EtOH.

4.5.1. 5-*n*-Butyl-3-[(hydroxyimino)methyl]-2-(4-methoxyphenyl)indole (8a). Colorless crystals (67% yield), mp 138 °C. ^{1}H NMR (DMSO-d_6) δ 0.90 (t, ^3J = 7.3 Hz, 3H, –CH_2–CH_3); 1.32 (sext, ^3J = 7.3 Hz, 2H, –CH_2–CH_2–CH_3); 1.58 (quin, ^3J = 7.5 Hz, 2H, –CH_2–CH_2–CH_2–); 2.65 (t, ^3J = 7.5 Hz, 2H, –CH_2–CH_2–); 3.84 (s, 3H, –OCH_3); 7.02 (dd, ^3J = 8.3 Hz, ^4J = 1.5 Hz, 1H, indole-H^6); 7.13, 7.51 (AA′BB′, ^3J = 8.7 Hz, 4H, phenyl-H); 7.30 (d, ^3J = 8.2 Hz, 1H, indole-H^7); 7.88 (s, 1H, indole-H^4); 8.22 (s, 1H, –CH=N–); 10.63 (s, 1H, –OH), 11.53 (s, 1H, N–H). Anal. for $C_{20}H_{22}N_2O_2$; calcd C, 74.51; H, 6.88; N, 8.69; found C, 74.67; H, 6.84; N, 8.56.

4.5.2. 5-*n*-Butyl-3-[(hydroxyimino)methyl]-2-[4-(trifluoromethyl)phenyl]indole (8b). Colorless crystals (86% yield), mp 166 °C. ^{1}H NMR (DMSO-d_6) δ 0.91 (t, ^3J = 7.3 Hz, 3H, –CH_2–CH_3); 1.33 (sext, ^3J = 7.4 Hz, 2H, –CH_2–CH_2–CH_3); 1.59 (quin, ^3J = 7.5 Hz, 2H, –CH_2–CH_2–CH_2–); 2.67 (t, ^3J = 7.5 Hz, 2H, –CH_2–CH_2–); 7.10 (dd, ^3J = 8.3 Hz, ^4J = 1.6 Hz, 1H, indole-H^6); 7.37 (d, ^3J = 8.3 Hz, 1H, indole-H^7); 7.81, 7.92 (AA′BB′, ^3J = 7.9 Hz, 4H, phenyl-H); 7.94 (s, 1H, indole-H^4); 8.29 (s, 1H, –CH=N–); 10.84 (s, 1H, –OH); 11.81 (s, 1H, N–H). Anal. for $C_{20}H_{19}F_3N_2O$; calcd C, 66.66; H, 5.31; N, 7.77; found C, 66.71; H, 5.32; N, 7.71.

4.6. Materials and reagents for bioassays

Drugs and biochemicals were obtained from Sigma (Deisenhofen, Germany). The microplate-based tubulin polymerization assay (Cytoskeleton®) was purchased from Tebu-bio (Offenbach, Germany). MDA-MB 231 and MCF-7 breast cancer cells were obtained from the American Type Culture collection (ATCC, Rockville, MD, USA). Buffer solutions used: PEM: 0.1 M Pipes–NaOH, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.6–6.7; PEMG: 0.1 M Pipes–NaOH, 0.8 M monosodium L-glutamate, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.6–6.7; PBS: 8.0 g/L NaCl, 0.2 g/L KCl, 1.0 g/L $Na_2HPO_4 \times H_2O$, 0.2 g/L KH_2PO_4.

4.7. Determination of anti-proliferative activity

Hormone-independent human MDA-MB 231 breast cancer cells were grown in McCoy-5a medium, supplemented with L-glutamine (73 mg/L), gentamycin sulfate (50 mg/L), $NaHCO_3$ (2.2 g/L), and 5% sterilized fetal calf serum (FCS). At the start of the experiment, the cell suspension was transferred to 96-well microplates (100 µL/well). After the cells grew for 4 days in a humidified incubator with 5% CO_2 at 37 °C, medium was replaced by one containing the test compounds (200 µL/well). Control wells (16/plate) contained 0.1% of DMF that was used for the preparation of stock solutions. Initial cell density was determined by addition of glutaric dialdehyde (1% in PBS; 100 µL/well) instead of test compound. After incubation for about 4 days, medium was removed and 100 µL of glutaric dialdehyde in PBS (1%) was added for fixation. After 15 min, the solution of aldehyde was decanted. Cells were stained by treating them for 25 min with 100 µL of an aqueous solution of crystal violet (0.02%). After decanting, cells were washed several times with water to remove adherent dye. After addition of 100 µL of EtOH (70%) plates were gently shaken for 2 h. Optical density of each well was measured in a microplate autoreader EL 309 (Biotek) at 578 nm.

