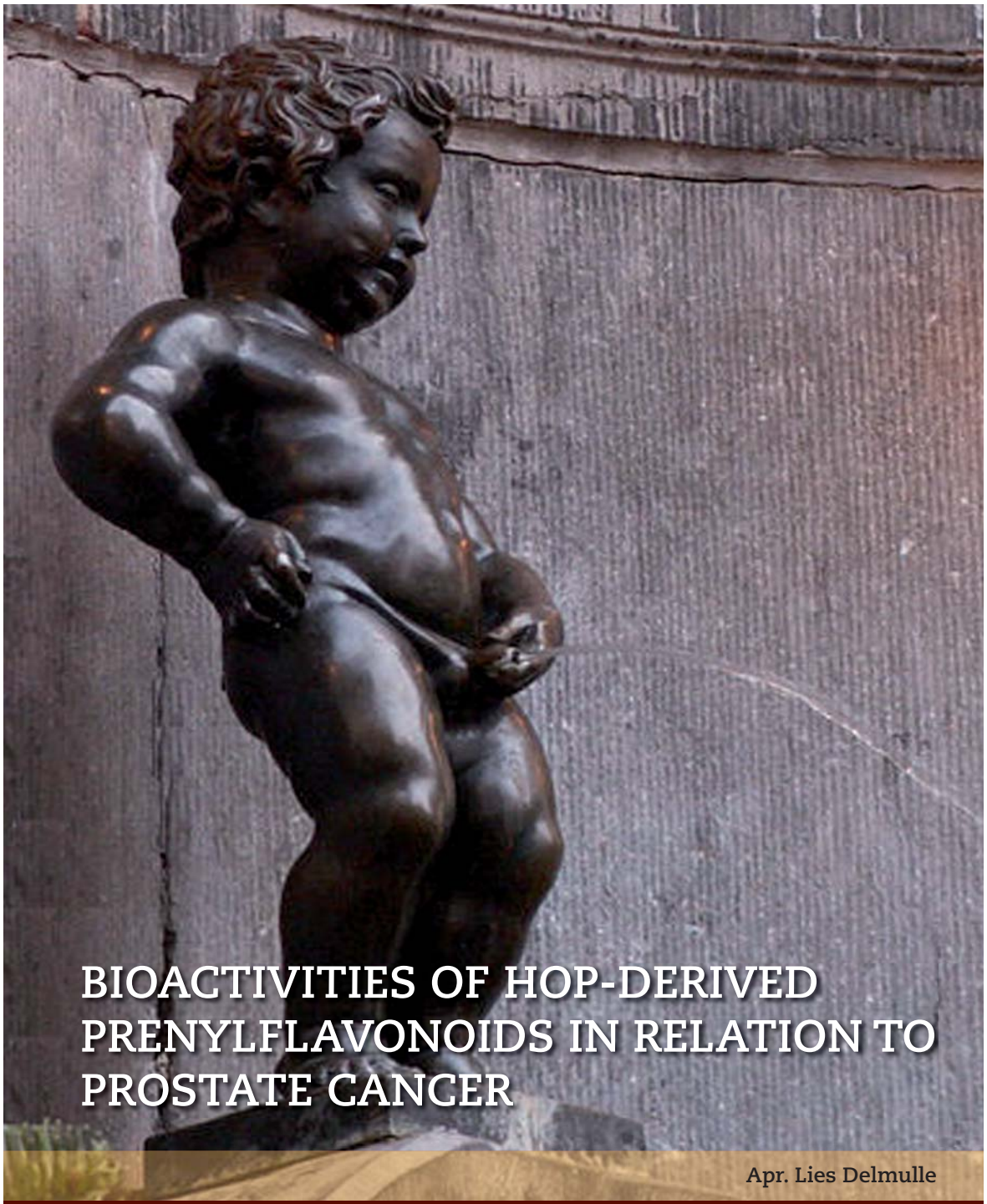

BIOACTIVITIES OF HOP-DERIVED PRENYLFLAVONOIDS IN RELATION TO PROSTATE CANCER

Apr. Lies Delmulle

UNIVERSITEIT GENT

FACULTEIT FARMACEUTISCHE WETENSCHAPPEN
Vakgroep Geneesmiddelenleer | Laboratorium voor Farmacognosie en Fytochemie

Thesis submitted to the Faculty of Pharmaceutical Sciences in order to obtain the degree of Doctor in Pharmaceutical Sciences

PROMOTOR: Prof. Dr. D. De Keukeleire

Academic year 2006-2007





ACKNOWLEDGEMENTS

This Ph.D. project would never have been succeeded without the help, the support, and the patience of a number of persons and, to some of them, I would like to express my sincere gratitude.

First and foremost, I would like to thank the promoter of my Ph.D. project, Prof. Dr. Denis De Keukeleire, for offering me unique research opportunities, for conveying his knowledge on hop and hop-derived prenylflavonoids, and, above all, for his confidence to let me work in a serene environment and in full scientific freedom.

This Ph.D. work would not have been succesful without the essential contribution of Prof. Dr. Em. F. Comhaire, who hosted me for more than a year in his laboratory at the Ghent University Hospital, Faculty of Medicine and Heath Sciences, Department of Endocrinology. The expertise present in this laboratory undoubtly improved my scientific skills and I would very much like to thank in this respect the colleagues, who assisted me personally during this period and shared their scientific knowledge: Willem, Frank, Sabrina and Katelijne.

During the execution of the experimental work, I was offered the opportunity to discover the exciting world of cell cultures thanks to Prof. Dr. V. Castronovo, who kindly invited me to work for almost a year in his Metastasis Research Laboratory at the University of Liège. I would like to thank particularly Akeila and Naïma, who assisted me on a daily basis and shared their broad experience on the *in vitro* bio-assays using cancer cell lines and on cancer research in general. Also, I am grateful to all members of the laboratory: Stephan, Marie-Hélène, Frédéric† (I am sorry to hear that you are no longer with us and herewith I would like to express my sincere condolences to the family), David, Cédric, Michaël, Sabine, Pascale who made my stay in Liège unforgettable! I would also like to thank the other members of the department for the pleasant atmosphere.

I would like to express my special gratitude to Prof. Dr. P. Vandenabeele from Ghent University, Faculty of Sciences, Flanders Interuniversity Institute for Biotechnology, Department for Molecular Biomedical Research, Molecular Signalling, and Cell Death Unit, who allowed me to work in his laboratory for several months to conduct studies on the

mechanisms of cell death. Dr. Tom Vanden Berghe assisted me during this research and shared all his expertise on apoptosis and necrosis. I acknowledge his help greatly and I add many thanks to the colleagues, who made that period very pleasant!

I am also very grateful to Prof. Dr. M. Bracke from Ghent University, Faculty of Medicine and Health Sciences, Department of Radiotherapy and Nuclear Medicine, for the scientific conversations and the help during the preparation of my IWT defences.

Thanks are due to Prof. Dr. J. Sumpter from Brunel University, UK, who kindly provided a yeast assay to assess estrogenic activity and to Dr. Wim Vanden Berghe from Ghent University, Faculty of Sciences, Department of Molecular Biology, Laboratory for Eucaryotic Gene Expression and Signal Transduction, who kindly delivered the program Clonewin to develop oligonucleotide primers.

Undoubtly, the close contacts with the colleagues of the Laboratory of Pharmacognosy and Phytochemistry at the Faculty of Pharmaceutical Sciences, Ghent University, were conducive to my scientific education and therefore I recognize the invaluable help of Dr. Arne Heyerick, Julie, Frederik, Kevin, Ulrik, Bram, Lieve, and Barbara. In particular, I would like to thank Ina for the nice moments we shared together.

I express my gratitude to IWT-Vlaanderen (Institute for the Promotion of Innovation by Science and Technology in Flanders, Brussels) for granting a pre-doctoral fellowship.

Last but not least, people in my personal environment must be recognized for their continuous support throughout my research and afterwards for their encouragements to finish the writing of my thesis. Mum and dad, thank you very much for everything! Nele, Alex, Stain&Stan, Yvon&Elvis&Canis, Ingrid&Fripoen, and especially Muriel, I would not have succeeded without you!! And of course I'm grateful to my phantastic loyal dog, Denka, for the warm huggs and long relaxing walks!

Orroir, September 2007.

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CONTENTS

ABBREVIATIONS

6PN	6-prenylnaringenin
8PN	8-prenylnaringenin
A	absorbance
Acetyl(Ac)-DEVD-AMC	7-amino-4-methylcoumarin, N-acetyl-L-aspartyl-Lglutamyl-L-valyl-l-aspartic acid amide
ACS	American Cancer Society
AFB1	aflatoxin B1
AFC	7-amino-4-trifluoromethylcoumarin
ALL	acute lymphoblastic leukaemia
AMC	7-amino-4-methylcoumarin
AR	androgen receptor
ARE	androgen response element
ATCC	American Type Culture Collection
BERKO	ERβ-knockout
bFGF	basic fibroblast growth factor
BLAST	basic logical alignment search tool
BPH	benign prostatic hypertrophy
CAM	chorioallantoic membrane
CAT scan or CT scan	computer-assisted tomography scan
CAU	corrected absorbance units
COX	cyclooxygenase
CPRG	chlorophenol-red-β-D-galactopyranoside
DCC-FBS	dextran-coated charcoal-stripped foetal bovine serum
DCC-FBS	dextran-coated charcoal-stripped foetal bovine serum
DCX	dehydrocycloxanthohumol
DGAT	diacylglycerol acyltransferase
DHT	5α-dihydrotestosterone
DMX	desmethylxanthohumol
DRE	digital rectal examination
DX	dehydrocycloxanthohumol

EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	estrogen receptor
ERE	estrogen response element
EROD	7-ethoxyresorufin O-deethylase
ERα	estrogen receptor α
ERβ	estrogen receptor β
E-screen	cell proliferation assay
FBS	foetal bovine serum
FBS	foetal bovine serum
FGF-II	fibroblast growth factor-II
FP	forward primer
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
HBME	human bone marrow endothelial cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hFOB	human fetal osteoblast cells
HPLC	high performance liquid chromatography
HRT	hormone replacement therapy
IGF	insuline growth factor
IGFBP-1	insulin-like growth factor binding protein 1
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
IX	isoxanthohumol
KGF	keratinocyte growth factor
LBD	ligand binding domain
LDL	low-density lipoprotein
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
LUTS	lower urinary tract symptoms
LUTS	lower urinary tract symptoms
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MulVRTase	murine leukemia virus reverse transcriptase
NCBI	national centre for biotechnological information
NEAA	non-essential amino acids
NMR	nuclear magnetic resonance
PARP	poly ADP ribosyl polymerase

PBS	phosphate buffered saline
PC	prostate cancer
PCR	polymerase chain reaction
PIN	prostatic intraepithelial neoplasia
PSA	prostate-specific antigen
QR	quinone reductase
RIE	relative induction efficiency
ROS	reactive oxygen species
RP	relative potency
RP	reversed primer
RT-PCR	reverse transcriptase polymerase chain reaction
SERM	selective estrogen-receptor modulator
SHBG	sex hormone-binding globulin
SHBG	sex hormone binding globulin
SRB	sulforhodamine B
T	testosterone
TBARS	thiobarbituric acid-reactive substances
TBE	tris boric acid EDTA
TBH	tert-butyl hydroperoxide
TCA	trichloroacetic acid
TF	tissue factor
TFs	tissue factors
TGF-β	transforming growth factor-β
TK	tyrosine kinase
TNF	tumor necrosis factor
TRUS	transrectal ultrasound
VEGF	vascular endothelial growth factor
VEGF	vascular endothelial growth factor
WST1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate
X	xanthohumol
XB	xanthohumol B
YES	yeast estrogen screen
zVAD-fmk	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone

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PURPOSE

Benign prostate hypertrophy (BPH) and prostate cancer (PC) are highly common among aging men causing bothersome symptoms or developing into life-threatening diseases. Both disorders become clinically manifest after 50 years of age, however, preliminary stages in their development can be recognized many years earlier. Aging is, therefore, a risk factor for the diseases. Disease of the prostate has generally been perceived as being associated with particular voiding problems considered by many men to be merely features of the aging process. The past decade has witnessed a major change in the attitude towards the various diseases of the prostate gland. The increasing incidence of BPH and PC has been related to the identification of a large number of prevalent, but previously undetected cases in the population through widespread screening using the prostate-specific antigen-test.[1] Consequently, the overall prognosis for prostate cancer patients has dramatically improved over the years. Unfortunately, whereas the treament and relief of BPH has noticeably progressed, the treatment of invasive PC has remained elusive. In an era of increasing life expectation, the prevention of diseases affecting the aging population has gained momentum and a lot of research is being carried out on the potential of chemopreventive agents present in the diet.

Epidemiological data show that the mortality risk associated with prostate cancer is much higher in European and American countries compared to Asian countries, even though the incidences are comparable. Migrants, who move from areas of low risk for particular cancers to areas where the risk is higher, have an increased risk for developing clinical PC and this suggests that environmental factors such as diet rather than genetical factors are involved. It has been recognized that certain constituents of the Asian diet and of the Mediterranean region protect against the development of PC and the lack of these constituents in the Western diet seems most important in the observed risk differences. Because diet seems associated with a lower mortality risk of PC in Asian people and because the consumption of soy is one of the most striking differences between Western and Eastern diets, an association with phytoestrogens that are present in signficant amounts in soy has been suggested.

Phytoestrogens are plant-derived compounds that possess weak estrogenic activities. In contrast to the notion that estrogens are essential in female hormonal regulations, the role of estrogens in the male neuro-endocrine system has only recently been accepted. The detection of the estrogen receptors ERα and ERβ in the male gonads and the prostate has been crucial in this respect. Phytoestrogens could influence hormone-dependent diseases including BPH and PC by their varying bioactivities such as roles in production, metabolization and biological activities of endogene hormones, the activities of intracellular enzymes, growth factors, protein synthesis, cell proliferation, differentiation and adhesion and processes such as angiogenesis.

In view of the prevention of the development and progression of benign and malign prostate disorders by altering dietary habits or by the intake of food supplements, it seems timely to study phytoestrogen-containing plants for their chemopreventive activities regarding BPH and PC. Hop (*Humulus lupulus* L.) is a rich source of prenylflavonoids and, in particular, 5 compounds are most interesting for their bioactivities including estrogenicity. *8-Prenylnaringenin* proved to be the most potent phytoestrogen known to date, while *xanthohumol* is devoid of any estrogenicic properties. *6-Prenylnaringenin, isoxanthohumol* and *desmethylxanthohumol* demonstrated very weak estrogenic activities.

This Ph. D. project deals with investigations into the chemopreventive or therapeutic potential of hop-derived prenylflavonoids with respect to prostate diseases, in particular PC. We aimed to get insights in how the hop-derived prenylflavonoids xanthohumol (X), desmethylxanthohumol (DMX), isoxanthohumol (IX), 8-prenylnaringenin (8PN) and 6-prenylnaringenin (6PN) influence the pathogenesis of PC and in how the underlying mechanisms operate. Therefore, we investigated the antiproliferative and cytotoxic properties in order to determine the relative potencies of the individual hop-derived prenylflavonoids in inhibiting the growth of human prostate cancer cells LNCaP.FGC, PC-3, and DU145, and to assess the sensitivities of the different cell lines to these compounds. LNCaP.FGC are unique androgen-sensitive human prostate carcinoma cells derived from a supraclavicular lymph node, that have retained functional differentiation of prostatic epithelial cells.[2] PC-3 and DU145 are androgen-insensitive prostate cancer cells derived from brain metastases of an untreated prostate cancer patient and from bone metastases of a patient with androgen-independent prostate cancer, respectively.[3,4] These cell lines have been widely used as models for prostate cancer in both basic research and chemopreventive studies.

As the antiproliferative effects of the prenylflavonoids on cancer cells may be mediated through hormonal - in particular estrogenic - mechanisms, we additionally assessed the estrogenic activity by a growth stimulatory experiment and an estrogen-inducible yeast screening assay (yeast estrogen screen; YES).

Agents with potential antiangiogenic properties could be promising anticancer drugs. Therefore, we investigated the hop-derived prenylflavonoids *in vitro* for their antiangiogenic activities using a human bone marrow endothelial cell line (HBME).

Finally, more research was performed to get insights into the underlying mechanisms by which the hop-derived prenylflavonoids influence cell proliferation by investigating the kinetics of their time-dependent antiproliferative activities and the morphological changes of the treated cells for typical apoptotic features.

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1 Hop (*Humulus lupulus* L.)

1.1 Botanical overview

1.1.1 Taxonomy and ethymology

According to the most recent International Code of Botanical Nomenclature (St Louis Code) adopted by the 16th International Botanical Congress in 2000, the current name for the family, comprised only of the two genera *Humulus* L. and *Cannabis* L., is Cannabaceae. Other names still applied to this family are Cannabinaceae and Cannabidaceae.[1] The name of the genus, ***Humulus***, is thought to originate from *humus* (ground, earth) referring to the rich ground in which the plant flourishes. Others believe that it refers to the manner of growing close to the ground when the plant is not supported. And even others assert that *Humulus* comes from *umidus* or moist because of the preferential growth in moist earth. Undoubtedly, ***lupulus*** is derived from the Latin *lupus* or wolf, because of the characteristic of hops to strangle neighbouring plants while climbing up, in an analogous way as a wolf strangles his prey. The word ***hops*** has been used since the 8th century and it is believed to be deduced either from the Anglo-Saxon *hoppan,* which means 'to climb' or from the old-german *hopfo*, which means 'to raise', thus both referring to the growing characteristics.

The genus *Humulus* L. comprises three species: *Humulus lupulus* L., *Humulus japonicus* Sieb. & Zucc. (*Humulus scandens* (Lour.) Merr.), and *Humulus yunnanensis* Hu. *Humulus lupulus* L. is the most common species with approximately 40 varieties (cultivars). It was first domesticated in central Europe and is currently naturalized throughout the northern temperate regions of the world as well as some temperate regions in Australia, South-Africa, and South-America. *Humulus japonicus* Sieb. & Zucc. is grown as an ornamental plant and is of no economical use. It is endemic to East-Asia. *Humulus yunnanensis* Hu. can only be found in the Yunnan Province in China and, apart from the botanical description, no data are available about this species, which is due to its very limited occurrence, but also because this species is very often wrongly identified as *Humulus lupulus* L.

Wild hops are found ubiquitously. There are accounts by early European settlers in North-America of wild hops being present when they arrived there.[2,3] Wild American hops readily interbred with European cultivars brought by the settlers.[3]

The names *H. americanus* Nutt., *H. neomexicanus* Rydb., *H. volubilis* Salisb., *H. vulgaris* Gilib., *Lupulus communis* Gaertn., and *L. humulus* Mill. are probably synonyms for various *H. lupulus* varieties including var. *lupulus* (European hops), var. *cordifolius* (Miq.) Maxim in Franch. Et Sav. (*H. cordifolius* Miq.) native to East-Asia, var. *lupuloides* E. Small, native to the Missouri-Mississippi river basin, var. *brachystachyus* Zapalowicz and var. *neomexicanus* Nelson et Cockerell (*H. neomexicanus* Rydberg) native to South-West United States. Over 100 hop cultivars have been selected, mainly for their high contents of bitter acids, while others were selected for many generations primarily for their contents of volatile oil.[2,3]

1.1.2 Botanical description

Figure 1.1 *Humulus lupulus* L. (Photo copyright Henriette Kress, http://www.henriettesherbal.com).

The hop plant is a tall dioecious perennial climbing vine growing very fast from spring through summer.[4] It can reach heights from 3 m up to 10 m sometimes growing up to 30 cm per day. In autumn and winter, the aboveground annual parts of the plant die. Every year, in early spring, numerous shoots are produced from the remaining expanding rootstock, from which new plants can sprout having a rough square to hexagonal stem that swings clockwise around trees and attaches by fine two-pointed hairs. Most of these shoots are removed by

pruning and only 3 to 6 are trained-up strings, when they have reached a length of about 1 m. As the root system of adult plants can extend to more than 1.5 m in depth and 2 m laterally, rich and deep soils are required for efficient growing. The vigorous and fast growth from April to July requires large amounts of fertilizer and a regular supply of water either by natural rainfall or via irrigation systems.

Figure 1.2 *Humulus lupulus* L. Stamens on the male plant (Photo copyright Henriette Kress, http://www.henriettesherbal.com).

The leaves of the hop plant are rough, pale-green, tri- to pentalobate or heart-shaped with a long stalk and sawed edges. They can grow up to 15 cm long, are singular, cross-wise opposed, with a hand-shaped nervure and they possess little supportive leaves that are single-lobed. During July and August, the male plants carry small flowers (stamens) standing in groups in the axil of the leaves. The flowers of the female plants, appearing from the 3rd growing season, develop into greenish-yellow, ovoid catkins formed of membranous scales (bract and bracteoles), which partially overlap, and they have fine veins. When at the end of the summer the stalks wither, the female inflorescence have reached ripening and can be harvested as ovoid, brownish, scaled hop cones or strobiles. The inside of bract and bracteoles contains oleoresin glands that appear as small yellow-orange granules and constitute lupulin, a resinous, yellow-orange powder. Lupulin contains numerous compounds that are of economical interest especially in the beer brewing industry and, therefore, only the female hop plants are being cultivated. In some hop growing areas, particularly England, male plants are cultivated together with the females in order to produce seeds in the hop cones following pollination and, accordingly, to improve crop yields. However, in other parts of the world, it is forbidden to cultivate a male plant, and, in Belgium, wild males must be removed within 5 km from a female plant, because fertilized hop cones contain hard greasy seeds that adversely influence the beer quality.

Figure 1.3 *Humulus lupulus* L. Hop cones from the female plant (Photo copyright Henriette Kress, http://www.henriettesherbal.com).

When the hop cones are harvested, they have a moisture content of 75% to 80% (w/w) and, in order to prevent deterioration before storage or processing, reduction to less than 12% (w/w) is necessary. This moist reduction, achieved by hot air dryers, is very critical with respect to the hop quality. High temperatures, especially above 65°C, may accelerate oxidative decomposition of major constituents. Dried hops are recommended to be stored at low temperatures prior to processing or further use. The hop plant is commercially productive between 12 and 20 years.

1.1.3 Plant origin

Hop originates very likely from East-Europe. The plant has been cultivated for more than a millenium. At present, it grows wild in the hedges and wood skirts of the northern temperate climate zones of the world including Europe, North-America, and West-Asia, extending roughly from 35 to 55 degrees of latitude in humus-rich, moist soils. The material of commerce comes exclusively from female plants cultivated primarely in the United States, Germany, Great Britain, Czech Republic, China, but also in South-Africa and Australia[3].

1.2 Use of hops

1.2.1 Traditional use

Hops have been reputed for their bioactivities since ancient times. This was expressed in the treatment of a variety of diseases and in the widespread use as a health-promoting plant. In the 1st century after Christ, Plinius mentioned the breeding of hops in the roman gardens for the culinary use of the shoots, which were served as a delicacy in spring. Today, the shoots are still eaten and prepared in many different ways as kind of special asparagus, in soups or even raw in salads, but they are very expensive. The young leaves can also be eaten usually mixed in salads.

There was presumptive evidence of the use of hops for its calming, sleep-inducing, and libido-diminishing effects already during early medieval times. The pillows of restless children were stuffed with hop cones to induce sleep, give pleasant dreams or to reduce pain as they suffered from ear or nerve pains. Early medieval texts mention the use of hops for their diuretic, blood-purifying, and menstruation-promoting effects. In the 12th century,

Hildegard von Bingen stated that barley beers should be prepared with hops. She asserted that hops used as a spice cause a sad and melancholic mood. The North-American Indians used hops successfully for sleeplessness and pain and as a gyneacological remedy against breast and uterus problems.

Since the 14th century in the Netherlands and in Belgium, and since the 16th century in England, hops became one of the most important ingredients for beer brewing, because of the contributions to the bitter taste and overall flavor, and the natural preservative effects. However, in England, the use of hops in brewing was initially barred and the parlement stated that hops was 'a wicked weed that would spoil the taste of the drink and endanger the people'.

In the 17th century, hops were advized by Culpeper for the treatment of jaundice, liver and stomach diseases, headache, and infections of the skin, and as a blood-purifyer. The digestion-enhancing effect of hops had been described earlier by Paracelsus (2nd century) and hops had been used as a stomach tonic since then.