For hormone-sensitive MCF-7 human breast cancer cells a similar procedure to that described for MDA-MB 231 cells was applied with modifications: Cells were grown in EMEM supplemented with sodium pyruvate

(110 mg/L), gentamycin sulfate (50 mg/L), $NaHCO_3$ (2.2 g/L), phenol red, and 10% FCS for 5 days.

4.8. Flow cytometry

MDA-MB 231 cells were grown to 70–80% confluence on the bottom of a 75 cm^2 culture flask. Test substances were dissolved in DMF and diluted to the required concentrations. The content of DMF in medium and in controls after 1000 times dilution was set to 0.1%. For each concentration one culture flask was used. Cells were exposed to test substances for 24 h. This incubation time was necessary to obtain a sufficient number of cells (5×10^6 cells). After incubation cells were trypsinized, centrifuged (250*g*) in an excess of serum containing medium for 10 min, and washed with PBS.

The pellets of cells were resuspended in 1 mL of PBS and 9 mL of 70% EtOH (ice-cold). EtOH and PBS were removed by centrifugation and the cells were washed with PBS. After the addition of 0.5 mL of PBS and 0.5 mL of DNA extraction buffer the cells were transferred into Eppendorf cups (2 mL), incubated for 5 min at room temperature, and centrifuged again. The cells were stained with 1 mL of propidium iodide solution for at least 30 min at room temperature and then analyzed by flow cytometry using a FACS-Calibur™. For analysis, data files for four parameters were collected for 8000–15,000 events from each sample. The flow rate was adjusted to ca. 300 cells/s for sufficient accuracy. Data were analyzed by the WinMDI 2.8 software.

4.9. Tubulin polymerization assay on microplates

This assay was performed according to the vendor's protocol. Stock solutions of test compounds in DMSO were diluted 1:10 with G-PEM buffer. Ten microliters of this solution was added to the corresponding well of a microplate which was warmed in a microplate reader (Tecan) to 37 °C. Ice-cold tubulin was suspended in cold G-PEM buffer + glycerol (5%) and depolymerized at 0 °C within 1 min. Within 30 s, 100 μL of this solution was added to the warm solutions of the test compounds on the microplate and the plate returned to the reader. Absorbance at 355 nm was recorded at 37 °C for 30 min. Reference drugs were taxoterre and colchicine; control wells contained only the solvent (1%).

4.10. Tubulin polymerization assay in cuvettes

4.10.1. Isolation and purification of calf brain tubulin. The cortex of one or two fresh calf brains in ice-cold PEM buffer (1 mL/g tissue, +16 mg DTE/100 mL buffer solution) was homogenized in portions. After centrifugation (90 min; 20,000*g*) at 2–4 °C, the supernatant was carefully decanted. The concentrations of GTP and ATP were adjusted to 0.1 and 2.5 mM, respectively. After stirring gently at 37 °C for 30 min the solution was transferred to centrifugation tubes and carefully underlayered with a pre-warmed (37 °C) sucrose solution (10% in PEM buffer solution containing 1 mM GTP, approx 10% of the transferred volume). After centrifugation at 37 °C for 45 min (20,000*g*) the pellets were weighed and suspended in ice-cold PEM buffer solution (3 mL/g) and homogenized in a Teflon-in-glass potter. After standing in ice for 30 min, the suspension was centrifuged at 2 °C for 30 min (40,000*g*). The supernatant was separated and adjusted to 1 mM GTP. By incubation at 37 °C for 15 min tubulin was polymerized once again. After centrifugation at 37 °C for 30 min microtubules were obtained as shiny gel-like pellet. The yields ranged from 2 to 6 g per brain. Aliquots were frozen in liquid nitrogen and stored at −70 °C. Purity was checked by polyacrylamide gel electrophoresis.

4.10.2. Temperature-induced tubulin polymerization. The pellet of frozen microtubules was warmed to 37 °C in a water bath. After addition of the 20-fold volume of ice-cold PEMG buffer it was homogenized. Depolymerization was completed by keeping the mixture at 0 °C for 30 min, followed by centrifugation at 2 °C (30 min; 30,000*g*) to remove insoluble protein. Each reaction tube contained 0.46 mL of the supernatant and 20 μL of the DMSO solution of the test compound in varying concentrations. Reaction mixtures were preincubated at 37 °C for 15 min and chilled on ice followed by addition of 20 μL of a 25 mM GTP solution in PEMG buffer to each tube. Reaction mixtures were transferred to cuvettes of a UV spectrophotometer connected to two different temperature controller. First, the temperature inside the cuvettes was held at 2 °C. The cuvette holder was then switched to the second temperature controller at 37 °C and the absorption was measured over a period of 20 min at 350 nm. Absorption at the start of the reaction was used as baseline. Three-independent experiments were performed for the determination of IC_{50}-values. Each experiment had two control reaction mixtures; their mean value was defined as 100% and their turbidity readings were generally within 10% of each other.

4.11. Confocal laser scanning microscopy

U-87 MG cells were seeded into 8-well Lab-Tek Chamber Slides (Nunc, Wiesbaden, Germany). At 75% confluence the culture medium was replaced with medium containing vincristine (10 nM), 2-phenylindole-3-carbaldehyde **5j** (50 nM), or corresponding vehicle (EtOH). The cells were incubated at 37 °C for 3 h. After removal of the culture medium the cells were fixed with 4% paraformaldehyde solution in PBS for 20 min at room temperature. Thereafter each well was washed three times with PBS supplemented by 0.5% BSA. For permeabilization cells were incubated with PBS (+0.5% BSA) containing 1% Triton X-100 (Serva, Heidelberg, Germany) for 10 min at room temperature and washed three times with PBS (+0.5% BSA).

Nuclei and chromosomes were stained with SYTOX-Green® nucleic acid stain (Molecular Probes, Eugene, OR, USA). Microtubules were stained using mouse anti-human α-tubulin primary antibody (Dianova, Hamburg, Germany) and Cy5-conjugated goat anti-mouse secondary antibody (Molecular Probes). All

antibodies were used in a 1:200 dilution in PBS, containing 0.5% BSA. A Carl Zeiss Axiovert 200M LSM510 confocal laser scanning microscope was employed for acquisition of fluorescence images.