The calming and sleep-inducing activities of hops that were well-known among hop pickers could have been caused by transferring the hop resins from hand to mouth or by inhalation of hop essential oils. Among female hop pickers, menstrual disturbances were commonly reported, while male pickers could suffer from gyneacomasty.[2,4]

1.2.2 Modern use

Nowadays, the main use of hops is for beer brewing. Hops are responsible for the bitter taste of beer and the typical 'hoppy' aroma. Barley malts often have a sweety, unpleasant flavor and the hop bitterness is of the utmost importance for beer to balance this sweetness. However, at first, this use was not really appreciated. Originally, hop was only added for its natural preservative value protecting beer against microorganisms and deterioration of taste stability, thereby enhancing the keeping quality of beer when brewing became an industry. Another advantage of the use of hops in beer brewing refers to the beer foam-stabilising properties. The hop constituents responsible for these beneficial effects in the process of beer brewing have been studied extensively.

Due to its prominent mild sedative and weak hypnotic effects, a great number of hop-containing preparations are on the market, mostly in combination with other sedative herbs

such as *Valeriana officinalis* (valerian), *Melissa officinalis* (lemon balm), *Passiflora incarnata* (passion flower). These preparations are used for anxiety, nervousness, and insomnia, which stems from the anecdotal evidence of sleepiness among hops pickers in the past. The German Commission E Monograph lists hops as an approved herb for "mood disturbances such as restlessness and anxiety and for sleep disturbances".[5] Although there have been no meaningful clinical trials to support hops as a sedative, several studies have demonstrated that formulas combining hops with other sedative herbs are effective for insomnia.[6,7] Combinations of hops with valerian and passion flower or lemon balm are also approved by the German Commission E as sedative and sleep-promoting formulas. Reports have indicated that preparations of hops have sedative-like activity in frogs, pigeons, mice, goldfish, and golden carp.[8] Clear understanding of the biochemical mechanism and the conclusive identification of compounds responsible for such activity have not yet been achieved. Sikorski and Rusiecki[9] reported that both humulone and lupulone were "strongly sedative to pigeons and small birds, and somewhat less active on mice". Most attention has been given to volatile constituents of the hop essential oil. A degradation product of humulone and lupulone, 2-methylbut-3-en-2-ol, has been suggested as the active compound and it was shown to be sedative in mice.[10,11] Wohlfart et al. could not detect this compound in fresh hops and they observed that it reaches a maximum concentration after 2 years of storage at room temperature. Thus, if this compound can fully explain the sedative activity attributed to hops, it must be formed from hop constituents such as bitter acids that would then be considered as "pro-drugs". Hänsel and Wohlfart[12] asserted that the sedative effect of hops was not due to its content of myrcene, which was since shown to have an analgetic activity in mice.[13] As mentioned above, hops also has its use in the culinary field as well as in cosmetics being an ingredient for relaxing bath oils and others.

Hop extracts and/or compounds have been reported to be active in the following assays: antioxidant and/or chemoprevention, antimicrobial (particularly against Gram-positive bacteria), antigonadotropic, and cytotoxic. Recently, a lot of attention has been given to the health-beneficial effects of hop polyphenols. Research carried out on the alleged hormonal activity from hops has led to the identification in 1999 of a strong estrogenic compound, 8-prenylnaringenin.[14] The discovery of this compound provided the explanation for the reported hormonal disturbances in female hop pickers in the past. 8-Prenylnaringenin was shown to be one of the most potent phytoestrogens currently known. Our laboratory has expanded its

research on the possible beneficial activities of hop polyphenols, especially of prenylated chalcones and flavanones.

1.3 Hop chemistry

Several hundreds of constituents have been identified in hops[15,16] as secondary metabolites that are concentrated in the lupulin glands. Among the many different classes of compounds present in lupulin, hop bitter acids, hop essential oil, and hop polyphenols are the most important ones, not only for the commercial uses of hops, but also as chemical markers to distinguish between different hop cultivars.[17]

Table 1.1 Average composition of dried hop cones

Compounds	Percent (w/w)
_-Acids	2-19
_-Acids	2-10
Hop oil	0.5-3.0 (v/w)
Polyphenols	3-6
Monosaccharides	2
Amino acids	0.1
Proteins	15
Lipids and fatty acids	1-5
Pectins	2
Ash - salts	10
Cellulose - lignins	40-50
Water	8-12

1.3.1 Presence of hop-derived prenylflavonoids

A mixture of prenylated, geranylated, oxidized, and/or cyclized chalcones, is secreted by the lupulin glands along with the hop bitter acids and the hop essential oil.[14,18,19,20,21,22,23] The chalcone xanthohumol (**X**, **Figure 1.4, 1**) is the most abundant prenylated flavonoid in fresh and properly preserved hops present in concentrations exceeding 1%.[20,21,22] A majority of the known flavonoids from hops can be considered as derivatives of 2',4,4',6'-tetrahydroxy-3'-prenylchalcone, commonly known as desmethylxanthohumol (**DMX, Figure 1.4, 3**), which is found in hops in much smaller amounts compared to X. With the exception of two geranylated compounds, which have only be detected by MS,[20,21] all known

constituents of hops from this series are prenylated at either the 6-position or the 8-position or both. X isomerizes readily by an intramolecular Michael-type addition to the flavanone isoxanthohumol (**IX, Figure 1.4, 2**), the reaction reportedly proceeding more rapidly with increasing pH.[19,20,21] Isomerization of the chalcone DMX to a mixture of 6-prenylnaringenin (**6PN, Figure 1.4, 4**) and 8-prenylnaringenin (**8PN, Figure 1.4, 5**) is the so-called conversion of Nastainczyk,[24,25] which may occur spontaneously during hop storage.

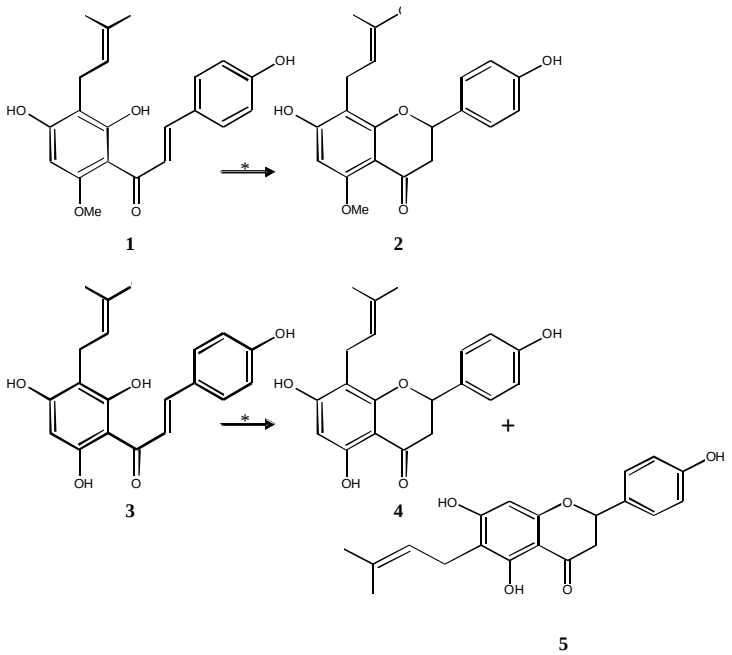

Figure 1.4 Hop-derived prenylflavonoids. (*Intramolecular Michael-type addition). 1, Xanthohumol (X); 2, Isoxanthohumol (IX); 3, Desmethylxanthohumol (DMX); 4, 8-Prenylnaringenin (8PN); 5, 6-Prenylnaringenin (6PN).

It must be stressed that the name of the compound 8PN used in the English hop literature is misleading for a variety of reasons. Naringenin is a chiral natural product usually with a *S*-configuration at the stereocenter C-2. The prenylated flavanone 8PN from hops is a racemate, which should be noted as (±)-8PN.[22,24] The natural chiral compound 8PN has been identified in *Sophora flavescens* Ait., a fabaceous shrub native to Asia, and this compound is known in the literature as 8-isopentenylnaringenin[26] or sophoraflavanone B.[18] To prevent

misunderstanding, it may be preferable to use the name 'hopein' coined by Prof. Dr. D. De Keukeleire[27] when referring to the hop constituent 8PN.

1.3.2 Biosynthesis of hop-derived prenylflavonoids

The lupulin glands of hops produce exclusively free flavonoids of the chalcone type. Furthermore, the lupulin glands are able to transfer methyl and prenyl functions to phenolic rings like chalconaringenin and phloroglucinol. Prenylflavanones such as IX, 8PN, and 6PN are formed in the absence of chalcone isomerase. The type of reaction resulting in ring closure of the chalcone structures to form flavanones is referred to as an intramolecular Michael-type addition. This isomerization may occur in any chalcone containing a free 2'- or 6'-hydroxy group and, when hydroxyls are present both at the 2'-position and the 6'-position, two regioisomers are produced.

1.3.3 Biological activities of hop-derived prenylflavonoids

A wide range of biological activities such as antiallergenic, antiinflammatory, antiviral, antifungal, antibacterial, antioxidative, antiproliferative, and anticarcinogenic effects have been observed with various natural flavonoids.[28] Recent interest in hop polyphenols stems from the results of research on other beverages, especially wine, in which scientifically based arguments have highlighted the beneficial effects of consumption of polyphenols during moderate drinking of - in particular - red wine. The well-known health-protective properties of tea, especially of green tea, have repeatedly been shown to be caused by specific polyphenols.

1.3.3.1 In vitro estrogenic activity

The recurring suggestion that hops has a powerful estrogenic activity has been obvious from its traditional use in baths to treat gynaecological disorders[29,30,31,32,33] and from reports of menstrual disturbances amongst female hop pickers. A number of journal articles[29,34,35] and patents exist concerning the use of the estrogenic properties of hops. Koch and Heim reported in 1953 that hops contain 'the equivalent of 20-300 µg estradiol/g'.[36] Chury published in 1961 that the estrogenic activity for a saponified ethanol extract of hops was approximately 200-, 150- and 50-times the activities observed for peas, red clover, and cabbage, respectively.[37]

However, even though the estrogenic properties of hops were well established, the hop fraction or the individual compounds responsible for the estrogenic activity remained unknown.

In 1972, Nastainczyk reported the isolation of a compound named 'hops pro-estrogen', which is isomerized to a mixture called 'hops estrogen'.[25] In 1988, Hänsel and Schulz identified Nastainczyk's 'hops pro-estrogen' as 3'-prenylnaringenin chalcone (now better known as DMX). Furthermore, they revealed that the 'hops estrogen' was a mixture of 8PN and 6PN. The researchers asserted, without experimental data, that 6PN was not estrogenic, but they did not state explicitly that 8PN is the active estrogen. However, they were the first to correctly report the chemical structure of the principal estrogenic component of hops. Following this report, contradictory literature reports on the estrogenically active fraction were published, probably due to the variable nature of the extracts and the variety of assays used.

In 1997, De Keukeleire et al. examined the estrogenic activity of various hop fractions and individual compounds using a sensitive *in vitro* bioassay based on the ability of compounds with estrogenic activity to stimulate alkaline phosphatase activity in a human endometrial adenocarcinoma Ishikawa-Var I cell line.[38] Estrogenic activity could only be found in the polyphenolic extracts and X, the compound until then believed to account for the potent estrogenic activity of hops, was shown to be inactive.

The report in 1999 by the same research group in collaboration with Milligan et al. from King's College (London) may be regarded as the beginning of the modern, unambiguous understanding of the *in vitro* estrogenic activity of hops.[14] Milligan et al. identified the estrogenically active compound as 8PN by using 2 sensitive and specific *in vitro* bioassays (Ishikawa-Var I assay and an estrogen-inducible yeast screen from *Saccharomyces cerevisiae*) and proved that it was the most potent phytoestrogen known. The estrogenic activity of 8PN was completely blocked in Ishikawa cells by the anti-estrogen ICI 182,780, demonstrating that 8PN exerts its estrogenic effect by interaction with the estrogen receptor(s). 6PN displayed only very weak estrogenic activity (<1/100 of 8PN) and IX was weakly active in the Ishikawa Var I assay (1/100 of 8PN), but not active in the yeast cells. X was inactive in both assays. The relatively high estrogenic potency of 8PN was reflected in its ability to interact with the estrogen receptors in a competitive binding assay with rat uterine cytosol. IX

showed no activity in the receptor binding assay suggesting that its weak activity in the Ishikawa cell assay may have reflected metabolic conversion to 8PN.

In 2000, again by the same group of investigators, the endocrine properties of various hop flavonoids, to which humans are exposed, were investigated.[39] 6PN, 6,8-diprenylnaringenin, and 8-geranylnaringenin exhibited some estrogenic activity with a potency less than 1% compared to 8PN. The other compounds investigated (X, IX, 3'-geranylchalconaringenin, 6-geranylnaringenin, 4'-*O*-methyl-3'-prenylchalconaringenin) showed no estrogenicity. 8PN competed strongly with 17β-estradiol for binding to both ERα and ERβ, whereas 8-geranylnaringenin showed only weak competition. None of the other compounds were able to displace [^{3}H]-17β-estradiol from either receptor. There was no evidence of either progestogenic or androgenic bioactivity in the polyphenolic hop extracts or for pure prenylflavonoids. However, Zierau et al. demonstrated in 2003 strong antiandrogen activities for 8PN using a yeast-based androgen receptor assay.[40] 8PN was able to inhibit the androgenic effects induced by 10^{-8}M 5α-dihydrotestosterone (DHT) at concentrations above 5 x 10^{-6}M. In an androgen receptor activity assay based on the analysis of prostate-specific antigen concentrations in the supernatants of treated PC3(AR)$_2$ cells, 8PN had no detectable antiandrogenic effect. Zierau's results could not provide evidence for an antiandrogenic effect of 8PN, but suggest the ability of this compound to act through the androgen receptor.

The strong estrogenic properties of 8PN were confirmed by subsequent *in vitro* investigations.[39,41,42,43,44,45,46] Kitaoka et al. demonstrated that a prenyl group at position 8 is important for binding to the ER.[26] They also demonstrated that there were no significant differences in ER-binding potencies between the enantiomers of 8PN. However, Schaefer et al.[47] found that the 2*S*-enantiomer shows a 2- to 3-fold higher affinity for both ERs compared to the 2*R*-enantiomer, *in vitro* and *in vivo*. They reported that 8PN is the first known ERα-selective phytoestrogen exhibiting >2-fold higher affinity for ERα than ERβ measured by an *in vitro* competitive binding assay. Transactivational analysis revealed a >3.6-fold higher estrogenic activity of 8PN at ERα than at ERβ, which is in strong contrast to the ERβ-selective activation by coumestrol and genistein. It was proven that, on ERα, 8PN is 10-fold more potent than coumestrol, 100-fold more potent than genistein, and only 70-times weaker than 17β-estradiol. *In vitro*, 8PN acts as a pure agonist exhibiting an estrogenic activity profile comparable to estrone.

Bovee et al. tested the estrogenic potency of several phytoestrogens with ERα and ERβ using a rapid yeast bioassay stably expressing human ERα and ERβ.[48] The ranking of the estrogenic potency with ERα was: 17β-estradiol >> 8PN > coumestrol > zearalenone >> genistein >> genistin > naringenin. The ranking with ERβ was: 17β-estradiol >> coumestrol > genistein > zearalenone > 8PN >> naringenin > genistin. Effenberg et al. demonstrated that 6PN, X, and IX, but not 8PN show ERβ-preference.[49] *In vitro* evaluation of the estrogenic activity of hop extracts showed significant competitive binding to ERα and ERβ, induction of alkaline phosphatase activity, upregulation of progesterone receptor mRNA in cultured endometrial cells, and upregulation of presenilin-2, another estrogen-inducible gene.[50]

1.3.3.2 In vivo estrogenic activity

The relative *in vivo* potency of the estrogenic 8PN was examined in an acute *in vivo* test using uterine vascular permeability as an endpoint.[46] 8PN and coumestrol were considerably less potent (<1%) in inducing a rapid increase in uterine vascular permeability within 4 h of administration compared to 17β-estradiol. The dose-response relationship for 8PN was similar to that of coumestrol and a large stimulatory effect was produced by 100 nmoles 8PN. The amount of genistein to produce the same effect was at least 10-fold greater. The administration of 100 µg ml^{-1} 8PN to ovariectomized mice in their drinking water produced a significant estrogenic increase in vaginal mitosis after 72 h, which could be compared to the effect produced by the administration of 100 ng ml^{-1} 17β-estradiol. However, as opposed to 17β-estradiol, 8PN had no effect on uterine mass and epithelial mitosis.

Schaefer et al. assessed the *in vivo* estrogenic potency of 8PN for ERα by classic uterine and vagina growth assays in juvenile rats and reported that 8PN has a affinity for ERα, which is 20,000-fold less potent than that of 17β-estradiol, while, *in vitro*, 8PN was found only 70-fold less potent than 17β-estradiol in binding ERα[47].

Diel et al. also investigated the *in vivo* estrogenic potency of 8PN by carrying out a three-day uterotrophic assay on ovariectomized young female rats.[51] A single dose of 8PN (10 mg/day/kg body mass) significantly stimulated uterine wet weight accompanied by a proliferative response, which was comparable to a single dose of 17β-estradiol (0.03 mg/day/kg body mass). The treatment resulted in a statistically significant increase of the uterine epithelial height as well as of the vaginal epithelial height. In response to treatment

with 8PN, the same change in gene expression pattern in the uterus and in the liver was observed as in reponse to treatment with 17β-estradiol, but it wass less pronounced. Conversely, 8PN was found to be more potent than 17β-estradiol in inducing expression of insulin-like growth factor binding protein 1 (IGFBP-1). This report again provides overwhelming evidence that 8PN has largely to be regarded as a pure estrogen agonist.

Noteworthly is the work of Zierau et al., who compared the estrogenic effect of the phytoestrogens naringenin, 8PN, and 6-(1,1-dimethylallyl)naringenin in 2 mammalian *in vitro* systems and in fish as *in vivo* system.[52] They observed strong differences. In the *in vivo* assay, no estrogenic effects of the naringenin-type flavonoids were observed, while the mammalian *in vitro* systems showed a similar and graded response to the test compounds.

1.3.3.3 Effect on bone resorption and uterus growth

Bone remodelling is a dynamic process, which is maintained by a balance between bone formation and bone resorption. It has long been recognized that the acute loss of estrogen at the onset of menopause is responsible for an accelerated bone loss in women, called Type I osteoporosis,[53] which is characterized by an imbalance in bone remodelling and leads to an increased fracture risk. Hormone replacement therapy (HRT) is currently the most common therapy used for peri- and postmenopausal women to prevent bone loss. However, while HRT has a positive effect on bone, climacteric symptoms, and the cardiovascular system, there are also increased risks of breast and endometrial cancers after some period of application.[53,54] Certain plant-derived compounds show selective estrogen-receptor modulator (SERM) activity. Such SERMs display the desirable estrogenic effects, but lack the undesirable side effects and may, therefore, be alternatives to conventional HRT.

Tobe et al. made the interesting observation that bone resorption is inhibited by X in the pit formation assay.[55] A dose-dependent inhibition was observed both for X (IC_{50} 1–10 μM). Miyamoto et al. examined the effect of 8PN *in vivo* on uterus and bone metabolism in ovariectomized rats as a model to mimic hormone-dependent osteoporosis in menopausal women.[56] The rats were treated subcutaneously with 30 mg/kg/day for 2 weeks in comparison with 17β-estradiol at 0.01 mg/kg/day. They found that 8PN and 17β-estradiol completely protected from ovariectomy-induced bone loss and that 8PN showed a lower stimulation of

the uterus. However, the differences between both treatment groups were small and did not reach statistical significance.

Hümpel et al. studied the effects of 2*S*-8PN and racemic 8PN in adult ovariectomized rats using a treatment of 28 days.[57] They demonstrated that 8PN can completely protect from ovariectomy-induced bone loss, while exhibiting minimal trophic effects on uterus and endometrium. At equivalent bone-protective doses of 17β-estradiol and 8PN, the phytoestrogen has a 10-fold lower stimulatory effect on uterus and endometrium. In addition, it was demonstrated that, in the *in vivo* situation, no differences in potency or quality of effects were noted between the 2*S*-enantiomer and racemic 8PN. Moreover, the bone tissue-specific effect of 2*S*-8PN was confirmed in a transgenic reporter mouse model.

Effenberger et al. demonstrated that 8PN, 6PN, X, and IX exert estrogen-like activities on bone metabolism by examining their effects to modulate markers of differentiation and gene expression in normal human fetal osteoblast cells (hFOB) and human osteosarcoma cells (U-2 OS) stably transfected with either ERα or ERβ.[49] The compounds exerted their dose-dependent activity effect through ER stimulation.

1.3.3.4 Anticancer activity

Several hop-derived prenylflavonoids have been investigated for a specific activity or for a range of activities concerning cancer prevention. Each effect can be classified into three major cancer-development phases: initiation, promotion, and progression.

A/ Initiation phase

Many chemicals in the environment act as carcinogens following activation by cytochrome P450.[58] Accordingly, compounds that inhibit P450-mediated reactions of pro-carcinogens could be useful as cancer-chemopreventive agents at the initiation stage of carcinogenesis by blocking carcinogen activation. Other than inhibiting these phase I-enzymes, compounds could exert carcinogen-protective effects by stimulating detoxification (stimulation of phase II-enzymes) of the carcinogens formed in phase I.

Henderson et al. studied the *in vitro* effects of some hop-derived prenylflavonoids on cDNA-expressed human CYP enzymes.[59] X almost completely inhibited the 7-

ethoxyresorufin *O*-deethylase (EROD) activity of CYP1A1 and CYP1B1 at 10 μM. The other prenylflavonoids (DMX, IX, 8PN, 6PN) decreased the EROD activity of the enzymes in varying inhibitory degrees at the same concentration. In general, prenylated flavanones were found to be more active than non-prenylated flavanones or isoflavones in inhibiting these two human P450 enzymes expressed in insect cells. In contrast, 8PN and IX proved to be the most potent inhibitors of CYP1A2 acetanilide 4-hydroxylase activity (> 90% inhibition at concentrations of 10 μM) and of the CYP1A2 metabolism of aflatoxin B_1 (AFB_1) and both compounds decreased the covalent binding of radiolabelled AFB_1 to microsomal protein in a concomitant manner. This proved that prenylflavanones are more inhibitory to human CYP1A2 than prenylchalcones. Furthermore, the number and the position of the prenyl group(s) on the flavanone structure seemed important regarding the inhibitory activity. However, no consistent structure-activity relationship was found in the inhibitory activity of the hop-derived prenylflavonoids towards different forms of human P450. X, IX, and 8PN were poor inhibitors of CYP2E1 and CYP3A4, thereby proving the important feature that the hop-derived prenylflavonoids not only inhibit some important CYP enzymes, but are also selective in their inhibition.

Miranda et al. examined the chalcones X and DMX, and the flavanones IX, 6PN, and 8PN for their ability to induce the phase II-enzyme, quinone reductase, in wild-type mouse Hepa 1c1c7 cells.[60] All compounds were found to be potent inducers of the enzyme, X being most potent. None of the compounds were found to induce CYP1A1 nor to repress the expression of the enzyme. Furthermore, it was demonstrated that X, 8PN, and IX are potent inhibitors of the metabolic activation of the heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). The compounds could potentially act as chemopreventive agents against cancer induced by heterocyclic amines activated by CYP1A2.[61]

Gerhäuser et al. also examined the modulation of the activity of enzymes involved in carcinogen metabolism (phase I-enzyme CYP1A) and detoxification (phase II-enzyme quinone reductase, QR) and the antioxidant properties of X and IX.[62] Both X and IX were identified as potent inhibitors of CYP1A activity *in vitro*, with IC_{50} values of 0.022 ± 0.002 μM and 0.30 ± 0.13 μM, respectively. X was found to induce the specific activity of QR in a dose-dependent manner with a concentration-dependent value of 1.67 ± 0.23 μM. Loss of the α,β-unsaturated keto by cyclization to the flavanone IX reduced the QR-inducing potential. X

and IX were identified as effective antioxidants with a capacity to scavenge physiologically relevant reactive oxygen species (ROS), including hydroxy and peroxy radicals for X and IX, and superoxide anion radicals for X.

Dietz et al. studied the effect of a hop extract on QR and found that X was most effective at inducing QR with a concentration required to double the specific activity of QR of 1.7 ± 0.7 μM.[63] They also investigated the reporter activity mediated by the androgen response element (ARE) in HepG2-ARE-8 cells after incubation with X for 24 h. X increased ARE-reporter activity in a dose-dependent manner. Moreover, it was shown that X alkylates human Keap1 protein most likely on a subset of the 27 cysteines of Keap1. This suggests that X induces QR by covalently modifying the Keap1 protein. Liu et al. developed a new screening method for the discovery of potential cancer chemoprevention agents based on mass spectrometric detection of alkylated Keap1 and they confirmed that X is a potent inducer of QR and that it reacts with Keap1.[64]

B/ Promotion phase

As potential antitumor-promoting mechanisms, X demonstrates antiinflammatory properties by inhibition of the constitutive form of cyclooxygenase Cox-1 and, more importantly, the inducible Cox-2 activity, which is linked to carcinogenesis with IC_{50} values of 16.6 ± 1.8 μM and 41.5 μM, respectively. IX was inactive with respect to both enzymes (IC_{50} > 100 μM).[62]

C/ Progression phase

Miranda et al. investigated 6 hop-derived prenylflavonoids for their ability to inhibit the proliferation of human breast cancer (MCF-7) cells, colon cancer (HT-29) cells, and ovarian cancer (A2780) cells *in vitro*.[65] A dose-dependent growth inhibition of the MCF-7 cells was observed with X, IX, and dehydrocycloxanthohumol (DCX). The IC_{50} values were 3.47, 6.87, and 4.69 μM, respectively, after 4 days of exposure. X and DCX were even more cytotoxic against ovarian cancer (A2780) cells with IC_{50} values of 0.52 and 5.00 μM, respectively, at 2 days of exposure. The viability of colon cancer (HT-29) cells was hardly decreased; X, IX, and 4 other prenylflavonoids showed a weak growth-inhibitory effect with IC_{50} values greater than 10 μM. X was the most cytotoxic prenylflavonoid in these studies, indicating that modification of its structure (cyclization of the prenyl function, demethylation,

and isomerization of the chalcone nucleus) leads to partial loss of cytotoxicity against the 3 human cancer cells *in vitro*. None of the 6 prenylflavonoids was toxic to normal (rat hepatocyte) cells at 10 μM. These findings demonstrate that specific prenylflavonoids from hops have preferential growth-inhibitory effects on tumor cells, which cannot be explained by a general toxic effect.

The effect of 8PN was investigated on aggregation, growth, and invasion in the human MCF-7 breast cancer cell line family.[43,66] MCF7/6 cells showed a poor tendency to aggregate in slow aggregation assays. Treatment with 8PN at 10 μM and 1 μM had a beneficial effect on the aggregation, as was evident from the formation of larger and more compact aggregates. A significant growth-stimulatory effect was observed in cultures treated with varying concentrations of 8PN. 8PN had no effect on invasion in the embryonic chick heart assay. 8PN had similar effects as 17β-estradiol: it promotes cell-cell adhesion and growth without an effect on invasion. Xanthohumol was found to inhibit invasion of human breast cancer MCF7/6 and T47-D cells and to induce apoptosis of these cell lines[67].

Antiproliferative mechanisms of X to prevent carcinogenesis in the progression phase include inhibition of DNA synthesis (inhibition of DNA-polymerase-α activity *in vitro*, as well as thymidine incorporation into newly synthesized DNA *in vivo*) and induction of cell cycle arrest in the S-phase and apoptosis in an ER-negative MDA-MB-435 mammary carcinoma cell line. Finally, X could induce terminal cell differentiation in transformed cells as a mechanism of prevention that is effective in late stages of carcinogenesis. These anticarcinogenic effects were verified *in vivo* demonstrating that X prevents carcinogen-induced preneoplastic lesion in mouse mammary gland organ culture in the nanomolar range (IC_{50} value of 0.02 μM).[62]

DMX has been investigated for its antiproliferative and apoptotic activities. It was found to be antiproliferative and to induce apoptosis via the mitochondrial pathway in BJAB cells (Burkitt lymphoma cell line) and in primary lymphoblasts of childhood acute lymphoblastic leukaemia (ALL).[68] Pepper et al. demonstrated that 8PN inhibits angiogenesis induced by basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) or the synergistic effect of the two cytokines in combination, with an IC_{50}-value of 3-10 pM using bovine microvascular endothelial cells derived from the adrenal cortex (BME cells) and from the thoracic aorta (BAE cells).[69] The *in vitro* inhibitory effects of 8PN were comparable

to those found for genistein. Results from early chorioallantoic membrane (CAM) assays showed reductions in both vessel lengths and vein diameters, with a similar potency in the 8PN and in the genistein groups.

1.3.3.5 Effect on lipid metabolism

Tabata et al. showed that a methanol extract of hop exerted an inhibitory activity against rat liver diacylglycerol acyltransferase (DGAT), an enzyme involved in lipid metabolism.[70] Bioactivity-guided fractionation identified two active chalcones, X and XB (xanthohumol B), which inhibited DGAT with IC_{50} values of 50.3 µM and 194 µM, respectively, in rat liver microsomes.

Miranda et al. examined the ability of hop-derived prenylflavonoids (X, DMX, IX, 6PN, 8PN) to inhibit *in vitro* Cu^{2+}-mediated oxidation of human low-density lipoprotein (LDL) by measuring the formation of conjugated dienes and thiobarbituric acid-reactive substances (TBARS) and the oxidation of tryptophan residues.[71] It was found that especially the prenylated chalcones and, to a lesser extent, the prenylated flavanones displayed antioxidant activities and thus provide protection against oxidative modification of LDL. Moreover, it was shown that the presence of a prenyl group or a methoxy group enhanced the antioxidant activities of the chalcones. A prenyl group also appeared to be important in the antioxidant activities of flavanones at a high concentration (25 µM).

Rodriguez et al. studied the ability of hop-derived prenylflavonoids to inhibit lipid peroxidation by measuring the inhibition of metal-ion (iron)-dependent and independent (*tert*-butyl hydroperoxide - TBH) lipid peroxidation in rat liver microsomes.[72] Also, the ability of these compounds to protect isolated rat hepatocytes from injury caused by TBH was examined. Again, the antioxidant activity of these compounds was established and the importance of a prenyl group for the antioxidant activity of hop chalcones was confirmed. At 25 µM, prenylated chalcones such as X and DMX may have antioxidant activities comparable to the flavonol quercetin and better than those of the flavanone naringenin and the isoflavone genistein. Moreover, X was found to protect rat hepatocytes from TBH-induced injury.

Stevens et al. demonstrated that X and some other chalcones and flavanones inhibit peroxynitrite-mediated oxidation of LDL at low micromolar concentrations.[73] IX

unexpectedly showed a slight prooxidant effect instead of an inhibitory effect on LDL oxidation.

1.3.3.6 Antimicrobial effects

Mizobuchi and Sato investigated the antimicrobial activity of X, IX, 6PN, and 8PN. X and both flavanones were active against the fungi *Trichophyton mentagrophytes* and *T. rubrum* with minimum inhibitory concentrations in the range from 3.13 to 12.5 µg/ml.[74] Moreover, all compounds showed moderate activities against the fungus *Mucor rouxianus* and the bacterium *Staphylococcus aureus*. None of the flavonoids were active against the fungi *Candida albicans* and *Fusarium oxysporum* or against the bacteria *Escherichia coli*. Naringenin showed little activity against the above microorganisms. This indicates that introduction of a prenyl group is required for antifungal activity, probably because it renders the flavonoid skeleton sufficiently lipophilic to pass the cell membrane of the microbes or it could also be involved in more specific binding to microbial enzymes.

1.3.3.7 Conclusions

Due to the pure estrogen agonistic effect on the estrogen receptors and the consequent effect on bone metabolism, which is very comparable to that of 17β-estradiol but without the strong uterus stimulation, 8PN is a very suitable candidate to compete with HRT in menopausal women. First clinical results from a phase Ia-study in menopausal women[75] have demonstrated excellent tolerance and first signs of effectiveness after single oral doses. Heyerick et al. demonstrated in a prospective, randomized, double-blind, placebo-controlled study that the daily intake of a hop extract, standardized on 8-PN, exerted favorable effects on vasomotor symptoms and other menopausal discomforts and could, therefore, provide an attractive addition to the alternative treatments available for relief of hot flushes and other menopausal discomforts.[76] Especially X has been shown to be a very potent cancer-chemopreventive compound *in vitro* with an exceptional broad spectrum of inhibitory mechanisms at the initiation (by blocking the cytochrome P450-mediated activation of procarcinogens and by inducing the carcinogen-detoxifying enzymes such as quinone reductase), promotion (antiinflammatory properties), and progression (by inhibiting proliferation and angiogenesis) stages of carcinogenesis.

1.3.4 Metabolization of hop-derived prenylflavonoids

Although flavonoids are abundantly present in the human diet and in animal feeds, little is known about their metabolism. The two major sites of flavonoid biotransformation are the liver and the colonic flora.[77] Since the CYP superfamily is abundant in the liver and small intestine, a role in the metabolism of flavonoids is evident. Yilmazer et al. studied the *in vitro* biotransformation of X by rat liver microsomes from rats untreated or treated with various P450-inducing agents.[78] Three major polar metabolites were produced from either untreated rats or phenobarbital-pretreated rats. Liver microsomes from isosafrole- and β-naphthoflavone-pretreated rats formed another major non-polar metabolite, which was identified as dehydrocycloxanthohumol (DX). The formation of these metabolites involved the role of CYP enzymes. Yilmazer suggested that modification of the prenyl substituent, induced by epoxidation of the double bond, constitutes a major metabolic pathway of prenylflavonoids.[79] Miranda et al. demonstrated the antiproliferative activity of metabolite DX, which is also found in hops and beer, in human breast cancer MCF-7 cells at concentrations of 0.1 and 1.0 μM.[65] In addition two major glucuronides were found with either rat or human liver microsomes. Coldham et al. investigated the metabolic activation of IX to 8PN using hepatic microsomes and a sensitive yeast assay.[80] They found that the estrogenic activity of IX remained unchanged after incubation with the microsomes and that evidence for metabolization of IX into 8PN is lacking.

Nikolic et al. studied the metabolism of 8PN by human liver microsomes and identified a total of 12 metabolites.[81] Biotransformations occurred on the prenyl group and the flavanone skeleton. The most abundant human liver microsomal metabolites were oxidation products of the prenyl group. Among these, hydroxylation of one of the terminal methyls of the prenyl group was more abundant than the epoxidation of the double bond. Yilmazer et al., however, did not observe oxidation of the prenyl methyl groups when investigating the metabolism of X in the rat.[78] It was suggested that this pathway did not occur for the more rigid, open structure of X or that it was such a minor metabolite that is was undetectable. Nikolic et al. also observed that the oxidation of the prenyl methyl group was predominant at low concentrations and proportionally less dominant at higher substrate concentrations.[81] Yilmazer et al. used relatively high substrate concentrations (100 μM), which could be another explanation why this pathway was not observed in the study of the metabolization of

X.[78] Zierau et al. examined the estrogenic activity of the two most abundant metabolites of 8PN.[82] Using the yeast estrogen receptor assay, a dose-response analysis of the two metabolites was performed in comparison to 17β-estradiol and 8PN. Both metabolites induced an estrogenic response with an EC_{50} value of approximately one order of magnitude less potent than 8PN. Using a bioluminescent estrogenic assay, both metabolites proved to induce luciferase activity in a dose-dependent manner, inducing a response comparable to the response induced by 10^{-8}M 17β-estradiol when applied in a concentration of 10^{-5}M. Also, the metabolites were less active than the parental compound 8PN. Jang et al. reported that an isomeric metabolite of 8PN is a moderately potent, but a highly selective inhibitor of human cyclooxygenase-2.[83]

Nikolic et al. investigated the metabolism of X and IX by human liver microsomes.[84] They found that hydroxylation of a prenyl methyl group was the primary route of oxidative metabolism forming either *cis*- or *trans*-hydroxylated metabolites of IX, but only the *trans*-isomer of X. The double bond in the prenyl group of both compounds formed an epoxide, which was opened by an intramolecular reaction with the neighboring hydroxyl group. The potent phytoestrogen 8PN was detected as a demethylation product of IX. However, the analogous demethylation reaction was not observed for X. Since X can be converted to IX through acid-catalysed cyclization in the stomach, X might contribute to the *in vivo* levels of estrogenic 8PN.

Possemiers et al. studied the bioavailabilities of X, IX, and 8PN, and the transformation potency of the intestinal microbial community.[85] They found that, after incubation of IX with fecal samples, the estrogenic properties of some samples increased due to conversion of IX to 8PN. *Eubacterium limosum* was identified to be capable for this *O*-demethylation of IX. These results show that, although there are great interindividual variabilities, the intestinal microbial community could more than 10-fold increase the exposure concentration of 8PN, if hop products containing IX (most abundant hop-derived prenylflavonoids in beer) are consumed.

1.4 Hops legislation

Humulus lupulus L. is listed, acording to the Royal Decree of the 29th of August 1997 on the production and trade in food products composed of or containing plants or plant

preparations, in list 3. Article 4. §1 of this decree states that it is forbidden to produce or sell, in *pre-dosed form*, food products composed of or containing one or several of the plants mentioned in list 2 or list 3, if no prior notification has been filed with the Federal Agency of Medicine and Health Products in accordance with the clearly determined provisions.

The foodstuffs specified in article 4, containing *Humulus lupulus* L., may only be marketed under the following condition: the absorption of the daily recommended amount indicated on the labelling or in advertising may not give rise to the quantity of 8PN ingested being greater than 400 µg. By way of derogation to Article 4 (§1) (5), the results specified must be available for each batch of products. At this moment, there are no restrictions for the use of hops in non-predosed forms such as aromas, juices or teas (bulk or bags).

1.5 Side effects and toxicity

Humulus lupulus L. is believed to be non-toxic. However, as with all plants, it may cause allergic reactions in sensitive individuals. *Humulus lupulus* L. is known to cause a contact dermatitis in hop pickers. Additionally, contact dermatitis has been attributed to the rough hairs on the stem and secretions of the yellow glandular hairs on hops.[86] No clinical cases of allergy or anaphylaxis resulting from therapeutic use of hops have been published.

1.6 Contra-indications and drug-botanical interactions

Hops should not be used in patients suffering from depression and, moreover, long-lasting intake of hops may cause depressive moods. Limited evidence from one animal study suggests that hops may potentiate the effects of sedative drugs.[87] Because of the estrogenic activity, hops is contra-indicated during pregnancy or in case of history or presence of hormonal-dependent diseases including breast and prostate cancer. However, the latter is a safety scenario, because it has not been argumented that phytoestrogens negatively influence these diseases. On the contrary, the evidence collected from the literature point out a variety of possible protective effects of phytoestrogens, but, indeed, more research, particular clinical studies, will have to be performed before final conclusions can be made.

2 Benign prostate hypertrophy and prostate cancer

The prostate is a walnut-sized gland of the male reproductive system (**Figure 2.1**). It is located in front of the rectum just below the urinary bladder and is wrapped around the upper part of the urethra **(Figure 2.2)**. The prostate gland contains three areas or zones (**Figure 2.3**). The central area of the prostate, which appears as 2 small pear-shaped lobes surrounding the proximal urethra (**Figure 2.3, 2**), is called the *transition zone* (**Figure 2.3, 3**). This zone makes up 5% of the total prostate volume. It is the area in which benign prostatic hypertrophy occurs and 15 to 20% of the prostate cancers begin. The *central zone* (**Figure 2.3, 1**) is the second-largest element of the prostate (25% of the total gland volume), which surrounds the transition zone until the bladder base. The ejaculatory ducts (**Figure 2.3, 8**), where semen and sperm are combined before ejaculation, lay in the middle of this zone. About 5-10 % of prostate cancers originate in this area. The *peripheral zone* (**Figure 2.3, 7**) occupies about 70% of the prostate and it is the zone in which the majority of prostate cancers occur. It is also the most common site of chronic prostatitis. This area surrounds the two other zones from apex posterior to base. During a digital rectal exam, the peripheral zone is the only zone that can be felt.

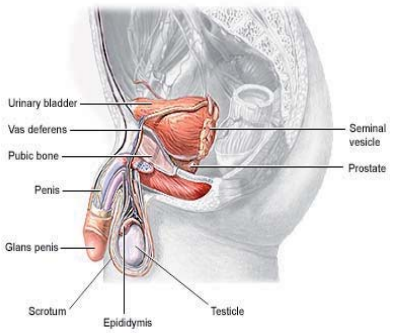

Figure 2.1 The male reproductive system (http://health.allrefer.com/pictures-images/).

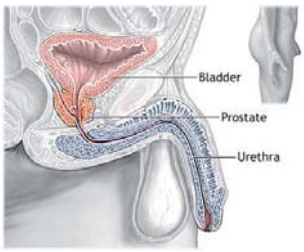

Figure 2.2 The prostate (http://health.allrefer.com/pictures-images/).

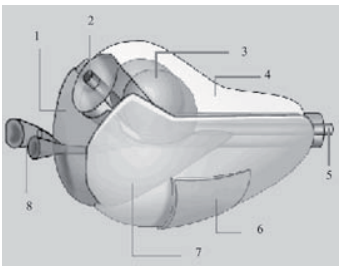

Figure 2.3 Zones of the prostate. 1, central zone; 2, 5, urethra; 3, transitional zone; 4, fibromuscular zone; 6, capsule; 7, peripheral zone; 8, ductus deferens (http://health.allrefer.com/pictures-images/).

There are different types of tissues found in the prostate. The *epithelial component* is primarily glandular tissue. These small glands are surrounded by a layer of tissue called the *stroma,* which is comprised by smooth muscle and connective tissue. On the outside, the prostate gland is surrounded by a capsule of fibrous tissue called the *prostatic capsule* (**Figure 2.3, 6**). In the normal prostate, the relationship between the stromal and the epithelial component is approximately 2:1.[88]

The primary function of the prostate gland is the secretion of the seminal fluid, which functions to nourish and to protect the sperm against the acid pH of the vagina, thereby increasing the chances that fertilization will occur. This seminal fluid is produced by the glandular cells from the prostate and the seminal vesicles, a pair of glands attached to the prostate. Sperm is produced in the testicles and carried through the vas deferens to the prostate, where sperm mixes with semen. During orgasm, this fluid is ejaculated first by a connection to the urethra called the ejaculatory ducts, which lay in the central zone of the prostate, and then through the urethra out of the body. The stroma contains approximately 30% muscular tissue, which contracts during ejaculation to help move fluid into the urethra.[89]

In the normal human prostate, the androgen receptor (AR) is almost equally distributed between stroma and epithelium, with an elective accumulation in the periurethral zone. The stromal AR is a primary target of androgen activity and its functionality appears to be particularly important during prostate development for maintaining the function of the normal prostate. It is needed for stromal-to-epithelial interactions that include various growth factor loops.[90] The estrogen receptor α (ERα) is expressed at low levels predominantly in the stroma of the normal human prostate.[91] The estrogen receptor β (ERβ) predominantly localizes in the epithelium and to a lesser extent in the stroma. Although the reports vary in their assessment of the ERα and ERβ status, it is generally accepted that there are higher levels of ERβ than ERα in the normal human prostate.[92]

2.1 Benign prostatic hypertrophy

There are a variety of prostate disorders that commonly afflict men of all ages. The most common prostate disease is benign prostatic hypertrophy or BPH (**Figure 2.4**). BPH is the formation of a benign tumor in the prostate gland. Per definition is a *tumor* or *neoplasm* an abnormal mass of tissue that results from excessive cell division (mitotic activity). A *benign* tumor is a *not*-cancerous, *not*-malignant tumor, which is *not* potentially fatal and relatively harmless, because it does not invade nearby tissue or spread to other parts of the body.[89]

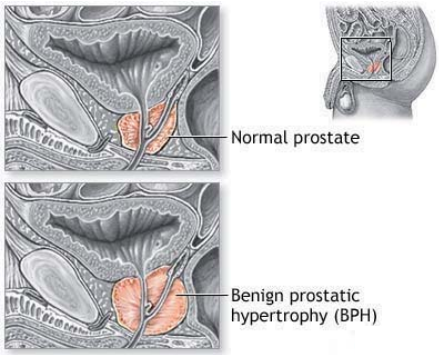

Figure 2.4 Normal prostate versus BPH (http://health.allrefer.com/pictures-images/).

The prostate gland resulting from BPH is generally composed of stromal elements. In patients with BPH, the stromal-to-epithelial ratio increases to 5:1 and the prostate gland can increase in size from 20 g, which is the average size of the prostate in younger men, to as large as 150 g.[87] This stromal hyperplasia is considered due to the synergistic effects of both androgens and estrogens, receptors for both being present in the stromal compartment of the prostate.

2.1.1 Incidence and prevalence

Many studies have attempted to assess the prevalence of BPH and few have estimated the incidence. Assessing the occurrence of BPH is difficult due to the lack of a standardized case definition. Based on autopsy studies, the prevalence of histologically diagnosed BPH increases from 8% in men aged 31 to 40 years to up to 40-50% in men aged 51 to 60 years, and to more than 80% in men older than 80 years. Based on clinical criteria, 4 to 25% of men aged 40 years and older suffer from BPH. Although the observed prevalence of clinical BPH varies depending on the definition of BPH, all studies confirm that the prevalence of BPH strongly increases with age.[93,94,95,96,97,98,99]

2.1.2 Risk factors

Age, normal androgenic function, and family history are known risk factors for BPH. Other potential risk factors include race, ethnicity, geographic location, and obesity.[88,93]

2.1.3 Causes

The aetiology of BPH has not yet been completely clarified despite its wide diffusion. Different factors are thought to be involved in the development of prostate hyperplasia: sexual hormones, stromal-epithelial interactions, stem cells, growth factors, insulin, and prolactin.[100] Among these, sexual hormones, in particular testicular androgens and estrogens, are recognized as playing the most important roles both in the physiological growth of the prostatic gland and in the development of BPH. It is believed that the condition is associated with very particular hormonal changes that occur as men age (see: 3.1).

2.1.4 Signs and symptoms

Patients with BPH often express urinary symptoms such as urge incontinence, dribbling, slow stream, and difficult voiding as a direct result of the pressure exerted by the enlarged prostate gland on the urethra thereby squizing it. These symptoms are called lower urinary tract symptoms (LUTS). LUTS, however, are not specific for BPH alone. Identical symptoms can also be caused by other urological conditions (e.g., urinary tract infections, detrusor instability) or other non-urological conditions such as heart failure and diabetes mellitus. The correlation between the severity of LUTS, prostate size, and the degree of obstruction is weak. Men with large prostates may be symptom-free, while those with small or normal-sized prostates may sometimes have symptoms that are more severe than in men with larger prostates.[88,93,101]

LUTS are generally classified into voiding symptoms (hesitancy, poor urinary flow and need to strain, incomplete bladder emptying, terminal or postmicturition dribbling) and storage symptoms (frequency, urgency, nocturia, urge incontinence). To quantify the severity and the extent of the LUTS, men are generally asked to complete symptom questionnaires.[88,93,94]

2.1.5 Diagnosis

The diagnosis of LUTS/BPH is made on the basis of medical history, physical examination including a digital rectal examination, urinalysis and uroflow measurements, and/or transrectal ultrasound (TRUS).[93,101]

2.1.6 Current treatment methods

The primary goals of treatment for BPH are to reduce the symptoms, to improve the urinary flow, and, to prevent progression. Not every patient with LUTS/BPH is treated and some patients are followed with the so-called *watchful waiting strategy*. Watchful waiting involves lifestyle changes such as avoiding alcohol, coffee, and certain drugs (e.g., diuretics, decongestants).[88,93,101] The choice between watchful waiting and treatment depends on a number of factors such as the severity of the symptoms, the prostate size, and the urinary flow rates. Generally, watchful waiting is recommended in patients with mild symptoms. Patients with moderate or severe symptoms are *pharmacologically treated* or undergo *prostate surgery*. The decision to opt for a specific treatment regimen is based on LUTS symptom severity and the patient characteristics such as age and medical conditions.[88,93]

The pharmacological treatments of obstructive and irritative symptoms associated with BPH include hormonal and non-hormonal therapies.[100,102,103,104,105] Hormonal or endocrine therapy with androgens and estrogens used in the past may reduce symptomatology of BPH, but the serious side-effects related to this kind of therapy are unacceptable. Studies on the pathogenic mechanism of BPH highlighted that pharmacological agents including antiandrogens, antiestrogens, and inhibitors of 5α-reductase and of aromatase may be effective in the therapy of BPH.[105] Furthermore, it is very important to take into consideration that, for an effective treatment of BPH, a reduction of both the glandular and stromal elements must be achieved. Thus, both androgens and estrogens seem to be involved in the regulation of prostate (over)growth. Therefore, a combination of androgen and estrogen deprivation might be a more promising approach than any single treatment.[106] Non-hormonal therapy includes treatment with α1-blockers that bind to α1-adrenoreceptors in the bladder neck and in the smooth muscle tissue of the prostate causing relaxation and thus improving urinary flow. Some of these drugs may also provoke apoptosis of the prostate epithelium.[88,93,101]

Recently, results from large randomized controlled trials have been published showing that treatment with 5α-reductase inhibitors and, especially, the combination of 5α-reductase inhibitors with an α-blocker were not only beneficial in the relief of urinary symptoms, but were also able to prevent disease progression in terms of acute urinary retention, prostate surgery, and urinary tract infections.[107,108]

In traditional medicine, various plant extracts are used in the treatment of urological disorders related to BPH. These extracts contain several active constituents that are endowed with antiandrogen and antiestrogen activity, inhibitory activity on prostaglandin synthesis, antiinflammatory and immunomodulatory effects, cholesterol-lowering activity, and interaction with sex hormone binding globulin (SHBG).[109,110] The pharmacological effects exerted by extracts are not directly related to the activity of the single components, but, probably, to their additive and/or synergistic actions. The exact mechanism for most plant extracts remains unclear and their efficacy needs to be further investigated in well-designed, randomized, controlled trials.[93,111] Positive monographs on Sabal fructus (*Serenoa repens* (Bartram) JK Small, fruit), Urticae radix (*Urtica dioica* L., *U. urens* L. and/or on their hybrids, root), and Cucurbita peponis (*Cucurbita pepo* L., seed) have been published by the Commission E for the Phytotherapeutic Substances of the German Federal Health Office. These extracts are reported to improve urological disturbances in patients with mild and moderate BPH, while the size of the prostate is, in most cases, not influenced by the phytotherapeutical agents. The extracts are reported to be well tolerated and to have minimal side-effects with a very good risk/benefit ratio.[105] Next to these extracts, research into herbal products used for BPH has also focused on pygeum (African prune tree, *Pygeum africanum* Hook. f., *Prunus Africana* Hook. f.). Herbs such as *Panax ginseng* (also known as Korean ginseng or panax) have a history of traditional use as “male tonics” and are still used by herbalists to treat prostatic enlargement.[112] In Italy, plant extracts are prescribed in almost 50% of cases, whereas α-blockers and hormonal therapies are both used around 5%.[109] Plant extracts of the fruit of *S. repens* and from the bark of *P. africanum* Hook. f. constitute the active principles of pharmaceutical proprietary products currently used in the symptomatic treatment of BPH.