References and notes

1. von Angerer, E. *Curr. Opin. Drug Discovery Dev.* **2000**, *3*, 575–584.
2. Tron, G. C.; Pirali, T.; Sorba, G.; Pagliai, F.; Busacca, S.; Genazzani, A. A. *J. Med. Chem.* **2006**, *49*, 3033–3044.
3. von Angerer, E. *Exp. Opin. Ther. Patents* **1999**, *9*, 1069–1081.
4. Prinz, H.; Ishii, Y.; Hirano, T.; Stoiber, T.; Camacho Gomez, J. A.; Schmidt, P.; Dussmann, H.; Burger, A. M.; Prehn, J. H.; Gunther, E. G.; Unger, E.; Umezawa, K. *J. Med. Chem.* **2003**, *46*, 3382–3394.
5. Liou, J. P.; Chang, J. Y.; Chang, C. W.; Chang, C. Y.; Mahindroo, N.; Kuo, F. M.; Hsieh, H. P. *J. Med. Chem.* **2004**, *47*, 2897–2905.
6. Romagnoli, R.; Baraldi, P. G.; Pavani, M. G.; Tabrizi, M. A.; Preti, D.; Fruttarolo, F.; Piccagli, L.; Jung, M. K.; Hamel, E.; Borgatti, M.; Gambari, R. *J. Med. Chem.* **2006**, *49*, 3906–3915.
7. Brancale, A.; Silvestri, R. *Med. Res. Rev.* **2007**, *27*, 209–238.
8. De Martino, G.; Edler, M. C.; La Regina, G.; Coluccia, A.; Barbera, M. C.; Barrow, D.; Nicholson, R. I.; Chiosis, G.; Brancale, A.; Hamel, E.; Artico, M.; Silvestri, R. *J. Med. Chem.* **2006**, *49*, 947–954.
9. Dupeyre, G.; Chabot, G. G.; Thoret, S.; Cachet, X.; Seguin, J.; Guenard, D.; Tillequin, F.; Scherman, D.; Koch, M.; Michel, S. *Bioorg. Med. Chem.* **2006**, *14*, 4410–4426.
10. Mahboobi, S.; Sellmer, A.; Eichhorn, E.; Beckers, T.; Fiebig, H. H.; Kelter, G. *Eur. J. Med. Chem.* **2005**, *40*, 85–92.
11. Kuo, C. C.; Hsieh, H. P.; Pan, W. Y.; Chen, C. P.; Liou, J. P.; Lee, S. J.; Chang, Y. L.; Chen, L. T.; Chen, C. T.; Chang, J. Y. *Cancer Res.* **2004**, *64*, 4621–4628.
12. Liou, J. P.; Chang, Y. L.; Kuo, F. M.; Chang, C. W.; Tseng, H. Y.; Wang, C. C.; Yang, Y. N.; Chang, J. Y.; Lee, S. J.; Hsieh, H. P. *J. Med. Chem.* **2004**, *47*, 4247–4257.
13. Liou, J. P.; Mahindroo, N.; Chang, C. W.; Guo, F. M.; Lee, S. W.; Tan, U. K.; Yeh, T. K.; Kuo, C. C.; Chang, Y. W.; Lu, P. H.; Tung, Y. S.; Lin, K. T.; Chang, J. Y.; Hsieh, H. P. *ChemMedChem* **2006**, *1*, 1106–1118.
14. Mahboobi, S.; Pongratz, H.; Hufsky, H.; Hockemeyer, J.; Frieser, M.; Lyssenko, A.; Paper, D. H.; Bürgermeister, J.; Böhmer, F. D.; Fiebig, H. H.; Burger, A. M.; Baasner, S.; Beckers, T. *J. Med. Chem.* **2001**, *44*, 4535–4553.
15. Wang, L.; Woods, K. W.; Li, Q.; Barr, K. J.; McCroskey, R. W.; Hannick, S. M.; Gherke, L.; Credo, R. B.; Hui, Y. H.; Marsh, K.; Warner, R.; Lee, J. Y.; Zielinski-Mozng, N.; Frost, D.; Rosenberg, S. H.; Sham, H. L. *J. Med. Chem.* **2002**, *45*, 1697–1711.
16. Pandit, B.; Sun, Y.; Chen, P.; Sackett, D. L.; Hu, Z.; Rich, W.; Li, C.; Lewis, A.; Schaefer, K.; Li, P. K. *Bioorg. Med. Chem.* **2006**, *14*, 6492–6501.
17. Thorpe, P. E. *Clin. Cancer Res.* **2004**, *10*, 415–427.
18. Chaplin, D. J.; Horsman, M. R.; Siemann, D. W. *Curr. Opin. Investig. Drugs* **2006**, *7*, 522–528.
19. Gastpar, R.; Goldbrunner, M.; Marko, D.; von Angerer, E. *J. Med. Chem.* **1998**, *41*, 4965–4972.
20. Romagnoli, R.; Baraldi, P. G.; Remusat, V.; Carrion, M. D.; Cara, C. L.; Preti, D.; Fruttarolo, F.; Pavani, M. G.; Tabrizi, M. A.; Tolomeo, M.; Grimaudo, S.; Balzarini, J.; Jordan, M. A.; Hamel, E. *J. Med. Chem.* **2006**, *49*, 6425–6428.
21. Watanabe, J.; Natsume, T.; Fujio, N.; Miyasaka, K.; Kobayashi, M. *Apoptosis* **2000**, *5*, 345–353.
22. De Martino, G.; La Regina, G.; Coluccia, A.; Edler, M. C.; Barbera, M. C.; Brancale, A.; Wilcox, E.; Hamel, E.; Artico, M.; Silvestri, R. *J. Med. Chem.* **2004**, *47*, 6120–6123.
23. Flynn, B. L.; Hamel, E.; Jung, M. K. *J. Med. Chem.* **2002**, *45*, 2670–2673.