2.2 Prostate cancer

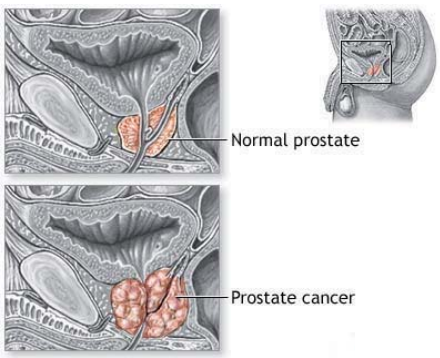

Figure 2.5 Normale prostate versus prostate cancer (http://health.allrefer.com/pictures-images/).

Prostate cancer (PC) is the presence of a *malignant tumor* of mostly glandular origin in the prostate gland. A *tumor* or *neoplasm* is defined as an abnormal mass of tissue that results from excessive cell division. As cancer is always *malignant*, it signifies growth of a tumor with a tendency to invade and destroy nearby tissue and spread to other parts of the body through the bloodstream and lymphatic system, a process called metastasis. The word *cancer* originates from the Latin word for crab, because the swollen veins around a surface tumor appear like the legs of a crab. Cancer can be classified either by the cell type or by its primary site. Although there are several cell types in the prostate, over 95% of the prostate cancers originate in the glandular tissue and these types of cancer are referred to as adenocarcinomas. About 70% of these adenocarcinomas are derived from the glandular tissue of the peripheric zone. Prostate cancer often starts out localized in the prostate, but can then spread into adjacent tissue structures and lymph nodes if not detected early. The most common site of metastatic disease is the bone, but it can also spread to the lung, liver, and other organs. The most common cause of death from prostate cancer continues to be hormone-refractory metastatic disease.[88]

2.2.1 Incidence and prevalence

According to the American Cancer Society (ACS), prostate cancer is the most common malignancy, other than skin cancer, and the second leading cause of cancer death in men, second only to lung cancer, in the United States.[113] In some Western countries, prostate cancer has become the leading cause of cancer deaths.

As our population ages, the impact of prostate cancer increases and the development of new treatments is necessary to control the disease. No other cancer rises in incidence and mortality rate with increasing age as rapidly as does prostate cancer. However, much of the

increased incidence has been related to public awareness and to the identification of a large number of prevalent, but previously undetected cases in the population through widespread screening using the prostate-specific antigen (PSA)-test.[114] The overall prognosis for prostate cancer patients has dramatically improved compared with years ago. Over the past 20 years, the overall survival rates for all stages of prostate cancer combined have increased from 67% to 97%. This means that more men are living longer after diagnosis.

Prostate cancer has a disproportional impact on certain segments of the human population. African-American men have the highest incidence of prostate cancer and are twice as likely to die from it than other Americans. Asian and native American men have the lowest incidence. It is observed that, as people migrate from Asia to the United States, the low rates observed in Asia begin to rise with time and with subsequent generations towards those observed commonly in the general population in the United States. Furthermore, as Asian countries adapt Western-style diets, the incidence of prostate cancer has started to rise.[115,116,117,118] These observations suggest an environmental connection (e.g., high-fat diet, smoking).

2.2.2 Risk factors

The lifestyle risk factors accompanying prostate cancer include a lower risk associated with high intake of fruits, vegetables, fibre, and soy products. Alternatively, a higher risk is associated with increased intake of red meat, animal fats, dairy products, and steroid exposure, as well as body mass and birth weight. Other risk factors include age, race and nationality, exposure to sex steroids and genetic predisposition or family history.[115,116,117,118,119]

2.2.3 Signs and symptoms

In contrast with BPH, early prostate cancer usually does not cause any symptoms, because the cancerous gland is not necessarily enlarged. However, the tumor may spread from the prostate to surrounding areas and metastasize to nearby lymph nodes, bones or other organs. At first, the symptoms are often similar to those of BPH. However, while the cancer spreads, symptoms will get worse going from frequent urination (especially at night) with a weak or interrupted urinary flow to inability to urinate, blood in the urine or semen and the

presence of pain or stiffness in the back, hips, upper thighs or pelvis during ejaculation and urination (dysuria). All these signs should raise suspicion.[89]

2.2.4 Diagnosis

The diagnosis of early prostate cancer is done by a combination of PSA and digital rectal examination (DRE). Once a tumor is found a transrectal ultrasound (TRUS)-guided biopsy will be performed to determine if the tumor is benign or cancerous. Computer-assisted tomography scan (CAT scan or CT scan) and bone scans are carried out to visualize metastasis of the cancer.

2.2.5 Current treatment methods

The treatment strategy for prostate cancer depends on the stage of the disease and the patient's age and overall health. When cancer is confined to the prostate gland (local prostate cancer), the disease is usually curable. The urologist will choose between the watchful waiting strategy or surgery. In the case of advanced or metastatic prostate cancer, however, the disease can not be cured and a palliative therapy will be initiated. This includes *endocrine treatment* all or not in combination with chemotherapy.

Endocrine therapy is a softer form of therapy supporting the patient for prolonged periods with minimal side-effects, hence, patients can live more comfortably than do many patients who are receiving chemotherapy.[120] The main forms of endocrine therapy for prostate cancer interfere in some manner with the basic pathway of androgen stimulation of prostate cancer cells (**Figure 2.6**). Androgen deprivation can be achieved by bilateral orchiectomy (surgical castration), estrogens, LHRH agonists, antiandrogens or a combined androgen blockade.

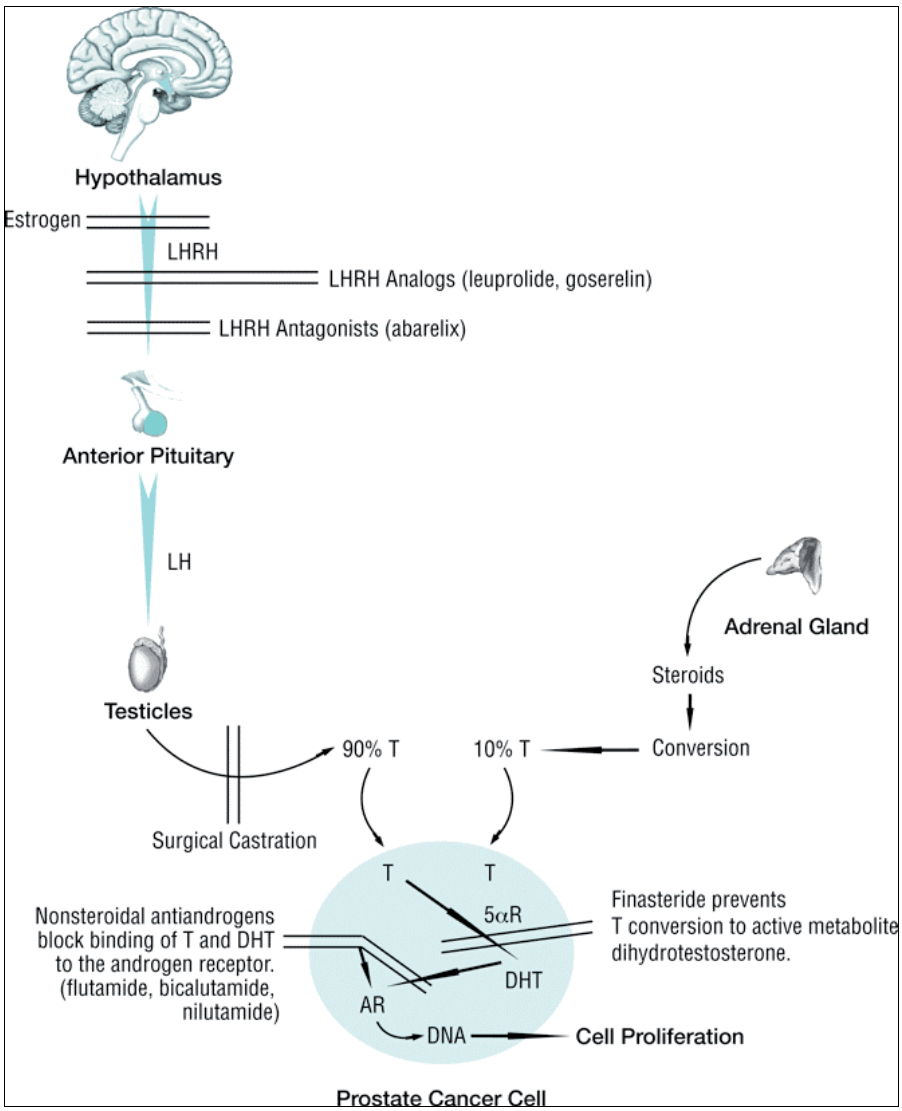

Figure 2.6 Key physiologic pathways of endocrine therapy for advanced prostate cancer. Each form of therapy interferes in some way with the basic pathway of androgen stimulation of prostate cancer cells. LHRH, luteinizing hormone-releasing hormone; LH, luteinizing hormone; T, testosterone; DHT, dihydrotestosterone; 5αR, 5α-reductase; AR, androgen receptor; DNA, desoxyribonucleic acid (http://www.postgradmed.com/issues/1996/09_96/taub.htm).

PC patients and those with BPH are increasingly exploring the use of evidence-based CAM (Complementary Alternative Medicine) treatments,[121] especially due to the risk of mortality and long-term morbidity associated with surgical procedures.[122] Moreover, hormonal manipulation, α-adrenergic blockers, and 5α-reductase inhibitors may often have unpleasant and undesirable side-effects.[123] These medications may also be ineffective due to the grade of tumor at presentation or hormone-insensitive profile of the tumor cell population.

Several phytomedicines are used for prostatic disorders. Some herbals hold potential promises, although much research is still required to investigate the mechanism of action and the compounds that account for this activity. Promising plants to treat prostate cancer are *Bidens pilosa* L., which is better known as common black-jack, *Hypoxis hemerocallidea* Fish. and C.A. Mey. (syn. H. *rooperi* S. Moore), commonly known as the African potato, *Prunus africanus* (Hook. f.) Kalkm., also known as *Pygeum africanum* Hook. f., *Serenoa serrulata* Hook. f., commonly known as saw palmetto, *Urtica dioica* L. or stinging nettle, summer pumpkin (*Cucurbita pepo* L.), and rye grass pollen (*Secale cereale*).

3 Chemoprevention of prostate diseases

Chemoprevention consists of the administration of drugs or other agents aimed at preventing the induction or inhibiting or delaying the progression of cancer. It is hypothesized that even a modest delay in the development of prostate cancer could substantially reduce the clinical impact of this very prevalent disease.[124] With its long latency time, high incidence, significant morbidity, and mortality, well-documented risk factors, hormone dependency, the availability of a widely accepted ideal marker (prostate-specific antigen, PSA), and precursor lesions such as prostatic intraepithelial neoplasia (PIN), prostate cancer is a relevant target for chemoprevention.[125] The development of chemopreventive strategies requires a proper knowledge of the pathogenesis of prostate diseases and identification of the agents that can interfere with the mechanisms involved in this pathogenesis.

3.1 Pathogenesis of benign prostate hypertrophy and prostate cancer

The prostate, a hormone-dependent gland, grows rapidly from adolescent to the early twenties,[126] when the gland attains its maximal size and homeostasis is established. A growth regulatory balance is then achieved between the processes concerned with cell proliferation and those related with programmed cell death or apoptosis.[127,128] This homeostasis, however, is not always established.

Studies carried out by Coffey et al. indicate that *microscopic benign prostate hypertrophy (BPH)*, hyperplasia of the epithelial cells, can be recognized in the prostates of men in the early twenties and the prevalence increases with age.[127] This focal process that specifically develops periurethrally in the transition zone of the gland leads to nodular hyperplasia. The process would be seen as a consequence of an imbalance in the growth-regulatory mechanisms with 5α-dihydrotestosterone (DHT) having a pivotal role.[115,127] The driving force for the development of this epithelial hyperplasia is, therefore, considered to be of androgenic nature. Bostwick et al. presume that microscopic BPH could lead to genetic instability and early cancer, possibly giving rise to transition-zone cancer, which constitutes approximately 15-20% of cancers of the prostate gland.[129] However, it is generally believed that men diagnosed with BPH do not have an increased risk of prostate cancer and that there is no causal connection between both diseases, but both conditions can and do exist together.

Although preliminary stages in the development can be recognized many years earlier, BPH only becomes clinically manifest after 50 years of age. Aging is, therefore, a risk factor for both diseases. Since early castration prevents the development of clinical BPH as well as cancer[125] and, since an enlarged prostate regresses following androgen withdrawal therapy, the pathogenesis of these diseases would seem to be mediated by androgens, either directly or passively. There is no doubt that androgenic steroids are important mitogenic factors within the prostate and a testicular source of testosterone (T) is a prerequisite to the pathogenesis of prostate disease.

Paradoxically, testicular function and T concentration would generally decline with aging. This becomes evident from the age of 45-50 years on, as far as the concentration of total T is considered. There is an earlier and stronger decline in the free T-fraction compared to the total T-concentration, as a result of the increased binding capacity for T in the serum. The decline in androgen concentrations with aging is also partially attributable to impaired hypothalamic secretion of luteinizing hormone releasing hormone (LHRH) and decreased stimulation by luteinizing hormone (LH), but there is also evidence of a primary testicular deterioration. The concentration of the plasma estrogens is, however, sustained by increased peripheral aromatization due to increased adipose tissue in later years. The result is an enhanced 'estrogenic status' in older men, which is believed to be the trigger for the development of BPH.[103,130,131]

T, the major circulating androgen hormone, diffuses across plasma membranes and is transformed into its active metabolite DHT within the prostatic gland by 5α-reductase. DHT binds to cell androgen receptors at the level of the epithelial and stromal structures, thereby promoting the synthesis of nuclear mRNA and proteins, and cell growth. Even though the concentration of T in the plasma decreases, it is reported that the DHT concentration within the prostate gland increases with aging and this phenomenon could be involved in the development of BPH.[103] This deposition and retention of DHT in the prostate could be explained by the abnormal enzyme activities present in BPH tissue compared to normal tissue: increased activity of 5α-reductase, decreased activities of 3α-hydroxysteroidreductase and 17β-hydroxysteroidoxidase, enzymes which control the reduction of DHT to androstanediol and the conversion of DHT to androstanedione, respectively (**Figure 3.1**).[105]

BPH is primarily a stromal disease from a morphometric point of view.[103,132] Estrogens that are formed from T and androstenedione in men by the aromatase enzyme system may contribute to the proliferation of stroma cells. Interaction between the stromal and the epithelial compartment is also involved in the development of BPH. In particular, the proliferation of stroma cells induced by estrogens could stimulate androgen-dependent epithelial growth.[100,104,133]

The observed estrogenic shift in aging men is also related to the increase in sex hormone binding globulin (SHBG), the synthesis of which is being induced especially by estrogens, but with a higher binding affinity for androgens than for estrogens. Consequently, the plasma concentration of free, biologically active T decreases and, therefore, the androgen/estrogen balance shifts further towards the estrogens.[134] SHBG also has a direct effect by binding to the receptor sites on cell membranes of the human prostate. Therefore, it could play an important role as regulator of biochemical cell functions.[135] Stromal growth factors seem to be involved in the prostate hyperplastic process. The basic fibroblast growth factor (b-FGF), which is present in higher amounts than normal in BPH tissue, is most important.[100,105]

Prostate cancer (PC) is a complex, multi-stage process encompassing the premalignant hyperplastic lesions and cancer initiation, through promotion and progression to the invasive metastatic disease. Premalignancy, described as atypical hyperplasia, a diffuse or multifocal proliferation of ductal or epithelial tissue,[136] is more prevalent in prostates associated with cancer. The clinically asymptomatic 'latent carcinoma' is a lesion that is originally recognized in approximately 30% of men aged over 50 years,[137] men of all races from both East and West.[138] It should be considered an early stage (a slowly growing lesion) in the development of the aggressive cancer phenotype and it would appear that a small proportion of these cancer foci progress to invasive disease in Western men, but less in their Eastern male counterparts. The recognition that prostate cancer progression is observed much less in the Asian male certainly deserves consideration and the important question is, therefore, whether dietary factors influence the development and progression of these early prostate cancer lesions.[130]

In the pathogenesis of prostatic cancer, it is generally considered that androgens would play at least a permissive role and there is a discussion on whether early prostate cancer is

specifically DHT-dependent[139] such that a 5α-reductase inhibitor would restrain the progression of the cancer. Androgen insensitivity is implicated in prostate cancer progression to the so-called 'androgen-independent' state. Androgen sensitivity may be greater than had hitherto been suspected and greater sensitivity may be conferred on cancer cells. Since the expression of the androgen receptor (AR) is maintained in most androgen-resistant prostate cancers, the presence of AR may offer a growth advantage, even in the presence of extreme low concentrations of androgens.[130]

In the prostate gland, androgens in particular, but also estrogens and glucocorticoids, modulate the production and the biological activities of the various growth regulating factors, the *intrinsic factors*. Steroid hormones should, therefore, be considered the *extrinsic factors*. These growth factors directly stimulate cell proliferation. The intercellular signalling systems, whereby a growth factor made by one type of cell is released to influence another cell type (paracrine effect), or, alternatively, affects the type of cell from which it is produced (autocrine effect), is orchestrated by the steroid hormones. The concept is that DHT is formed in the stromal compartment and then promotes the production of the keratinocyte growth factor (KGF) by the fibroblasts, which, through a paracrine effect, induces epithelial cell proliferation. As part of the homeostatic status, a growth-inhibitory factor, the transforming growth factor-β (TGF-β), inhibits proliferation and promotes apoptosis or cell death. The binding of the growth factors to cell membrane receptors induces, by way of the tyrosine-specific protein kinase (TK) enzyme, the intracellular signal transduction processes that activate genes such as the Fos and Jun proto-oncogenes, growth-related genes that encode for the Fos and Jun proteins, respectively. The Fos and Jun proteins as tissue factors (TFs) interact with their own specific (AP-1) recognition sites on the genome. The TFs are depicted in juxta-position to the DHT-AR complex on the genome, the Fos-Jun proteins associating as heterodimers on their recognition sites. As such, the modulating influence exercized by the DHT-AR complex on the growth factor activity relates to the capacity of the complex to alter the spatial orientation of DNA such that the change facilitates easier access of the TFs to their recognition sites. This interrelationship between the signalling pathways that is promoted by androgens and the other pathways mediated by peptide growth factors is referred to as '*cross-talk*' and is probably pivotal to the growth-regulatory control and the concept of steroid sensitivity.

The presence of a relative high concentration of the estrogen receptor α (ERα) in the smooth muscle cells of the stroma of the prostate suggests an important physiological role for estrogens in prostate development, as well as in prostate cancer pathogenesis. It is accepted that 17β-estradiol helps to sustain AR levels within the prostate and it has been suggested that estrogens may be essential for the androgen-mediated promotion of epithelial proliferation and, like DHT, may also inhibit apoptosis.[140] 17β-Estradiol probably promotes stromal hyperplasia by inducing the production of fibroblast growth factor-II (FGF-II). The *cross-talk* between estrogens and signalling pathways of growth factors, and the synergistic functional interaction between ERs and co-activators and co-repressors in juxta-position of the genome are probably different for both ERs. The ER-mediated signalling pathways seem more complex and could have a role in sustaining prostate cancer progression.[130]

3.2 Chemopreventive agents and targets for BPH and PC

Chemopreventive agents have the potential to influence one or more of the targets in the pathogenesis of prostate diseases. A significant proportion of the promising chemopreventive agents are phytonutrients from food, whose potential has been demonstrated in chemopreventive trials. The marked differences in the incidence of certain types of cancers between various countries or regions of the world suggest that particular dietary factors can influence the biological processes related to carcinogenesis. In the past decade, data have been accumulated supporting the role for such dietary factors and, although often contradictory, a pattern is recognisable, which directs attention to the potential benefits for healthcare of preventive studies related to the intake of these factors.[141] Geographical differences in cancer incidence attributed to the diet are supported by changes observed in the incidence rates in migrants, who move from areas of low risk for particular cancers to countries where the risk is higher.[118] Such changes are illustrated by data from the migration of Japanese and Chinese men and women to Hawaii and to mainland USA, whose risk of developing prostate or breast cancer (previously being low) increases to match that of the indigenous population within a few generations.[142,143,144]

Cancers of the prostate and breast, cardiovascular disease, and osteoporosis feature prominently as 'Western-type diseases'. The Western-style diet is characterized by high-meat and high-fat, and low-fruit and low-fibre intakes, which contrasts with the diet of the Asian communities that are low in animal fat and rich in starches, legumes, fruit and vegetables, all

of which have a high fibre content. Predictably, therefore, animal fat is considered a causative factor. More recently, however, it has been recognized that certain constituents of the Asian diet and that of the Mediterranean region protect against the development of these diseases and the lack of these constituents in the Western diet is the most important factor.[130,141]

The health-beneficial effects of these factors regarding prostate diseases indicate the ability to interfere with one or more targets of the pathogenesis process. Many of these health-protective constituents belong to the phytoestrogens that could potentially exert an influence on hormone-dependent diseases such as BPH and PC. Phytoestrogens are non-steroidal plant-derived compounds that structurally or functionally mimic mammalian estrogens and, therefore, are considered to play an important role in the prevention of cancers, heart disease, menopausal symptoms, and osteoporosis. Their structures relate closely to the structure of the endogenous estrogen 17β-estradiol in that they usually possess at least two phenolic groups adequately positioned. Phytoestrogens constitute a diverse group of compounds with regard to their chemical structures as well as their biological activities. In general, four main groups are distinguished, namely isoflavonoids, flavonoids, coumestans, and lignans.[145,146] Many plants contain phytoestrogens, although the amounts and the combinations of the various compounds vary significantly. For *isoflavones*, the main dietary source for humans is soybean, while *flavonoids* are more widely distributed in the plant kingdom, as they are found in several vegetables, fruits, berries, herbs, and also in green tea. For *coumestrol*, the only significant sources are sprouts of alfalfa and various beans. *Lignans* are present in fibre-rich foods such as flaxseed, unrefined grain products, rye, and some berries. However, their active forms (enterodiol and enterolacton) are only formed after metabolisation by the gut microflora.[147,148,149,150,151]

Clinical evidence points to the beneficial role of soya in inhibiting the growth of prostate cancer in Japanese men.[152,153] The advantages of a soya-based diet have been attributed to isoflavonoids.[154] There is an inverse relationship between the consumption of soya and the incidence of clinically significant prostate cancer,[155] furthermore between coumestrol and daidzein and prostate cancer risk.[156]

Although there are considerable experimental data regarding the antitumoral effects of several soya-derived products, large-scale epidemiological studies are sparse. A recently published work aimed to identify variables for prostate cancer mortality from data collected in

59 countries.[157] Prostate cancer was inversely associated with estimated consumption of cereals, nuts, oil seeds, fish, and, in particular, soya products. While dietary phytoestrogens have been implicated in prostate cancer prevention, they are now under investigation as a possible treatment for prostate cancer, in view of their multiple mechanisms of action that may or may not be related to their hormonal activity.

3.2.1 Interaction with hormonal regulation

Hormonal regulation is controlled by the hypothalamus and the anterior pituitary gland, which influence both the testicular activity and the adrenal activity (**Figure 3.1**). The two principal functions of the male hypothalamic-pituitary-gonadal axis are production of regulated quantities of physiologically relevant sex steroid hormones and production of healthy male gametes under diverse environmental conditions. The two corresponding activities of the testes are segregated anatomically. Androgen biosynthesis occurs in Leydig cells and spermatogenesis is accomplished in the seminiferous tubules. The hypothalamus and the anterior pituitary gland participate integrally in the regulation of these functions via respective secretion of gonadotropin-releasing hormone (GnRH) (formerly called luteinizing hormone-releasing hormone or LHRH) by specific brain stem neurons and subsequent stimulation of the release of the two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by specific adenohypophyseal cells. The amounts of the released hormones are dependent on the age and the hormonal status of the individual both before and after birth.

Circulating androgens exert a direct negative feedback on the pituitary gland and an indirect negative feedback on the hypothalamus after conversion into estrogens by the aromatase enzyme. Circulating estrogens exert direct negative feedbacks on the hypothalamus, pituitary gland, and on their own production from cholesterol in the Leydig cells. This decreases the frequency of the GnRH pulse generator and, consequently, the quantity and the quality of bioactivity of released LH molecules, thereby decreasing the production of steroids.

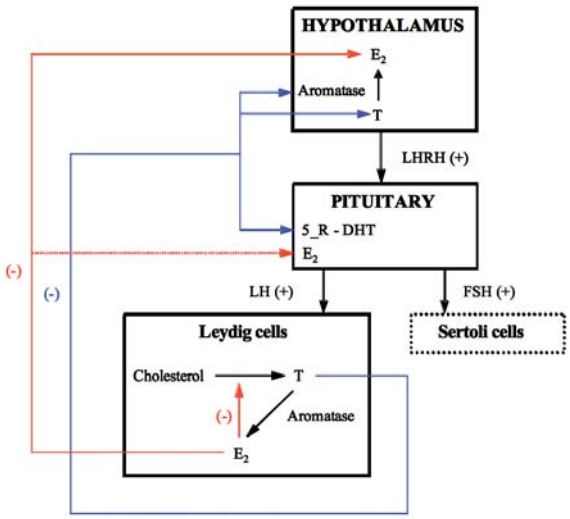

Figure 3.1 Hormonal regulation in men.
E_2: 17β-Estradiol, T: Testosterone, 5αR: 5α-Reductase, DHT: 5α-Dihydrotestosterone, LHRH: Luteinizing hormone-releasing hormone, LH: Luteinizing hormone, FSH: Follicle-stimulating hormone, (+): stimulation, (-): inhibition.

The protective effect of phytoestrogens could be related to the reduction in androgen production via the hypothalamic-pituitary-gonadal axis leading to a reduced gonadal T production, however, so far there is no evidence of this effect.[158]

3.2.2 Interaction with the steroid metabolism

About 95% of the total amount of T is produced in the testes by the Leydig cells. The remaining 5% is secreted by the adrenal glands, which are situated on top of the kidneys. Circulating T is bound by proteins in the plasma and the small unbound fraction passively diffuses into prostate cells, where it is converted to DHT by 5α-reductase located on the nuclear membrane. DHT, on its turn, binds to the androgen receptor in the cytoplasm and the DHT-AR complex is then transported to the nucleus to directly modulate gene expression and stimulate cell division.[130,159]

Estrogens are primarily produced from T by the aromatase enzyme. About 30-40% of the estrogens in plasma is synthesized and secreted by the Leydig cells in the testes, the remainder being derived from the peripheral aromatization of adrenal androgens by adipose

and muscular tissue. Because this adipose tissue is a major source of aromatase, the sensitive ratio of estrogens to androgens can be altered by the body mass and the degree of fat within the body.[160] **Figure 3.2** demonstrates the hormonal conversions and the relevant enzymes in men.

3.2.2.1 Inhibition of the human 5α-reductase enzyme

The conversion of T to its potent derivative DHT through the 5α-reductase enzyme activity represents a crucial step for androgen-mediated activity on the prostate gland. Compounds that inhibit or influence this conversion could indirectly diminish the androgenic stimulatory influence on the prostate.

3.2.2.2 Inhibition of the 17β-hydroxysteroid-dehydrogenase

17β-Hydroxysteroid-dehydrogenase regulates the reversible interconversion between testosterone and androstenedione, on the one hand, and, estrone and 17β-estradiol, on the other hand.[161] Compounds that affect this conversion could influence the steroid metabolism, thereby altering the hormonal influence on the prostate gland.

3.2.2.3 Inhibition of the human aromatase-enzyme system

The human aromatase-enzyme system regulates the production of estrogens from the C19-androgen precursors such as T and androstenedione. The system is an enzyme complex consisting of two components, a flavoprotein NADPH-cytochrome P-450 reductase, which transfers electrons from NADPH to the terminal enzyme, and a specific form of cytochrome P-450 known as aromatase cytochrome P-450.[162] Aromatization occurs in specific tissues. About 30% of the plasma estrogens is synthesized and excreted in the testes, where aromatization constitutes the last and one of the most important steps in the biosynthetic progression from cholesterol to estrogens. Aromatization also occurs in adipose tissue and, therefore, increases in aging men resulting in a change in hormonal balance and a increased risk to develop BPH. Therefore, specific inhibitors of the aromatase-enzyme system could be useful in controlling pathologic conditions associated with estrogens, especially BPH and maybe also PC.

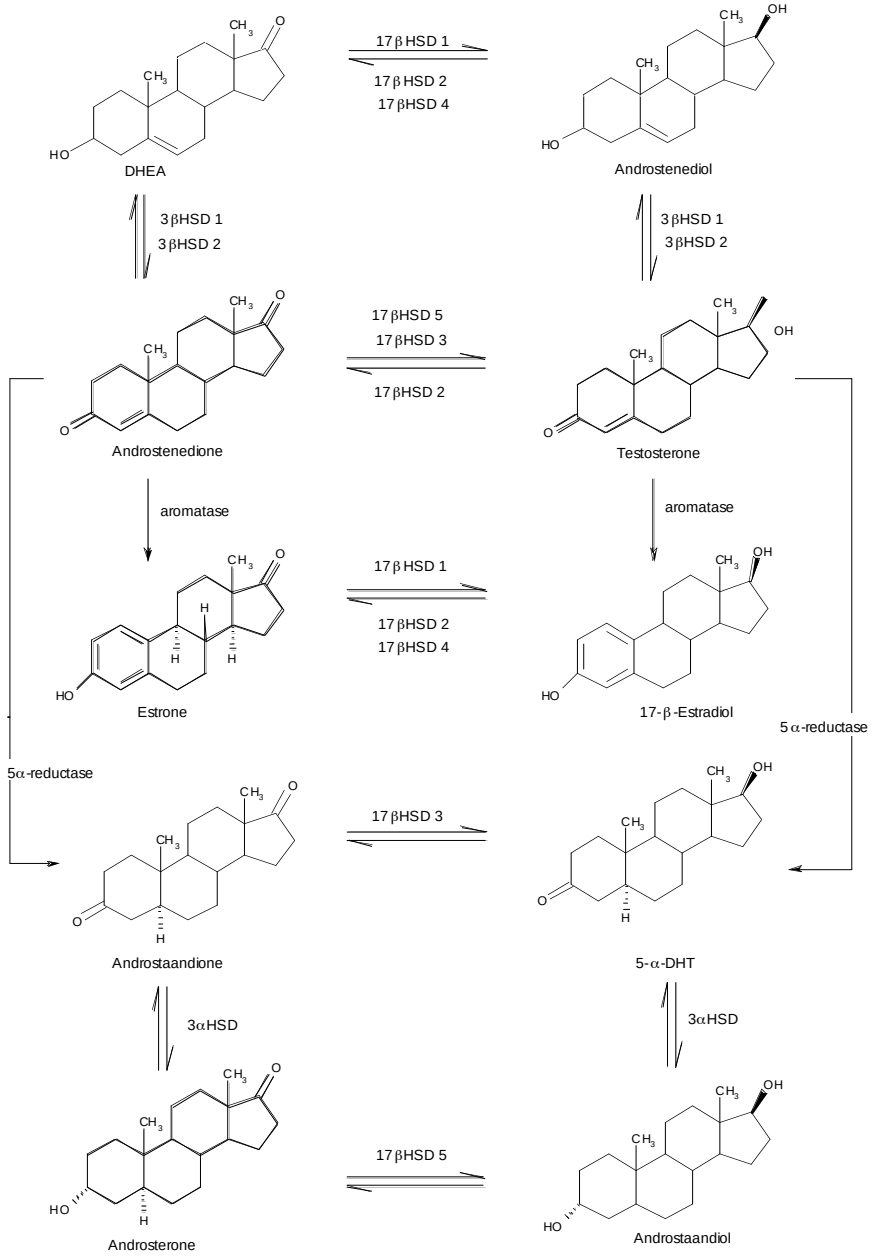

Figure 3.2 Steroid metabolism in men.
DHEA: Dehydroepiandrosterone, 5α-DHT: 5α-Dihydrotestosterone, 3αHSD: 3α-Hydroxysteroid-dehydrogenase, 3βHSD 1 and 2: 3β-Hydroxysteroid-dehydrogenase 1 and 2, 17βHSD 1-5: 17β-Hydroxysteroid-dehydrogenase 1-5.

Numerous *in vitro* studies have shown that phytoestrogens such as the lignane enterolactone and the soya-derived isoflavone genistein inhibit several of the key enzymes in estrogen and androgen biosynthesis including 17β-hydroxysteroid-oxidoreductases, aromatase, and 5α-reductase.[160,163,164,165] None of these actions have been confirmed *in vivo*. However, it was demonstrated that the levels of the indicators for the activities of 5α-reductase, namely 3α,17β-androstanediol and androsterone glucuronide, were much lower in young Japanese men compared to young men in the United States.[166] This suggests that phytoestrogens may modulate the production and the bioavailability of endogenous steroidal estrogens and androgens, hence, they could alter the exposure to these hormones resulting in hormone- or antihormone-like activity.

3.2.3 Interaction with the steroid receptors

Even though the mechanism of action is not fully understood, there is no doubt that the presence of steroid hormones, more specifically androgens and estrogens, are predominant in the development and evolution of BPH and PC. Compounds that can interfere with the hormonal activities could alter the hormonal influence on the prostate and, thereby, also the pathogenesis of BPH and PC.

Several *in vitro* studies have indicated that phytoestrogens act similarly to steroidal estrogens. They bind to estrogen receptors, induce estrogen-responsive genes, stimulate the growth of estrogen-sensitive cancer cells, and their activities can be blocked by pure antiestrogens.[167,168,169] However, phytoestrogens are generally weaker than the endogenous steroidal estrogens and it has been suggested that they could act as antiestrogens or partial agonists by competing with the more potent endogenous estrogens for binding to the ERs. With regard to their sensitive activities, phytoestrogens could be classified as selective estrogen receptor modulators (SERMs) that may function as agonists or antagonists depending on the tissue, ERs, and concentrations of circulating endogenous estrogens. Pure antiestrogenic activity has not been demonstrated for isoflavones or coumestrol and only few phytoestrogens with very weak intrinsic estrogenic activity including naringenin can be classified as such.[170]

Kuiper et al. have screened the binding affinities of several phytoestrogens to the ERs and showed that some phytoestrogens such as genistein and coumestrol are efficient ligands

for ERs, while other isoflavones or flavonoids bind with much less affinity.[171] Intriguingly, phytoestrogens prefer ERβ for binding, while most endogenous steroidal estrogens show no such difference.[169] This could allow unique, tissue- or organ-specific actions, different from those of endogenous hormones. Phytoestrogens stimulate the transcriptional activity of both ER subtypes at nanomolar concentrations. When phytoestrogens bind to the ERs, heat shock proteins dissociate, a change in conformation and homodimerisation occurs enabling subsequent binding to the estrogen response element (ERE), which functions to enhance the transcriptional potential of a gene. The EREs are typically located in the promoters of estrogen-responsive genes and upstream from the transcriptional start site. The ligand-activated, DNA-bound receptor then recruits co-activators, which serve as a bridge between the receptor and the general transcription machinery (**Figure 3.3**). The relative binding affinities of phytoestrogens for the ERs differ between studies, each displaying different agonist and antagonist activities. Moreover, the relative binding affinities of the phytoestrogens do not necessarily correlate with the affinities of the receptors for the ERE and the transcriptional activities of the receptor-ERE complex.[172,173,174,175]

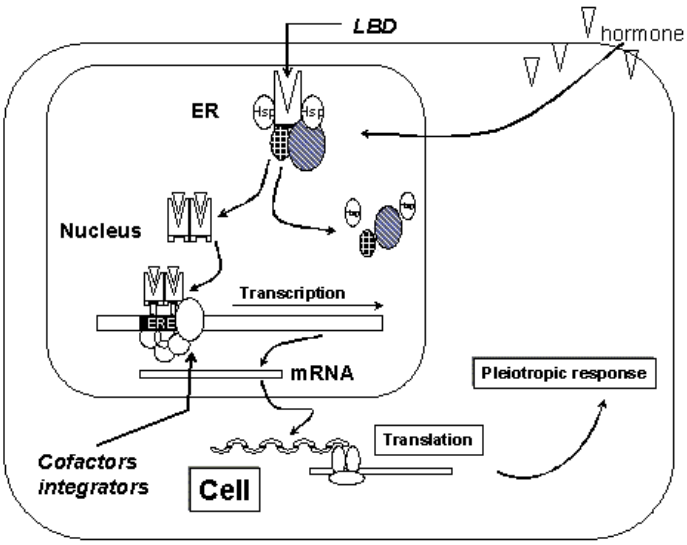

Figure 3.3 General estrogen receptor mechanism. On ligand binding (hormone), the estrogen receptor (ER) homodimerises, interacts with the estrogen response element (ERE) located in the promoter region of estrogen-activated genes and recruits co-regulator proteins, which serve as a physical bridge between the receptor and the general transcription machinery (www.andrology.be/life98/technical.php).

The potential of phytoestrogens to target the pathogenesis of PBH and PC could be expected from their preferential binding to the ERβ in the prostate epithelium, which is the predominant ER in the prostate, and is related to antiproliferative activities, but so far there is no evidence for this effect of phytoestrogens. The difference in binding affinity and activity of phytoestrogens for both ER types compared to endogenic estrogens could result in different cross-talk and different stromal-epithelial interactions at the prostate level that may change the overall hormonal influence of both androgens and estrogens on the prostate gland.

3.2.4 Non-hormonal interactions

3.2.4.1 Stimulation of the production of sex hormone-binding globulin

Of the total T in the plasma, only 2% is free, enters the prostate, and is biologically active. The stimulation of the production of sex hormone-binding globulin (SHBG) results in a decrease in the free fraction of T and an altered hormonal influence on the prostate. This phenomenon was demonstrated in vegetarian[176] and Japanese and Chinese men,[177] who had high levels of isoflavonoids in urine and plasma. However, if the concentration of free T drops under a critical level, the expression of the C-met oncogene is stimulated in the prostate cancer cells, which is associated with an increased invasive character.[178] This U-shaped connection between T levels and cancer invasiveness is a very critical balance, which should be taken into account when targeting androgen activities on the prostate. Phytoestrogens have been demonstrated to stimulate the production of SHBG *in vitro*.[179]

3.2.4.2 Interaction with tyrosine-specific protein kinases

Tyrosine-specific protein kinases are a part of the receptor proteins that are localized in the cell membrane. These receptors are necessary for signal transduction activated by several growth factors such as the epidermal growth factor (EGF), the platelet-derived growth factor, and the insuline growth factor (IGF).[180,181] Fosforylation of tyrosine plays an important role in cell proliferation and cell transformation. It was demonstrated that genistein is a tyrosine-kinase-specific inhibitor and, therefore, a compound which can influence the processes of cell proliferation and transformation.[182]

3.2.4.3 Influence on angiogenesis

Angiogenesis or neovascularization is concerned with the formation of new capillaries, a process involving proliferation and migration of endothelial cells. Normally, the process is restricted to wound healing, but is enhanced in association with tumor growth. Folkman reported that new capillary blood vesssels are necessary for a cancer to expand beyond 2 mm in size, having been involved in 27 doublings prior to that phase.[130] Angiogenesis, therefore, exercises an important role in cancer progression and is essentially seen as the growth of capillary sprouts of endothelial cells from pre-existing capillaries towards cancer foci, a process probably promoted by the production of growth factors. The fibroblast growth factor (FGF–II) and the vascular endothelial growth factor (VEGF), which is produced by neuroendocrine cells in the prostate, are potent angiogenic agents.[130] Genistein has been demonstrated to inhibit angiogenesis and endothelial cell proliferation, possibly through its capacity to influence the tyrosine-specific protein kinase of the growth factor receptor (VEGF-R) and other enzymes promoting cell growth such as topoisomerases I and II.[183]

3.2.4.4 Influence on DNA topoisomerases

DNA topoisomerases are enzymes that alter the conformation of DNA and are crucial to the process of cell division. By strand cleavage, strand passage, and religation, these enzymes untangle supercoiled DNA which simplifies the replication.[184] Topoisomerases are a well-researched target in the development of new anticancer drugs.[130] It has been shown that genistein inhibits the activity of DNA topoisomerase, which could induce apoptosis.[185,186,187]

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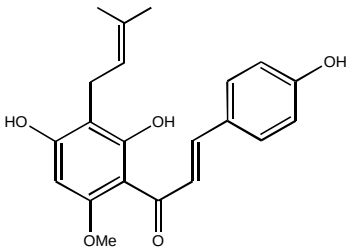
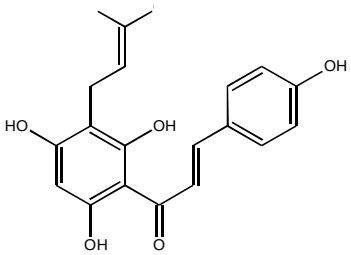
4. Isolation, purification and stability of hop-derived prenylflavonoids

4.1. Isolation and purification of xanthohumol and desmethyl-xanthohumol

Xanthohumol (X) and desmethylxanthohumol (DMX) (**Figure 4.1**) were isolated from spent hops, a residue obtained after supercritical carbon dioxide extraction of the hop variety Nugget (NATECO$_2$, Wolnzach, Germany).



Xanthohumol (X)



Desmethylxanthohumol (DMX)

Figure 4.1: Structures of the chalcones xanthohumol (X) and desmethylxanthohumol (DMX).

Spent hops (100 g) were extracted with MeOH (3 x 1 L) at ambient temperature. After filtration, the volume was reduced to about 500 ml (Büchi rotavapor R-200, 35°C), diluted with H_2O (1:1, v/v), and extracted with *n*-hexane (3 x 1 L). The upper layer was rejected and the remaining volume was reduced to about 500 ml, diluted with H_2O (1:1, v/v), and extracted with EtOAc (2 x 1 L). The organic layer was separated and evaporation of EtOAc afforded a residue (ca. 5 g) containing approximately 12% X and 1.2% DMX, as determined by HPLC. HPLC separations (Waters Alliance 2695 Separation Module, Milford, MA, USA) were achieved using a Varian Omnisphere C18 column (4.6 mm x 250 mm, 5 µm) at a flow rate of 0.9 ml.min^{-1} using a linear gradient from 45% MeOH to 95% MeOH in H_2O containing 0.05% formic acid over a time span of 29 min (conditions, see: **Table 4.1**). UV detection of X and DMX was done at 370 nm. The retention times of X and DMX were 26.9 min and 23.9 min, respectively.

The linear gradient was applied for semi-preparative isolation of X and DMX (residue dissolved in MeOH containing 0.05% formic acid [100 mg/ml]) using an Alltech Econosil C18 column (22 mm x 250 mm, 10 µm) at a flow rate of 10 ml.min^{-1} (conditions, see: **Table 4.2**). Fractions containing X and DMX were collected separately, the solvent was evaporated (Büchi rotavapor R-200, 35°C), and the residue was freeze-dried (Heto Lyolab 3000, connected to a Pfeiffer vacuum pump). Freeze-dried residues were dissolved in MeOH (50 mg/mL).

Final purification (Alltech Econosil C18, 22 mm x 250 mm, 10 µm) was achieved using isocratic elution (60% MeCN in H_2O containing 0.05% formic acid for X and 55% MeCN in H_2O containing 0.05% formic acid for DMX) (conditions, see: **Table 4.2**).

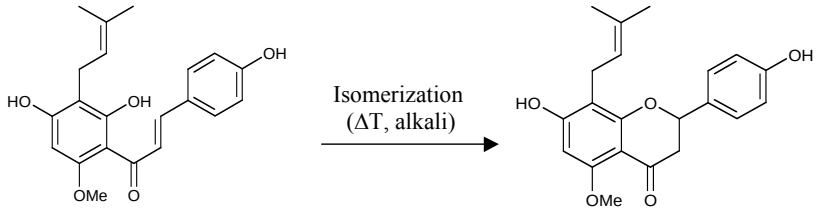
The identity of the pure compounds was confirmed by analytical HPLC (t_R and UV spectra) and ^{1}H-NMR (Varian 300 MHz). ^{1}H-NMR and UV spectra of the compounds were in accordance with those described in the literature[1,2].

Table 4.1: Conditions for analytical HPLC

HPLC apparatus	Waters Alliance 2695 Separation Module	
Sample	± 0.05 mg/mL MeOH, 0.22-µm filter	
Column	Varian Omnisphere C18; dp: 5 µm; i.d.: 4.6 mm; length: 250 mm	
Eluens	Solvent A: 0.05 % HCOOH in H_2O	
	Solvent B: 0.05 % HCOOH in MeOH	
Gradient range	45 % B in A to 95 % B in A	
Gradient course	0-3 min:	isocratic 45 % B in A
	3-32 min:	45 % B in A to 95 % B in A
	32-37 min:	isocratic 95 % B in A
	37-45 min:	95 % B in A to 45 % B in A
	45-47 min:	isocratic 45 % B in A
Flow rate	0.9 mL/min	
Injection volume	20 µL	
Detector	Photodiode Array Detector Waters 996	
Detection wavelength	X, DMX: 370 nm	
	IX, 8PN, 6PN: 295 nm	

4.2. Semi-synthesis and purification of isoxanthohumol

IX was prepared by refluxing xanthohumol (1 g) in alkaline (KOH, 0.18 M) MeOH (100 ml) for 1 h at 85°C (**Figure 4.2**). Acidification with HCl (0.18 M), removal of the solvent, addition of H_2O (150 ml), extraction with EtOAc (1 x 150 ml), evaporation of EtOAc, and lyophilization (Heto Lyolab 3000 with a Pfeiffer vacuum pump) furnished a residue, which was dissolved in MeOH at a concentration of 100 mg/ml and separated by semi-preparative HPLC (Alltech Econosil C18, 22 mm x 250 mm, 10 µm) using isocratic elution (40% MeCN in H_2O containing 0.05% formic acid) (conditions, see: **Table 4.2**). The purity (> 99%) was confirmed by analytical HPLC (Varian Omnisphere C18, 4.6 mm x 250 mm, 5 µm) (conditions, see: **Table 4.1**) and ^{1}H-NMR (Varian 300 MHz). The retention time of IX was 19.5 min. Comparison of the ^{1}H -NMR and UV spectra with literature data led to correct identification.[1,2]



Xanthohumol (X) **Isoxanthohumol (IX)**

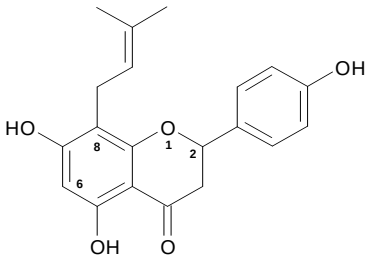
Figure 4.2: Isomerization of the chalcone xanthohumol (X) into the flavanone isoxanthohumol (IX).

4.3. Synthesis and purification of 8-prenylnaringenin and 6-prenylnaringenin

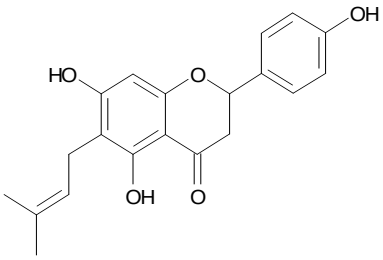
The flavanones 8-prenylnaringenin (8PN) and 6-prenylnaringenin (6PN) were synthesized according to a previously published method (**Figure 4.3**).[3]

Final purification of the mixture obtained after synthesis (Alltech Econosil C18, 22 mm x 250 mm, 10 µm) was achieved using isocratic elution (50% MeCN in H_2O containing 0.05% formic acid for 8PN and 6PN) (conditions, see: **Table 4.2**). The identity of the pure compounds and the purity (> 99%) were confirmed by analytical HPLC (Varian Omnisphere C18, 4.6 mm x 250 mm, 5 µm) and ^{1}H-NMR (Varian 300 MHz). The retention time of 8PN

and 6PN were 21.5 min and 25.4 min, respectively. Comparison of the ^{1}H -NMR and UV spectra with literature data led to correct identification.[1,2]



8-Prenylnaringenin (8PN)



6-Prenylnaringenin (6PN)

Figure 4.3: Structures of the flavanones 8-prenylnaringenin (8PN) and 6-prenylnaringenin (6PN).

Table 4.2: Conditions for semi-preparative HPLC

Prepurification		
HPLC apparatus	Gilson 322	
Sample	100 mg/mL MeOH, 0.22-µm filter	
Column	Alltech Econosil C18; dp: 10 µm; i.d.: 22 mm; lengte: 250 mm	
Eluens	Solvent A: 0.05 % HCOOH in H_2O	
	Solvent B: 0.05 % HCOOH in MeOH	
Gradient range	45 % B in A to 95 % B in A	
Gradient course	0-2 min:	isocratic 45 % B in A
	2-32 min:	45 % B in A tot 95 % B in A
	32-38 min:	isocratic 95 % B in A
	38-45 min:	95 % B in A tot 45 % B in A
	45-47 min:	isocratic 45 % B in A
Flow rate	10 mL/min	
Injection volume	0.5-1 mL	
Detector	Gilson dual wavelength UV detector (UV/VIS-156)	
Detection wavelength	X, DMX: 370 nm	
	IX, 8PN, 6PN: 295 nm	
Fraction collector	Gilson 206	

Final purification	
HPLC apparatus	Gilson 322
Sample	50 mg/mL MeOH, 0.22-µm filter
Column	Alltech Econosil C18; dp: 10 µm; i.d.: 22 mm; lengte: 250 mm
Eluens	Solvent A: 0.05 % HCOOH in H_2O
	Solvent B: 0.05 % HCOOH in MeCN
Isocratic system	X: 60 % B in A
	DMX: 55 % B in A
	IX: 40 % B in A
	8PN: 50 % B in A
	6PN: 50 % B in A
Flow rate	10 mL/min
Injection volume	0.5-1 mL
Detector	Gilson dual wavelength UV detector (UV/VIS-156)
Detection wavelength	X, DMX: 370 nm
	IX, 8PN, 6PN: 295 nm
Fraction collector	Gilson 206

4.4. Stability of the purified hop-derived prenylflavonoids

The pure hop-derived prenylflavonoids, X, DMX, IX, 8PN, and 6PN were tested for their stability in dry, solid form or dissolved in EtOH (200 mM) by storage at different temperatures (–20°C, 4°C and room temperature) during 1 year.

At regular time intervals, small fractions of the stored samples were analysed by HPLC (Waters Alliance 2695 Separation Module, Milford, MA, USA) using a Varian Omnisphere C18 column (4.6 mm x 250 mm, 5 µm) at a flow rate of 0.9 $ml.min^{-1}$ under isocratic conditions previously established for the semi-preperative purification of the compounds (conditions, see: **Table 4.2**).

In view of the use of prenylflavonoids in appropriate bio-assays, it was necessary to determine the stability of the pure hop-derived prenylflavonoids for long term-incubations at 37°C. Therefore, stock solutions of individual compounds were prepared in EtOH (200 mM), diluted with water (1:1000) and incubated at 37°C during 2 days. At the start of incubation and after every 24 h, fractions of the incubated samples were analysed by HPLC (Waters

Alliance 2695 Separation Module, Milford, MA, USA) using a Varian Omnisphere C18 column (4.6 mm x 250 mm, 5 µm) at a flow rate of 0.9 ml.min^{-1} in the isocratic conditions previously established for the semi-preparative purification of the compounds (conditions, see: **Table 4.2**).

The results demonstrate that the optimum storage conditions in dry, solid form are at a temperature of –20°C. Storage in solution promotes isomerization and especially DMX shows a time-dependent conversion at each temperature tested. Furthermore, even after storage in dry, solid form, the chalcone DMX proved to be instable. Thus, DMX must be purified just prior to use. The addition of a small quantity of acid (0.05 % HCOOH) improved the stability of this chalcone remarkably and the acidic conditions were routinely applied during storage of all prenylflavonoids. X, IX, 8PN and 6PN, were shown to be stable, as well in dry, solid form, as in solution.

Figure 4.4 shows the time-dependent conversion of DMX to the isomeric 8PN and 6PN following incubation at 37°C. After 48 h (mean incubation time of the bioassays), nearly 50% of DMX was converted into a mixture of 35% 6PN and 15% 8PN. This feature must be taken into account when interpreting the results of the bio-assays.

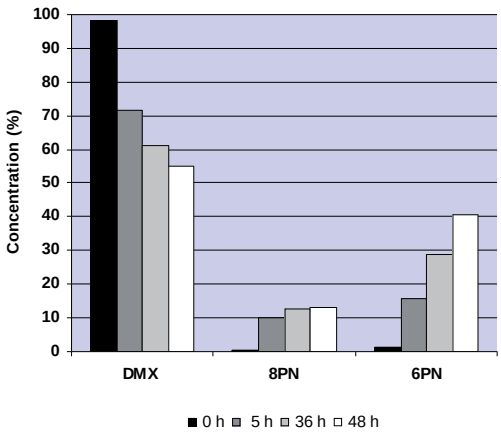


Figure 4.4: Stability of desmethylxanthohumol (DMX) and isomerization into 8-prenylnaringenin (8PN) and 6-prenylnaringenin (6PN) after incubation at 37°C during 48 h.

5. Antiproliferative, cytotoxic, antiangiogenic, and estrogenic properties of hop-derived prenylflavonoids

Development of cancer-chemopreventive and chemotherapeutic agents requires testing in *in vitro* cell culture systems prior to *in vivo* studies by appropriate animal model(s) that may be validated in final clinical trials. Models that emulate any or all of the key features of prostate cancer development and progression may prove to be valuable in understanding the mechanisms involved in carcinogenesis and in identifying useful chemopreventive agents.[4]

The need for representative *in vitro* models of prostate cancer chemoprevention and development has led to numerous attempts to establish cell lines from human prostate carcinoma and approximately 30 cell lines have been used for research purposes.[5] Molecular characterization and karyotype analysis of most commonly used cells have been described in recent studies.[6]

The LNCaP cells are unique human prostate carcinoma cells that have retained functional differentiation of prostatic epithelial cells.[7] LNCaP cells were derived from a supraclavicular lymph node of a prostate cancer patient. LNCaP cells are androgen-sensitive and they secrete prostate specific antigen (PSA) and prostatic acid phosphatase, and express the androgen receptor.[8] They have been widely used as an androgen-sensitive model for prostate cancer in both basic research and chemopreventive studies.

Other frequently applied cell lines are DU145 and PC-3 cells. DU145 cells are derived from brain metastases of an untreated prostate cancer patient, whereas PC-3 cells are derived from bone metastases of a patient with androgen-independent prostate cancer.[9,10] Both these cell lines are androgen-insensitive and they do not express PSA nor the androgen receptor.[11] These cell lines represent excellent models for the study of advanced prostate cancer and of the androgen-repressed phenotype of late events during prostate cancer progression. To study the chemopreventive or therapeutic potential of the hop-derived prenylflavonoids in prostate cancer (PC), a series of *in vitro* experiments was set up using the well-established human prostate cancer cell lines LNCaP.FGC, PC-3, and DU145. We aimed to get insights in how the hop-derived prenylflavonoids xanthohumol (X), desmethylxanthohumol (DMX), isoxanthohumol (IX), 8-prenylnaringenin (8PN) and 6-prenylnaringenin (6PN) influence the pathogenesis of PC, and in how the underlying mechanisms operate. These investigations

were performed in laboratories that possessed the expertise necessary for the purpose of each set of experiments.

The antiproliferative, cytotoxic, antiangiogenic, and estrogenic properties were studied at the laboratory of Prof. Dr. Em. F. Comhaire, Ghent University Hospital, Department of Endocrinology, Ghent, Belgium, and the laboratory of Prof. Dr. V. Castronovo, Metastasis Research Laboratory, University of Liège, Liège, Belgium.

5.1. Purpose

We aimed to determine the relative potencies of the individual hop-derived prenylflavonoids in inhibiting the growth of human cancer cells and to assess the sensitivities of the different cell lines to these compounds using the proliferation assays WST1 and SRB. Proliferation assays using human cancer cell lines are commonly recognized procedures to examine compounds of varying sources and diverse nature for their potential anticarcinogenic properties.

More specifically, we aimed at getting detailed insights into the effects of the chalcones X and DMX, and the flavanones IX, 8PN, and 6PN on the proliferation of different types of prostate carcinoma cells (PC-3, DU145, LNCaP.FGC). Each of these cell lines represents a different stage of the disease and has a different hormonal status and response. Therefore, the hormonal status of the cell lines, more specifically the presence of the estrogen recptor alpha (ERα) and beta (ERβ) mRNA transcripts, was evaluated using RT-PCR.

As the inhibitory effects of the prenylflavonoids on cancer cells may be mediated through hormonal - in particular estrogenic - mechanisms, we additionally assessed the estrogenic activity by a growth-stimulatory experiment and an estrogen-inducible yeast screening assay (yeast estrogen screen; YES) and we submitted the breast cancer cell lines MCF-7/6 and MDA-MB-231 in parallel to all experiments to serve as an estrogen-responsive reference control model.

Angiogenesis is an important process correlated to tumor growth and cancer development. Agents with potential antiangiogenic properties could be promising anticancer drugs. Therefore, we investigated hop-derived prenylflavonoids *in vitro* for their antiangiogenic activities on a human bone marrow endothelial cell line (HBME).

5.2. Introduction

Cancer in various forms is one of the leading causes of death in the human population and it has been well established that cancer incidence rates differ strikingly. Hormone-related cancers of breast and prostate have been reported to vary by as much as 5- to 20-fold between populations and migrant studies indicate that differences are largely attributed to environmental factors rather than genetics.[12,13]

Diet is probably one of the most important environmental factors influencing cancer risk and mortality, and intense research is being carried out to estimate the effects of the diet on human health. There are numerous natural products of plant origin (phytochemicals) that are of potential value as chemopreventive or therapeutic agents.

Hop (*Humulus lupulus* L.), an essential raw material for beer brewing, is a rich source of phenolic and polyphenolic compounds. Phenolic acids, catechins, proanthocyanidins, and flavonols represent the major classes and their relative proportions vary widely depending on the hop variety and the growing conditions.[14] Total concentrations of polyphenols in dried hop cones amount to few percentages. Xanthohumol (X), the most abundant prenylchalcone present in hops (concentrations up to 1%, w/w), is accompanied by its homolog, desmethylxanthohumol (DMX), albeit in lower concentrations.[1] Both chalcones are readily isomerized (intramolecular Michael-type addition reaction) to the corresponding flavanones, X giving rise to isoxanthohumol (IX) and DMX being converted into a mixture of 8-prenylnaringenin (8PN) and 6-prenylnaringenin (6PN). IX is the main prenylflavonoid in beer, since X is largely isomerized during particular stages in the course of brewing that occur at elevated temperatures.[15]

X, DMX, and IX, as well as some minor hop-derived prenylflavonoids, have been investigated for their ability to inhibit the proliferation of various human cancer cell lines *in vitro*.[16] A dose- and cell-type-dependent growth inhibition was observed for X and IX with IC_{50} values in the micromolar range for breast and ovarian cancer cells.

Recently, cancer-chemopreventive properties of X were demonstrated by cell- and enzyme-based *in vitro* bioassays using markers relevant for the inhibition of carcinogenesis during initiation, promotion, and progression.[17] Despite intensive studies on X and 8PN,[3] no data are available about their effects on prostate cancer cells.

In this study, X, DMX, IX, 8PN, and 6PN have been investigated using PC-3, DU145 and LNCaP.FGC prostate cancer cell lines. Because of the hormone-dependent nature of prostate cancer, we also aimed at correlating the observed antiproliferative effects with the estrogenic activity of the prenylflavonoids and with the effects observed with the reference breast cancer cell lines MCF7/6 and MDA-MB-231. For an adequate interpretation of the results, it was important to assess the hormonal status of all cell lines used. We investigated the presence of the ERα and ERβ mRNA transcripts in the human prostate (PC-3, DU145, LNCaP.FGC) and breast cancer (MCF7/6, MDA-MB-231) cell lines by means of RT-PCR.

Despite the well-known estrogenic activity of hops, mainly due to the presence of 8PN,[3] not all hop-derived prenylflavonoids investigated in this study have been comparatively examined for their estrogenic activity. Therefore, we additionally evaluated the estrogenic activities of these compounds using a growth-stimulatory experiment and an estrogen-inducible yeast screening assay (yeast estrogen screen; YES).[18] The correlation between antiproliferative effects, estrogenic activity, hormonal status, and hormone responsiveness of the different types of cell lines presents a matter of discussion.

Angiogenesis or neovascularization concerns the formation of new capillaries, a process involving proliferation and migration of endothelial cells. The normal process is restricted to wound healing, but is enhanced in association with tumor growth. Folkman reported that new capillary blood vessels are necessary for a cancer to expand beyond 2 mm in size having been involved in 27 doublings prior to that phase.[19] Angiogenesis, therefore, exercises an important role in cancer progression and is essentially seen as the growth towards foci of cancer of capillary sprouts of endothelial cells from pre-existing capillaries, a process that is probably promoted by cancerous production of growth factors. The fibroblast growth factor II (FGF-II) and the vascular endothelial growth factor (VEGF) (produced by neuroendocrine cells in the prostate) are potent angiogenic agents.[19]

Pepper et al. demonstrated that 8PN inhibits angiogenesis induced by basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) or by the two cytokines in combination, with an IC_{50} of between 3 and 10 μM using bovine microvascular endothelial cells derived from the adrenal cortex (BME cells) and from the thoracic aorta (BAE cells).[20] CAM (chorioallantoic membrane) assay results showed reductions in both vessel lengths and vein diameters.

Because of the observations for 8PN, we embarked on a first screening of antiangiogenic effects of the hop-derived prenylflavonoids by measuring antiproliferative and cytotoxic activities using a human bone marrow endothelial cell line (HBME).

5.3. Materials

5.3.1. Hop-derived prenylflavonoids

The hop-derived prenylflavonoids X, DMX, IX, 8PN, and 6PN were isolated and purified from spent hops as described in chapter 4.

5.3.2. Chemicals

17β-Estradiol (E_2; > 98% purity) and HEPES (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) were purchased from Sigma-Aldrich (Bornem, Belgium). Solvents (HPLC grade) were from Biosolve (Valkenswaard, The Netherlands). Cell culture media, L-glutamine, non-essential amino acids (NEAA), penicillin (10,000 IU/ml), streptomycin (10,000 µg/ml), foetal bovine serum (FBS), and sodium pyruvate were obtained from Invitrogen (Merelbeke, Belgium).

The RNeasy Mini Kit and the developed sets of primers were purchased from Qiagen (Leusden, The Netherlands). DNase-stock and DNase-buffer were from Fermentas (Sint-Leon-Rot, Germany). Agarose powder was purchased from Biozym Resource TM Line Agaroses (Landgraaf, The Netherlands). All PCR-materials (MulVRTase, Taq DNA-polymerase, dNTP mix, PCR-buffer, $MgCl_2$, ribonuclease inhibitor, TBE buffer, ethidium bromide, loading buffer Orange G, low range DNA-ladder from 100 to 1000 bp) were obtained from Fermentas (Sint-Leon-Rot, Germany). The 50 bp DNA-ladder was purchased from Invitrogen (Merelbeke, Belgium).

5.3.3. Product specifications

The enzyme MulVRTase (Murine Leukemia Virus Reverse Transcriptase) is a RNA-dependent DNA-polymerase. The Taq DNA-polymerase is a recombinant 94 kDa DNA-polymerase originally isolated from *Thermus aquaticus* and expressed in *Escherichia coli*. The dNTP mixture is a mixture of dATP, dCTP, dGTP, and dTTP. The PCR-buffer contained

500 mM KCl and 100 mM Tris-HCl. The concentration of the $MgCl_2$-solution was 25 mM. The ribonuclease inhibitor was purified from human placenta.

5.3.4. Cells and culture media

The mammary adenocarcinoma cell lines were obtained as follows: MCF-7/6 from Dr. H. Rochefort (Unité d'Endocrinologie Cellulaire et Moléculaire, Montpellier, France) and MDA-MB-231 from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). The established human prostate cancer cell lines (LNCaP.FGC, PC-3, DU145) and a human bone marrow endothelial cell line (HBME) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were maintained in cell specific media at 37°C in a humidified atmosphere of 5% carbon dioxide. The cells were passed at 70% confluency (i.e., two or three times a week). To reduce potential variations of cell characteristics, cells were used within 10 passages for all experiments.

The LNCaP.FGC, PC-3 and DU145 cells were cultured in Dulbecco's Modified Eagle's Medium DMEM/F12 (Ham) 1:1 (Dubecco's Mem Nut Mix F–12 (HAM)) with L–glutamine (365,00 mg/L) and 15 mM HEPES. The MCF-7/6 cells were cultured in Minimum Essential Medium (MEM) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA). The MDA-MB-231 and HBME cells were cultured in high-glucose (4500 mg/L) Dulbecco's Modified Eagle Medium (DMEM). HBME cells needed supplementation of 1 mM sodium pyruvate. All media were supplemented with 10% foetal bovine serum (FBS) and antibiotic agents (penicillin 10,000 IU/ml and streptomycin 10,000 µg/ml).

Experimental media differed from the maintenance media. Experiments with MCF-7/6, MDA-MB-231, DU145 and HBME cells were performed in high-glucose (4,500 mg/L) DMEM without phenol red; experiments with LNCaP.FGC and PC-3 cells were performed in phenol red-free RPMI 1640. Both media were supplemented with 10% steroid-free, dextran-coated, charcoal-treated FBS and 1% antibiotic agents (penicillin 10,000 IU/mL and streptomycin 10,000 µg/mL). In addition, 2 mM L-glutamine, 0.1 mM NEAA, and 1 mM sodium pyruvate were added to the DMEM medium and 1 mM sodium pyruvate and 10 mM HEPES were added to the RPMI 1640 medium.

5.3.5. Dextran-coated charcoal-stripped foetal bovine serum (DCC-FBS)

Charcoal was coated with dextran by stirring equal volumes (50 ml) of a 10% charcoal suspension in water and a 1% solution of dextran in water for 30 min, followed by centrifugation at 4200 x g for 10 min at 4°C. The supernatant was removed and the pellet was resuspended with FBS (same volume as the supernatant) and rotated overnight at 4°C. After centrifugation at 4200 x g for 15 min, the mixture was rotated again with a freshly prepared pellet of dextran-coated charcoal for 6 h at 4°C. This mixture was centrifuged at 4200 x g for 15 min at 4°C, the supernatant was centrifuged again at 550000 x g for 30 min, and the resulting solution was immediately sterilized by passage through a cellulose acetate filter with 0.22 µm pore size (Schleicher & Schuell, Dassel, Germany) and stored at -20°C.

5.4. Methods

5.4.1. Cell proliferation assays

5.4.1.1. WST1 tetrazolium assay

Mosmann developed a colorimetric method for the determination of proliferation and viability of cells.[21] This method assays proliferation by analysing the number of viable cells and is based on the cleavage of tetrazolium salts added to the culture medium. In recent years, various tetrazolium salts have been described. All salts are cleaved to their formazan products, a process catalyzed by the succinate dehydrogenase complex II of the inner mitochondrial membrane, an enzyme belonging to the respiratory chain of the mitochondria and involved in oxidative phosphorylation. However, other mechanisms could be involved as well. According to Slater et al., this system is active only in viable cells.[22] An increasing number of viable cells results in an increasing number of mitochondria and an increase in the overall activity of mitochondrial dehydrogenases. Thus, these assays reflect both the number of cells and the activity status of the mitochondria.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to its water-insoluble dark blue formazan poduct by reductive cleavage of the tetrazolium ring.[21,22]

The cellular reactions involved in this reduction are not completely understood. The involvement of active mitochondria, namely the mitochondrial succinate dehyrogenase system has been discussed.[21,23,24,25] It has been shown that cells with inactivated mitochondria are also able to produce the same amount of formazan product compared to cells with operative mitochondria.[26] Many non-mitochondrial dehydrogenases and flavin oxidases are able to reduce MTT.[27,28] Little attention has been given to the ability of extracellular factors to reduce MTT to its formazan product.[29]

Compared to MTT, WST1 is a more recently used tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate) which is cleaved to its formazan product (**Figure 5.1**). WST1 yields water-soluble cleavage products, thereby avoiding dissolution before colorimetric measurement.[21,23,30] The maximum absorbance for the formed formazan product is found between 420 and 480 nm.

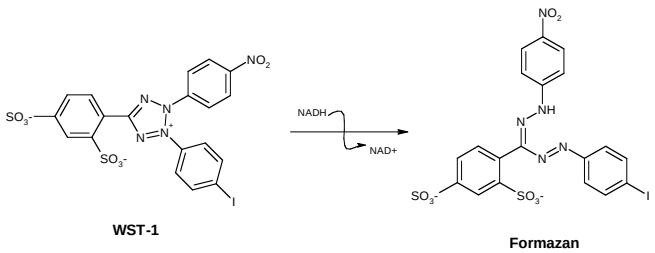


Figure 5.1. Reduction of WST1 to its formazan product.

Bruggisser et al. showed that the MTT tetrazolium assay may lead to false positive results when investigating natural compounds with an intrinsic reductive potential.[31] When testing the effects of kaempferol on breast cancer cell numbers (crystal violet staining) and viability (MTT tetrazolium assay), conflicting results were obtained. The cell number decreased but formazan product formation increased suggesting a direct interaction of kaempferol with MTT. This was highlighted in a cell-free system for kaempferol and resveratrol. Formazan product formation was immediately noticeable in the absence of cells. Additionally, antioxidants such as ascorbic acid, vitamin E, and *N*-acetylcysteine interfered with the MTT tetrazolium assay. When MCF7 and HS578 cells treated with kaempferol were washed before addition of MTT tetrazolium, direct reduction of the dye was significantly inhibited. These results indicate that the MTT tetrazolium assay may lead to false positive results when testing natural compounds with intrinsic reductive potential. Therefore,

application of tetrazolium assays in a screening system to detect the influence of natural products on the cell viability should be done with great care.

The cells were seeded into flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, France) at a density of 2,000 cells/well for MCF-7/6, 3,000 cells/well for DU145, 6,000 cells/well for PC-3 and HBME, 10,000 cells/well for MDA-MB-231, and 17,000 cells/well for LNCaP.FGC, in 100 µl experimental growth medium (see: 5.4.3.1 and 5.6.3.1).

After 24 h (or 48 h for LNCaP.FGC), the cells (20-30% confluent) were exposed for 48 h to culture media containing a serial dilution of the prenylflavonoids prepared in ethanol from a 200 mM stock solution to a final ethanol concentration of 0.1 % in each well. All experiments included a solvent control. Initial concentrations ranged from 10 nM to 200 µM, thereafter suitable concentrations were used to determine the IC_{50} values for each compound. All experiments were repeated three times with six replicas per condition. After two days, cell viability was measured using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene (WST1) assay (Roche Diagnostics, Vilvoorde, Belgium) according to the manufacturer's instructions. At each well containing 100 µL incubation medium, 10 µL of the WST1 reagent was added and the plate was incubated for another 3 h (determined experimentally) at 37°C. After 3 h, absorbances were measured at 450 nm using a microplate-spectrophotometer (Multiscan RC, Thermo Labsystems, Genesis Lite Software, Helsinki, Finland).

The average absorbance of the treated wells was compared with the average absorbance of the control wells to give a percentage of the control proliferation ([value/control]x100). IC_{50} values were calculated using Sigmaplot 8.0 (SPSS, Chicago, Illinois, USA) in a four-parameter logistic regression model. The experimental absorbances of X and DMX were corrected for their inherent absorption at 450 nm.

Possible interactions of the flavonoids with WST1 were investigated by incubation of WST1 with 100 µM of the prenylflavonoids in a cell-free environment under identical incubation conditions as described for the cell proliferation assay. More specifically, wells of a flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, France) were filled with 100 µL experimental medium containing the test compounds at a concentration of 100 µM and 10 µL of WST1. Plates were incubated at 37°C for 3 h and formation of formazan

salt was monitored at varying time intervals by measuring the absorbance at 450 nm using a microplate-spectrophotometer (Multiscan RC, Thermo Labsystems, Genesis Lite Software, Helsinki, Finland).

To be able to correct for interferences of inherent absorptions, 'correction' wells were used in each experiment containing cells, experimental medium, and X or DMX at various concentrations in the absence of WST1. More specifically, wells of flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, France) were filled with 100 µL experimental medium containing the test compounds at the same concentrations used for the WST1 proliferation assay. After incubation for 48 h at 37°C, the absorbances were measured at 450 nm using a microplate-spectrophotometer (Multiscan RC, Thermo Labsystems, Genesis Lite Software, Helsinki, Finland). The experimental values for X and DMX obtained by the WST1 tetrazolium assay were appropriately corrected for inherent absorbances.

5.4.1.2. SRB assay

The Sulforhodamine B (SRB) assay provides a sensitive method for measuring drug cytotoxicity in culture. SRB (**Figure 5.2**) is a bright pink aminoxanthene dye with two sulfonic groups. Under mildly acidic conditions, SRB binds to basic amino acid residues in trichloroacetic acid (TCA)-fixed cells to provide a sensitive index of the cellular protein content that is linear over a cell density range of at least 2 orders of magnitude. The cellular protein content is directly correlated to the number of cells in culture thereby indicating a growth-stimulatory or growth-inhibitory effect of the compound tested in the assay.

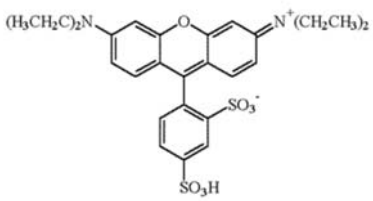


Figure 5.2. Structure of sulforhodamine B (SRB)

The SRB method appears to offer several advantages over the MTT assay for very large-scale drug screening. The SRB assay is simpler, faster, and more sensitive, provides a better linearity with the cell number, permits the use of saturating dye concentrations, is less

sensitive to environmental fluctuations, is independent of intermediary metabolism, and provides a fixed endpoint that does not require a time-sensitive measurement of the initial reaction velocity.[32]

The cells were seeded into flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, France) at a density of 2,000 cells/well for MCF-7/6, 3,000 cells/well for DU145, 6,000 cells/well for PC-3 and HBME, 10,000 cells/well for MDA-MB-231, and 17,000 cells/well for LNCaP.FGC, in 100 µl experimental growth medium (see: 5.4.3.1 and 5.6.3.1).

After 24 h (or 48 h for LNCaP.FGC), the cells (20-30% confluent) were exposed for 48 h to culture media containing a serial dilution of the prenylflavonoids prepared in ethanol from a 200 mM stock solution to a final ethanol concentration of 0.1 % in each well. All experiments included a solvent control. Initial concentrations ranged from 10 nM to 200 µM, thereafter suitable concentrations were used to determine the IC_{50} values for each compound. All experiments were repeated three times with six replicas per condition. After two days, cell viability was measured using the SRB assay. At each well, containing 100 µL incubation medium, 25 µL 50 % TCA was added to fix the cells and the plates were stored during 1 h at 4°C. After 1 h, the plates were washed 5 times with water and dried on the air overnight. To the dry wells, 50 µL SRB dye solution (0,4 % in 1 % acetic acid) was added and plates were incubated for 30 min. After 30 min, the plates were gently washed with 1% acetic acid (± 500 ml/plate depending on the intensity of color formation) until all the unbound color was washed off. Washed plates were dried on the air overnight. To dissolve the fixed dye, 200 µL Trisbuffer (2-Amino-2-(hydroxymethyl)-1,3-propanediol, 10 mM, pH 10.5) was added to each well and the absorbances were measured at 540 nm and 620 nm using a microplate-spectrophotometer (Multiscan RC, Thermo Labsystems, Genesis Lite Software, Helsinki, Finland). In each experiment, 'blank' wells (n=3) containing only incubation medium (no cells) were added to test for non-specific binding of SRB.

The average absorbance of the treated wells at 540 nm was corrected for the background absorbance at 620 nm and for the corrected 'blank' average absorption value. ([$Expvalue_{540nm}$-$Expvalue_{620nm}$]-[$Blankvalue_{540nm}$-$Blankvalue_{620nm}$]). These corrected average absorption values were compared with the average absorbance of the control wells to give a percentage of the control proliferation ([value/control]x100). IC_{50} values were calculated

using Sigmaplot 8.0 (SPSS, Chicago, Illinois, USA) in a four-parameter logistic regression model.

5.4.2. Estrogenicity assay

Many compounds of plant origin with the ability to bind to estrogen receptors have been identified. There is evidence that the consumption of some of these phytoestrogens may have beneficial effects, but it also seems possible that others may act as endocrine disrupters. For this reason, there is a need to characterize the estrogenic potency of these substances. *In vitro* test systems offer the possibility to screen compounds very efficiently. Routinely in use and widespread for the determination of estrogenicity are: (I) receptor binding assays, test systems where the binding affinity of a compound to the estrogen receptor is determined; (II) cell-proliferation assays (E-screens), (III) reporter gene assays, and (IV) analysis of the regulation of endogenous estrogen-sensitive genes in cell lines. All these test systems rely on the molecular mechanisms that are involved in the 'classical' estrogen action.[33]

5.4.2.1. Growth-stimulatory assay

The cell proliferation assay or E-screen is a widely used assay to determine the estrogenic potency of natural and environmental compounds. In these *in vitro* test systems, the ability of a substance to stimulate the growth of estrogen-dependent cell lines is measured. Most commonly, estrogen-dependent human breast cancer cell lines like MCF-7 or T47-D are used. To measure the proliferation of a cell population, either the synthesis of new DNA or changes in the metabolic activity of viable cells is determined. This method suffers from the fact that a considerable number of substances give positive results in the E-screen without exerting estrogenic activity.

Only the growth of hormone-dependent cancer cells is detected, although the growth of cancer cells can be stimulated in a similar way by cytokines, growth factors, mitogens, and nutrients. For this reason, the culture conditions of the cells may have a big influence on the results. In addition, it has been demonstrated that different MCF-7 stocks may possess wide variations with regard to their sensitivity to 17β-estradiol.[33]

The MCF-7/6 cells were seeded into flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, Meylan, France) at a density of 5,000 cells/well for the

experiment using culture medium supplemented with 2% dextran-coated charcoal-stripped foetal bovine serum (DCC-FBS), and 10,000 cells/well for the experiment using culture medium which was not supplemented with DCC-FBS in 100 µl experimental growth medium. The experimental medium was supplemented once with 2% and once without DCC-FBS (see: 5.4.3.2 and 5.6.3.2).

After 24 h, the cells (20-30% confluent) were exposed for 72 h to culture media containing a serial dilution of 8PN (1 fM to 200 µM) (for the experiment using culture medium supplemented with 2% DCC-FBS) or of 17β-estradiol (1 fM to 1 µM) (for the experiment using culture medium which was not supplemented with DCC-FBS) from 200 mM and 1 mM stock solutions, respectively, to a final ethanol concentration of 0.1 % in each well. All experiments included a solvent control and they were repeated three times with six replicas per condition. After three days, cell viability was measured using the WST1 assay and the SRB assay (see: 5.4.1.1 and 5.4.1.2, respectively).

To investigate the effect of X on the growth stimulatory concentrations of 8PN (1, 10 and 100 nM) (see: 5.6.2.1), MCF-7/6 cells were seeded into flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, Meylan, France) at a density of 5,000 cells/well in 100 µl experimental growth medium. The experimental medium was supplemented with 2% DCC-FBS (see: 5.4.3.2 and 5.6.3.2).

After 24 h, the cells (20-30% confluent) were exposed for 72 h to culture media containing a mixture of a specific concentration of 8PN (1, 10 or 100 nM) with any of the concentrations from a serial dilution of X (100 nM tot 200 nM) from a 200 mM stock solution. The final ethanol concentration in each well was 0.2 %. All experiments included a solvent control and they were repeated three times with six replicas per condition. After three days, cell viability was measured using the WST1 assay and the SRB assay (see: 5.4.1.1 and 5.4.1.2, respectively).

5.4.2.2. Recombinant yeast estrogen screen (YES)

In a reporter gene assay, the ability of a substance to activate the transcription of an estrogen-sensitive promotor is analyzed. Eukaryotic cells, either mammalian or yeast cells, are transfected with an expression vector encoding the human estrogen receptor and a reporter gene vector. The reporter gene vector is composed of an estrogen-sensitive promoter, which is

linked to a reporter gene. The reporter gene encodes for a protein with metabolic activity which can be easily quantified. The principle mechanism of the reporter gene assay is shown in **figure 5.3**.

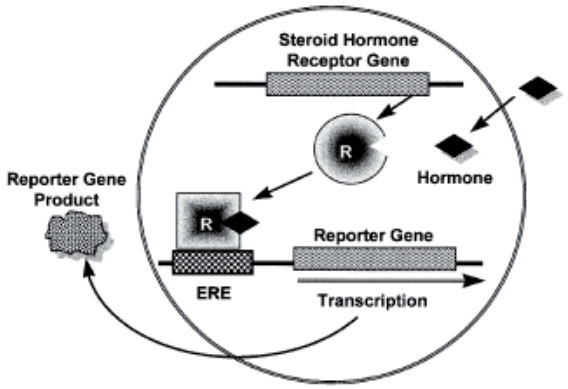


Figure 5.3. The functional principle of a reporter gene assay. R, estrogen receptor; ERE, estrogen response element.

The yeast estrogen screen (YES) is based on the stimulation of the transcriptional activity of the human estrogen receptors (ERα and ERβ) in recombinant yeast cells. An estrogen-inducible expression system was developed in yeast (*Saccharomyces cerevisiae*) in the Genetics Department of Glaxo Wellcome, Beckenham, Kent, to determine the estrogenic activity of compounds that interact with the human ER (**Figure 5.4**). Because yeast does not contain nuclear receptors, the DNA sequence of the human ER was integrated into the chromosome to effect expression of the receptor. Besides binding to an estrogen-active compound, the ligand-receptor complex is able to bind to the estrogen response element (ERE), which is situated on the integrated expression plasmides that carry the Lac-Z-gene. The Lac-Z gene measures the activity of the receptor by encoding to the enzyme β-galactosidase. Once excreted in the medium, this enzyme converts the yellow substrate, chlorophenol-red-β-D-galactopyranoside (CPRG), present in the medium, to the red reaction product, chlorophenol-red, which is measured spectrophotometrically at 540 nm. This reaction is very selective for compounds that bind to the ERs and, therefore, the absorbance is directly in accordance with the activity of the receptor.

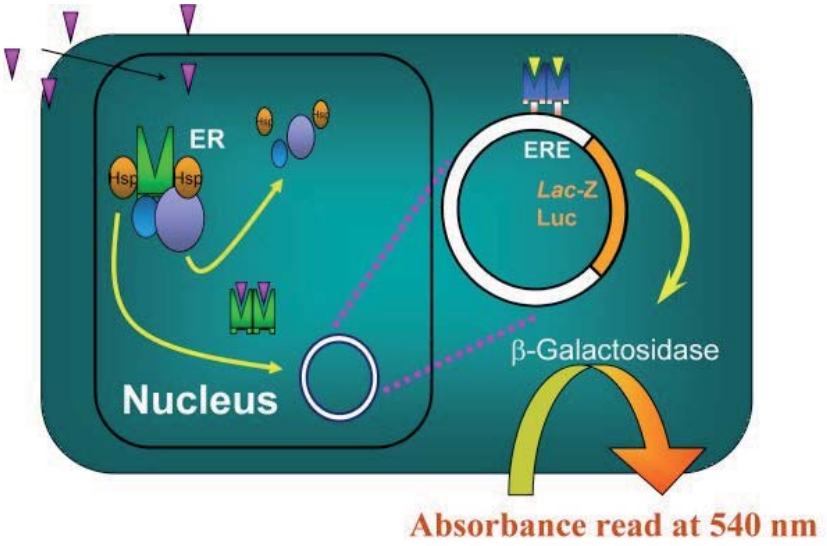


Figure 5.4. Estrogen-inducible expression system in yeast (adapted from Routledge et al., 1996). ER, estrogen receptor; ERE, estrogen response element.

The yeast system possesses several advantages compared to mammalian cells. Yeasts are easy to culture and, in contrast to mammalian cells, it is possible to incubate yeasts with non-purified naturally occurring extracts from plants or from the environment. Since there are no naturally occurring ERs in this species, yeast systems are very useful to analyse molecular mechanisms of initiation of transcription. However, analysis of the ER-induced transactivation in yeasts is a highly artificial test system as are all transactivition systems including those that are performed in mammalian cells.[34] The results show a wide variation depending on the reporter gene, promoter, and cell line used. Nevertheless, reporter gene assays are very useful and powerful tools to identify substances that are able to activate estrogen-dependent transcription and to determine their estrogenic potencies. Furthermore, they are suitable to characterize the agonistic and antagonistic properties of a substance. These assays give the possibility to analyse a high number of substances in a short period of time.[33]

A yeast assay to assess estrogenic activity was kindly provided by Prof. Dr. J. Sumpter (Brunel University, UK). Details of the YES have been previously described.[18] In brief, yeast cells, carrying the genetic code for the human estrogen receptor alpha together with

expression plasmids coding for secretory β-galactosidase under control of estrogen response elements (ERE) were incubated in media containing serial dilutions of the test compounds prepared in ethanol from a 2 g/L stock solution and chlorophenol red-β-D-galactopyranoside (CPRG). The estrogenic activity was determined from enzymatic hydrolysis of CPRG into chlorophenol by monitoring the absorbance at 540 nm. Because of the low activity of some of the compounds, the test was incubated for a longer period (5-6 days) than the usual three days.

Dose-response curves for β-galactosidase activity were obtained using corrected absorbance (A) units (CAU): CAU = $A_{(540nm)}$ compound – [$A_{(620nm)}$ compound – mean $A_{(620nm)}$ blank]. Curve analysis was performed using Sigmaplot 8.0 (SPSS, Chicago, Illinois, USA) in a four-parameter logistic regression model. The relative potency (RP) of a test compound is the ratio of that concentration of 17β-estradiol that caused the same CAU as the compound at its half-maximal response and the compound's EC_{50}. The relative induction efficiency (RIE) is the ratio between the (max-min) absorbance achieved with the test compound and that of 17β-estradiol (x 100). The detection limit is defined as the concentration of 17β-estradiol corresponding to an effect equal to the mean of the corrected absorbances of the blank rows plus three times the standard deviation. A compound was considered to exhibit estrogenic activity when, in at least two separate experiments, it caused dose-dependent (two consecutive concentrations) color formation above the detection limit.

5.4.3. Determination of the cell seeding density

5.4.3.1. Cell proliferation assay

The seeding density was determined experimentally by preparing 'blank' curves for each cell line separately. Therefore, a serial dilution of the suspended cells (MCF7/6, MDA-MB-231, PC-3, DU145 and HBME: from 1,000 cells/well to 10,000 cells/well; LNCaP.FGC: from 20,000 cells/well to 200,000 cells/well) were seeded into flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, Meylan, France) in 100 µl experimental growth medium and incubated under the same conditions as for the proposed cell proliferation assays (same experimental media, same incubation times) (see: 5.4.1), but without the stimulation of compounds. After 24 h (or 48 h for LNCaP.FGC), the cells were exposed for 48 h to fresh culture medium in the same way as fresh medium was provided to the cells during stimulation with the test agents. All experiments were repeated three times with six replicas per condition.

After two days, cell viability or protein content was measured using the WST1 assay or SRB assay, respectively (see: 5.4.1.1 and 5.4.1.2, respectively).

5.4.3.2. Growth-stimulatory assay

The seeding density was determined experimentally by preparing 'blank' curves for the breast cancer cell line MCF7/6. Therefore, a serial diluation of the suspended cells (from 500 cells/well to 10,000 cells/well) were seeded into flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, Meylan, France) in 100 µl experimental growth medium and incubated under the same conditions as for the proposed growth stimulatory assay (same experimental medium, same incubation times) (see: 5.4.2.1), but without the stimulation of compounds. The experimental medium was supplemented with varying concentrations of steroid-free, dextran-coated charcoal treated FBS (0 %, 2 % or 5 %). After 24 h, the cells were exposed for 72 h to fresh culture medium in the same way as fresh medium was provided to the cells during stimulation with the test agents. All experiments were repeated three times with six replicas per condition. After two days, the protein content was measured using the SRB assay (see: 5.4.1.2).

5.4.4. Determination of the presence of ERα and ERβ mRNA transcripts by RT-PCR

The presence of the ERα and ERβ mRNA transcripts in the human prostate (PC-3, DU145, LNCaP.FGC) and breast (MCF7/6, MDA-MB-231) cancer cell lines was determined by the reverse transcriptase polymerase chain reaction (RT-PCR). Therefore, mRNA was extracted from the cell cultures and, after conversion to its complementary doublestranded copy DNA (cDNA) by means of the reverse transcriptase enzyme MulVRTase, it was selectively amplified by the Taq DNA polymerase enzyme. The resulting copies of identical DNA-fragments were separated by gel electrophoresis, detected by staining with ethidium bromide, and identified by comparing with a standard.

5.4.4.1. RNA extraction

The specific mRNA-sequence that will serve as the template for RT-PCR (after conversion to cDNA) was isolated from the cell content by RNA extraction using the RNeasy procedure. Total RNA was extracted from the cell content of the cell cultures MCF7/6, MDA-MB-231, PC-3, DU145, and LNCaP.FGC using the RNeasy Mini Kit (Qiagen, Leusden, The

Netherlands). An optimized number of detached cells (5,000,000 cells) were centrifuged (10 min at 40 x g), the medium was gently removed, and the cell pellet was resuspended in 200 µL ice-cold PBS. After centrifugation (1 min at 2400 x g) and removal of PBS, 350 µL lysis buffer (10 µL β-mercaptoethanol in 1 mL RLT–buffer; ex-tempore preparation) was added to the loosened cell pellet and mixed well (incomplete loosening of the cell pellet may lead to inefficient lysis and reduced yields). The lysate was then homogenized by passing it 10-times through a 20-gauge needle (0,9 mm diameter) fitted to a RNase-free syringe. Then, 350 µL ethanol (70 %) was added to the homogenized lysate and mixed well by pipetting.

A maximum of 700 µL of the sample was applied to an RNeasy mini column and centrifuged for 15 s at 8000 x g. The column was washed a first time by application of 700 µL wash buffer RW1 and centrifugation for 15 s at 8000 x g, and a second time by application of 500 µL wash buffer RPE and centrifugation for 15 s at 8000 x g. Another 500 µL wash buffer RPE was applied to the column and the column was dried by centrifugation for 2 min at 8000 x g and for 1 min at 11200 x g.

To elute the RNA from the column, 100 µL of RNase-free water was pipetted directly onto the column and centrifuged for 1 min at 8000 x g. This first eluate was applied again onto the column followed by centrifugation for 1 min at 8000 x g thereby affording a second eluate, which was again applied onto the column and centrifuged for 1 min at 8000 x g.

If DNase needed to be used during the extraction protocol, the column was washed a first time by application of 350 µL wash buffer RW1 and centrifugated for 15 s at 8000 x g after application of a maximum of 700 µL of the sample to a RNeasy mini column and centrifugation for 15 s at 8000 x g. The dry column was completely covered with 80 µL of DNase solution (prepared by gently mixing 27.3 U DNase-stock with 52.7 µL DNase-buffer) and incubated for 15 min at room temperature. After incubation, the column was washed and the RNA was eluted from the column as described above.

The RNA concentration in the eluate was determined by measurement of the absorbances at 260 nm and 280 nm using a Beckman DU-600 UV-Vis Diode Array Spectrometer (DNA oligo quant-method) (Hainburg, Germany).

5.4.4.2. Development of selective oligonucleotide primers

The oligonucleotide primers provide a very selective targetted starting point for the reverse transcriptase and DNA-polymerase enzymes. The sequence of these primers can be found in the literature or can be developed using appropriate software such as the program "Primer 3"[35] or "clonewin" (a kind gift from Dr. Wim Vanden Berghe, Ghent University, Faculty of Sciences, Department of Molecular Biology, Laboratory for Eucaryotic Gene Expression and Signal Transduction). They are developed starting from the cDNA sequence from the human ERα and ERβ (see: the National Centre for Biotechnological Information site[36] or the 'Ensembl' site[37]). The sequences of human ERα and ERβ found at both sites have to be compared and only the overlapping part is used to develop the primers in order to bind to this part of the cDNA sequence. The optimum length of the primers is about 20 bp and the optimum length of the amplicon (part in-between the primers that will have to be synthesized by the enzyme) is 200 to 400 bp.

The primers should be developed such that they should be *selective* and provide a *difference* between the mRNA template and the (possibly present) genomic DNA. *Selective* primers are primers that have no affinity for other genes than the target genes. Thus, only the desired part of the target mRNA is reversed to cDNA and further amplified in the PCR reaction. The selectivity of the primers can be tested on the National Centre for Biotechnological Information (NCBI) site using the Basic Logical Alignment Search Tool (BLAST).

The developed primers should also be able to make a *difference* between mRNA and genomic DNA. Therefore, it is necessary that either both primers of a set of primers bind at the level of two different exons or either one or both primers from a set of primers bind at the overlap of two neighbouring exons. In the first case, the amplicon produced from the genomic DNA is much larger (or is too large to be produced) than the amplicon derived from mRNA, because of the presence of one or more introns between the exons that result in two different signals after separation by gel electrophoresis and staining with ethidium bromide. In the second case, there is no amplification of the genomic DNA, since the primers cannot bind at the overlap of two exons because of one or more intron(s) that lie in-between.

5.4.4.3. The RT-PCR reaction

The RT-PCR reaction was performed in 96 multi-eppendorf plates (Fermentas, Sint-Leon-Rot, Germany). Each eppendorf was filled with 20 µL RT-PCR reaction mixture resulting from addition of the non-enzyme and non-primer materials (PCR buffer, $MgCl_2$, dNTP, RNase-free water) to a recipient in a certain volume while mixing well by vortexing. This mixture was kept on ice during manipulation. Next, the enzymes (RNase–inhibitor, MulVRTase, Taq DNA-polymerase), the RNA extract of a cell culture, and the primers ('forward' and 'reverse' primers) were added in a certain volume and mixed well by vortexing. The amount of RNA necessary in one reaction mixture of 20 µL was experimentally determined to be 0.1 µg. The necessary volume of RNA extract to be added to the reaction mixture giving a concentration of 0.1 µg/20 µL RNA was determined by measurement of the RNA concentration for each extract separately.

An example of a reaction mixture is given in **figure 5.5**. The amounts of each reagent can vary depending on the concentration of the stock solution. The total volume was kept at 20 µL by adapting the amount of RNase-free water.

To control for the possible presence of genomic DNA, MulVRTase was removed from the reaction mix. To control for a positive RT-PCR reaction, the transcription of β-actin was examined in each reaction.

Product	Stock	Final	Dilution	Volume (µL)
PCR-buffer	10 X	1 X	10	2
MgCl2	25 mM	2.5 mM	10	2
dNTP	10 mM	300 µM	33.33	0.6
RNase inhibitor	20 U/µL	0.04 U/µL	500	0.04
FP	25 µM	200 nM	125	0.16
RP	25 µM	200 nM	125	0.16
MulVRTase	20 U/µL	0.06 U/µL	416.67	0.05
Taq DNA-polymerase	5 U/µL	0.05 U/µL	100	0.2
RNase-free water	-	-	-	X
mRNA template (extract)	-	-	-	Y (= 0.1 µg RNA)
			Final Volume	20

Figure 5.5: Example of a RT-PCR reaction mixture. MulVRTase: Murine Leukemia Virus Reverse Transcriptase, FP: Forward Primer, RP: Reversed Primer. Product specifications, see: 5.3.3.

A/ Thermogradient method

For every new set of primers, a thermogradient method was performed to determine the optimum annealing temperature at which a certain selective primer binds to its complement on mRNA or cDNA with the least possible chance of aspecific binding. Practice learned that the proposed annealing temperatures were not always the most optimum ones.

The following multistep optimized RT-PCR reaction program was used for the thermogradient method:

1. 30 min at 48˚C, the optimum temperature for the activity of the reverse transcriptase enzyme MulVRTase, which reversely transcribes mRNA into its cDNA complement.
2. 10 min at 91˚C, the temperature at which the denaturation process occurs and the doublestranded cDNA is separated. At this temperature, the Taq DNA-polymerase is activated.
3. 45 s at 91˚C, the denaturation step starting from the second cycle
4. 45˚C to 65˚C in 45 s, the thermogradient to determine the optimum annealing temperature for a specific set of primers (column 1: 45.0°C, column 2: 45.5°C, column 3: 46.5°C, column 4: 48.2°C, column 5: 50.5°C, column 6: 53.4°C, column 7: 56.7°C, column 8: 59.6°C, column 9: 61.8°C, column 10: 63.4°C, column 11: 64.6°C, column 12: 65.0°C).
5. 1 min at 72˚C, the extension step at which the nucleotides (dNTP mixture) are stringed together by Taq DNA-polymerase to build the complementary DNA strand on the template.
6. End of cycle 1 (step 3 to step 5) and starting again with step 3 for the second cyle. This is repeated 39 times to a grand total of 40 cycles.
7. 10 min at 75˚C, the final extension step at which loosely bonded DNA segments that are not fully complementary are broken.
8. 10˚C for indefinite time, which is the maximum temperature at which the reaction mixture remains stable overnight.

To determine the annealing temperature for one set of primers, the reaction mixture was tested at 12 different temperatures. Therefore, 12 x 20 µL (total of 240 µL) of reaction

mixture was necessary for one set of primers and one RNA extract of a certain type of cell culture.

B/ Fixed-temperature method

Once the optimum annealing temperature for a specific set of primers has been determined using the thermogradient method, this set of primers can be applied for further RT-PCR reactions using a simplified program. The steps are identical to the thermogradient program, except for step 4, which was set at the determined optimum annealing temperature for that particular primer set. The duration of this step was 45 s. Using this program, only one RT-PCR reaction mixture of 20 µL was necessary for one set of primers and one RNA extract of a certain type of cell culture.

5.4.4.4. Gel electrophoresis

An agarose gel of 2% was used to separate the amplicons present in the reaction mixture resulting from the RT-PCR reaction. Therefore, 2.8 g agarose powder was dissolved in 140 mL TBE-buffer 1x (tris boric acid EDTA) by stepwise heating in a microwave oven and gently stirring in-between the heating steps until a clear liquid solution was formed at the boiling temperature. Next, the solution was cooled to about 60˚C and poured into a casting tray containing a sample comb, and allowed to solidify at room temperature or in a refrigerator and form a gel. After solidifying, the comb was carefully removed.

The gel in its plastic tray was inserted horizontally into the electrophoresis chamber and covered with a minimum of TBE-buffer (0.5 x). This buffer needed to be replaced after about 7 electrophoresis runs. The samples to be loaded on the gels were prepared by adding 4 µL loading buffer Orange G to each eppendorf of the 96-multi-eppendorf plate in which the RT-PCR reaction occurred. The samples were then pipetted into the sample wells of the agarose gel. The outer sample wells of the gel were filled with 7.5 µL of a low range DNA-ladder from 100 to 1000 bp (Fermentas, Sint-Leon–Rot, Germany) or with 1 µL of a 50 bp DNA-ladder in 5 µL Orange G (Invitrogen, Belgium) (F**igure 5.6**). Gel electrophoresis was performed at 200 V for about 60 min. The gel was removed at the point where the loading buffer was at 2 cm from the edge of the gel at the kathode site.

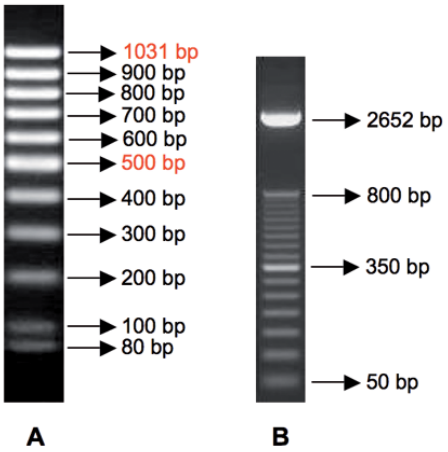


Figure 5.6: Illustration of the DNA-ladders separated by gel electrophoresis and visualized after ethidium bromide staining.
Ladder A: low range DNA–ladder. Ladder B: 50 bp DNA-ladder (signal every 50 bp).

After gel electrophoresis, the gel was removed and soaked in a solution of 50 µL ethidium bromide (10 mg/mL) in TBE-buffer (1 L) during 20 min. To visualize the DNA fragments, the gel was placed on an ultraviolet transilluminator and pictures were taken using a DC forte DC120 digital camera (Eastman Kodak, New York, USA). The intensity of fluorescence is proportional to the amount of DNA from the sample. This method does not allow quantification and it was, therefore, only used for a qualitative study of the presence of a certain mRNA fragment in the cellular extracts of different cell lines.

5.5. Statistical analysis

All additional statistics were performed using SPSS 10.0 (SPSS, Chicago, Illinois, USA).

5.6. Results

5.6.1. Cell proliferation assays

The cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene) and the SRB (sulforhodamine B) cytotoxicity test were applied for the quantification of cell proliferation rates and cytotoxicity. More specifically, these tests were used to determine the relative potencies of the individual compounds in inhibiting the growth of cancer cells and in assaying the sensitivities of the different cell lines to these compounds.

The WST1 colorimetric assay is based on the cellular reduction of WST1 to a formazan product by the mitochondrial dehydrogenases of viable cells and, thus, is used for the quantitation of cell viability and cell proliferation. This assay is very sensitive and gives results that directly correlate to the number of metabolically active cells in culture. The SRB assay uses an anionogenic aminoxanthene dye with two sulfonic groups, which binds to the proteins of fixed cells in culture. The results of this assay correlate directly to the number of cells in culture thereby providing an indication of the growth-stimulatory or growth-inhibitory effect of the compounds tested.

The antiproliferative effects of the chalcones, X and DMX, and the flavanones, IX, 8PN, and 6PN, were assessed using the human prostate cancer cell lines LNCaP.FGC, PC-3, and DU145. The human breast cancer cell lines MCF-7/6 and MDA-MB-231 served as a estrogen-responsive reference control model. All compounds caused, for all cell lines, a dose-dependent growth inhibition after two days of exposure. No significant differences between the results obtained with the WST1 assay and those obtained with the SRB assay were noted initially, hence, the WST1 assay was pursued further. The only small difference observed between both assays was the value of the absorbances at the highest concentrations of test agents. At these concentrations, the WST1 assay measures no activity, while the SRB assay measures a small absorbance due to the remaining cell debris from dead cells on the bottom of the wells in the 96-well tissue culture plates. The results of the WST1 assay are illustrated in **figure 5.7** and **figure 5.8**.

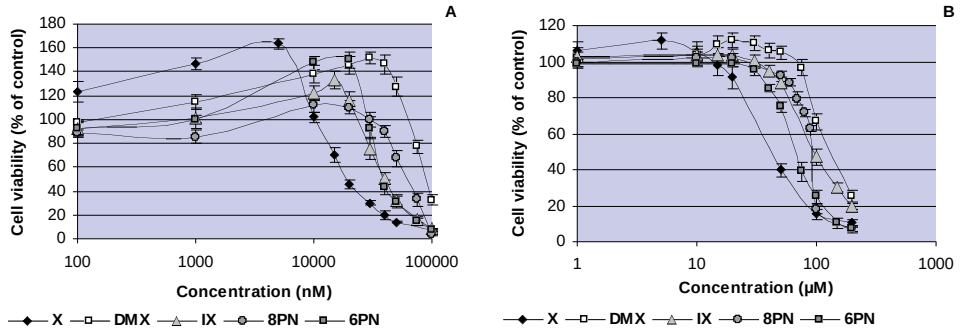


Figure 5.7: Dose-response relationships for hop-derived prenylflavonoids on the growth of human breast cancer cells (A: MCF-7/6, B: MDA-MB-231). Data are means ± SD (n=3). Materials and Methods, see: 5.3 and 5.4.1, respectively.
X: xanthohumol, DMX: desmethylxanthohumol, IX: Isoxanthohumol, 8PN: 8-prenylnaringenin, 6PN: 6-prenylnaringenin.

Chalcone X proved to be the most potent compound in inhibiting the growth of all cell lines (except for LNCaP.FGC), DU145 being the most sensitive (IC_{50} of 12.3 ± 1.1 µM), followed by MCF-7/6, PC-3, and MDA-MB-231 (IC_{50} of 12.4 ± 1.1 µM, 13.2 ± 1.1 µM, and 36.3 ± 1.1 µM, respectively). Flavanone 6PN appeared as the second most active compound (except for LNCaP.FGC) with a marked growth-inhibitory effect on PC-3 (IC_{50} of 18.4 ± 1.2 µM), followed by DU145, MCF-7/6, and MDA-MB-231 (IC_{50} of 29.1 ± 1.1 µM, 32.6 ± 1.0 µM, and 65.1 ± 1.0 µM, respectively). Flavanone 8PN showed its most pronounced antiproliferative effects on PC-3, LNCaP.FGC, and DU145 (IC_{50} of 33.5 ± 1.0, 42.8 ± 1.2, and IC_{50} of 43.1 ± 1.2 µM, respectively), while IX was more effective on the breast cancer cells MCF-7/6 (IC_{50} of 32.9 ± 1.1), but showing activities on prostate cancer cells comparable to those of 8PN (IC_{50} of 45.2 ± 1.1 µM on PC-3 and 47.4 ± 1.1 µM on DU145). However, IX proved to be the most potent inhibitor of LNCaP.FGC with an IC_{50} of 34.4 ± 1.0 µM. DMX was the least active compound in all cell lines (data are missing for LNCaP.FGC).

The calculated IC_{50} values (the dose resulting in a 50% inhibition of cell growth) are presented in **figure 5.9**.

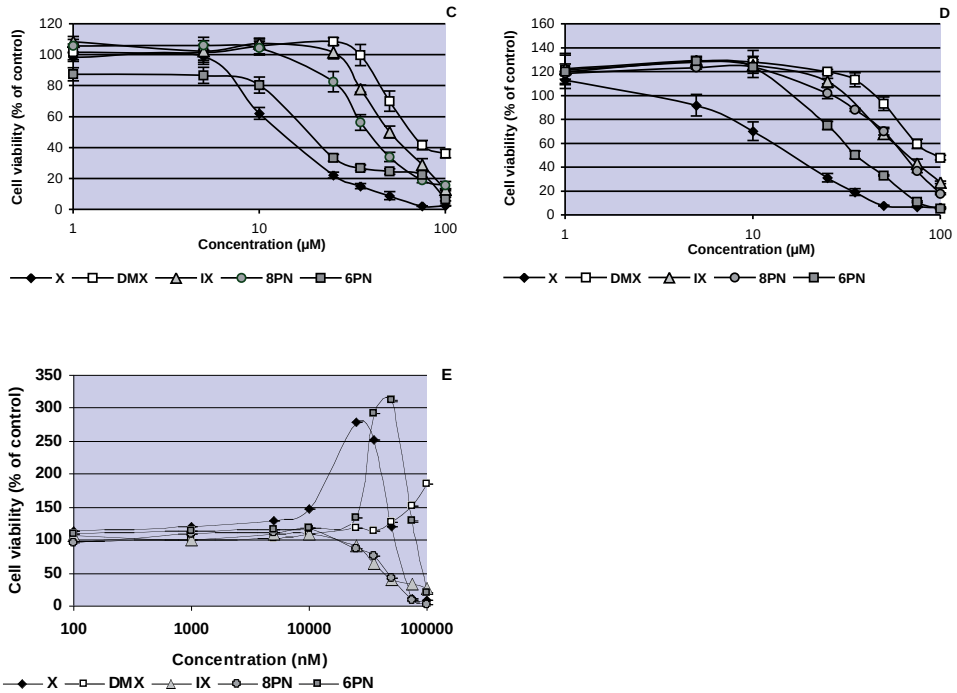
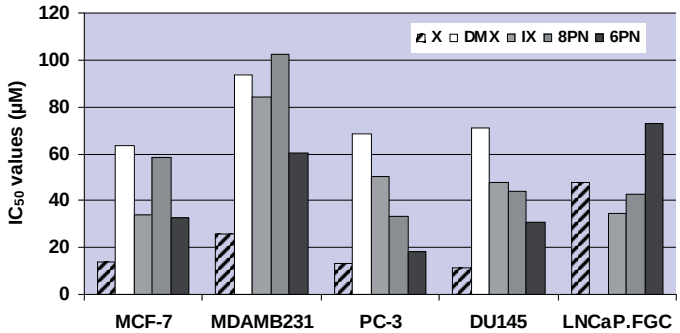


Figure 5.8. Dose-response relationships for hop-derived prenylflavonoids on the growth of human prostate cancer cells (C: PC-3, D: DU145, E: LNCaP.FGC) after 2 days of exposure. Data are means ± SD (n=3). Materials and Methods, see: 5.3 and 5.4.1, respectively.
X: xanthohumol, DMX: desmethylxanthohumol, IX: Isoxanthohumol, 8PN: 8-prenylnaringenin, 6PN: 6-prenylnaringenin.

On LNCaP.FGC prostate cancer cells, X and 6PN showed a biphasic dose-response behavior stimulating growth at low concentrations and inhibiting growth at higher concentrations. IX and 8PN, which are the most potent inhibitors for the growth of this cell line, inhibited growth at the same concentrations for which stimulation by X and 6PN was noted. DMX shows a similar trend as observed for X and 6PN. The highest concentrations tested for this compound on LNCaP.FGC are growth-stimulatory compared to the lower concentrations. The calculated EC_{50} values for growth stimulation of X and 6PN are 12.8 ± 1.1 and 29.6 ± 1.3, respectively. These compounds exhibit a very strong growth stimulation of about 300% compared to the control.



IC_{50} (SD) (µmol/L)	X	DMX	IX	8PN	6PN
MCF-7/6	12.4 (1.1)	68.3 (1.0)	32.9 (1.1)	56.0 (1.0)	32.6 (1.0)
MDA-MB-231	36.3 (1.1)	100.9 (1.0)	83.6 (1.0)	98.2 (1.0)	65.1 (1.0)
PC-3	13.2 (1.1)	49.9 (1.0)	45.2 (1.1)	33.5 (1.0)	18.4 (1.2)
DU145	12.3 (1.1)	53.8 (1.1)	47.4 (1.1)	43.1 (1.2)	29.1 (1.1)
LNCaP.FGC	47.7 (1.0)		34.4 (1.0)	42.8 (1.2)	72.7 (1.0)

Abbreviations: X: xanthohumol; DMX: desmethylxanthohumol;
IX: isoxanthohumol; 8PN: 8-prenylnaringenin; 6PN: 6-prenylnaringenin;
SD: standard deviation.

Figure 5.9: IC_{50} values of hop-derived prenylflavonoids in the WST1 proliferation assay for human breast cancer cells (MCF7/6, MDA-MB-231) and human prostate cancer cells (PC-3, DU145, LNCaP.FGC). Data are means ± SD (n=3). Materials and Methods, see: 5.3 and 5.4.1, respectively.

Biphasic dose-response behavior was noted for all compounds on MCF-7/6 breast cancer cells, whereby growth was stimulated at lower concentrations and inhibited at higher concentrations (**Figure 5.7, A**). The most potent growth stimulation was observed for X with a maximum effect at 5 µM. The other compounds were less active compared to X; 6PN gave the highest maximum growth stimulation at 20 µM, followed by DMX (30 µM), IX (15 µM), and 8PN (10 µM).

To determine the antiangiogenic potencies of the hop-derived prenylflavonoids, the antiproliferative effects of the chalcones X and DMX and the flavanones IX, 8PN, and 6PN were assessed using a human bone marrow endothelial (HBME) cell line. X proved to be a very potent inhibitor of the growth of HBME endothelial cells with an IC_{50} value of 3.2 ± 1.2 µM. IX and 6PN were the second most active growth inhibitors with IC_{50} values of 29.4 ± 1.1 µM and 39.8 ± 1.0 µM, respectively. 8PN and DMX were the least active growth inhibitors with IC_{50} values of 77.1 ± 1.0 µM and 72.7 ± 1.0 µM, respectively. Furthermore, compared to the breast and prostate cancer cell lines, where X also proved to be the strongest growth

inhibitor (except for LNCaP.FGC prostate cancer cells), the growth-inhibitory activity on endothelial cancer cell lines was much stronger. The results of the WST1 assay are illustrated in **figure 5.10.**

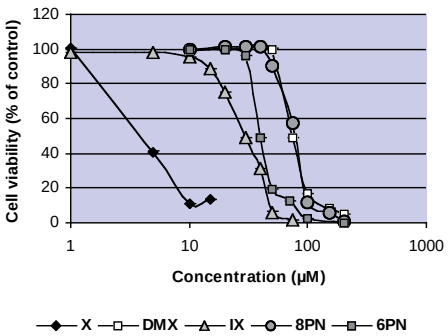


Figure 5.10: Dose-response relationships for hop-derived prenylflavonoids on the growth of human endothelial cancer cells (HBME). Data are means ± SD (n=3). Materials and Methods, see: 5.3 and 5.4.1, respectively.
X: xanthohumol, DMX: desmethylxanthohumol, IX: Isoxanthohumol, 8PN: 8-prenylnaringenin, 6PN: 6-prenylnaringenin.

The MTT assay as first described by Mosmann[21] was critically analyzed in a screening system.[31] Compounds with an intrinsic reductive potential reduce the MTT irrespective of any cellular mechanism and, therefore, may give false-positive results, unless the potentially interfering agents are removed by adequate washing. Bruggisser et al. concluded that the application of tetrazolium assays in a screening system to detect the influence on the cell viability demands precautions.[31] It is recommended to perform a pre-screening in cell-free systems to examine potential reductive activity before any cell culture experiment should be performed. When reduction occurs, washing procedures have to be implemented and results have to be interpreted carefully to avoid wrong interpretations. None of the hop-derived prenylflavonoids under study showed chemical interactions with the WST1 reagent. Therefore, possible false-positive results of this kind could be excluded.

The chalcones X and DMX are yellowish compounds, which absorb slightly at 450 nm. This inherent absorption may interfere with the absorption at 450 nm of the formazan product formed after incubation with the WST1 reagent. We found that X and DMX interfered with the absorbance of WST1 at the monitoring wavelength, hence, experimental values were corrected for the measured absorptions of these compounds at 450 nm.

5.6.2. Estrogenic activity assay

5.6.2.1. Growth-stimulatory assay

The effect of the hop-derived prenylflavonoids on the MCF7/6 breast cancer cells was determined to assess the estrogenic potencies of the compounds. As the MCF7/6 breast cancer cell line served as an estrogen-responsive reference control model in the proliferation assays (see 5.4.1 and 5.6.1), first results of the influence of the compounds on the growth of this cell line were already obtained. However, the growth-stimulatory effects observed required further investigations, since a strong growth-stimulatory effect for 8PN on this estrogen-responsive breast cancer cell line was expected, as 8PN has been characterized as a highly potent phytoestrogen.[3] Therefore, the MCF-7/6 breast cancer cells were incubated with 17β-estradiol (1 fM to 1 µM) or 8PN (1 fM to 1 µM) at varying concentrations of steroid-free, dextran-coated, charcoal-treated FBS and varying incubation times, in order to establish the most favorable conditions to reproduce a growth stimulatory dose-response curve.

A pronounced growth-stimulatory effect for 8PN was observed after three days of incubation with a concentration of 2% steroid-free, dextran-coated, charcoal-treated FBS. This effect was noted for 17β-estradiol, when the experimental media did not contain steroid-free, dextran-coated, charcoal-treated FBS. **Figure 5.11** illustrates the growth-stimulatory effects of both compounds on MCF7/6 cells carried out under the specific conditions as determined for each compound separately.

The calculated EC_{50} values for growth stimulation of 17β-estradiol and 8PN were 9.3 ± 0.5 pM and 4.2 ± 1.3 nM, respectively, indicating that 8PN is about 500 times weaker in stimulating growth as compared to 17β-estradiol. However, the comparison is not reliable, because of the different conditions used in both assays. We were able to confirm the biphasic dose-response curve for 8PN, which had been found indeed for all compounds in the cell proliferation assays (see: 5.6.1). At a concentration of 100 nM, maximum growth stimulation was achieved and the growth-stimulatory effect diminished at higher concentrations to finally result in a growth-inhibitory effect from a concentration of 10 µM.

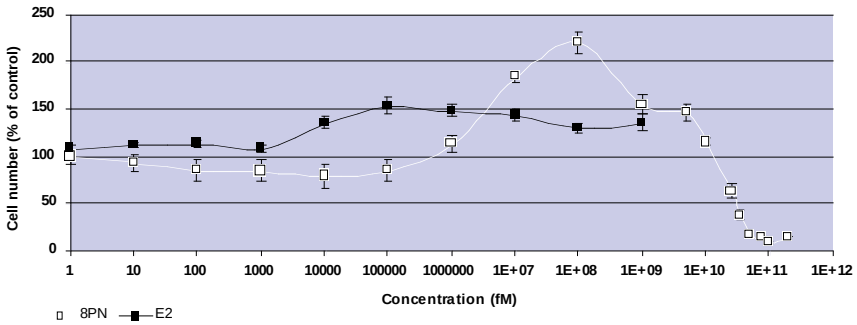
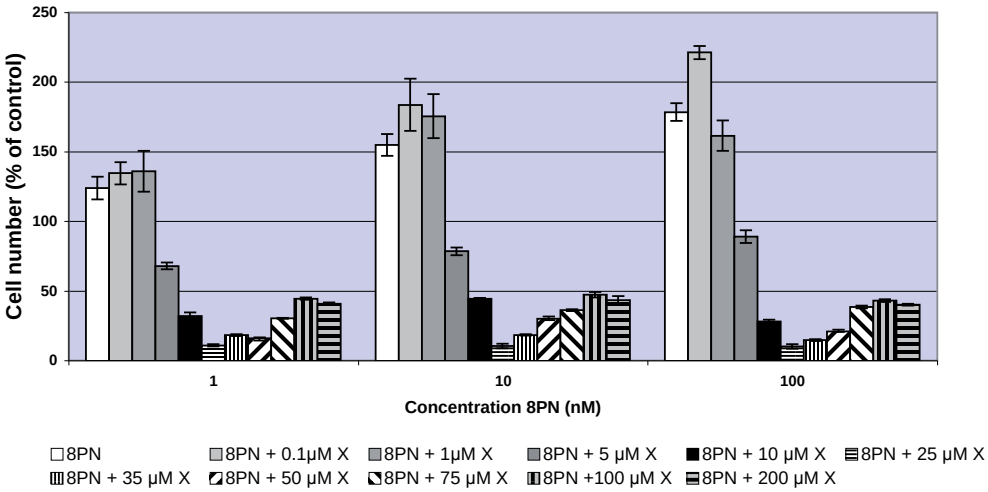


Figure 5.11: Growth-stimulatory effect of 17β-estradiol (E2) and 8-prenylnaringenin (8-PN) on MCF-7/6 breast cancer cells. Materials and Methods, see: 5.3 and 5.4.2.1, respectively.

A further study aimed at observing potential inhibitory effects of X known for its potent cancer chemopreventive activities,[17] on the growth-stimulatory effect of 8PN on MCF7/6 breast cancer cells. MCF7/6 cells were incubated with three concentrations of 8PN (1, 10 or 100 nM) that were effective in growth stimulation (**Figure 5.11**) and X was added in varying concentrations ranging from 100 nM to 200 µM. The results are illustrated in **figure 5.12**.

It was concluded that X inhibited the growth-stimulatory effect of 8PN at concentrations between 1 µM and 5 µM. Concentrations above 5 µM exhibited a growth-inhibitory effect as compared to the control (100%). Moreover, at a concentration of 0.1 µM X has an additional growth-stimulatory effect leading to an increased growth stimulation as was observed for 8PN. These findings are in accordance with the biphasic dose-response curves observed for X on MCF7/6 breast cancer cells in the cell proliferation assays (see: 5.6.1).



Figuur 5.12: Effect of xanthohumol (X) on the growth stimulation of 8-prenylnaringenin (8PN) on MCF-7/6 breast cancer cells. Materials and Methods, see: 5.3 and 5.4.2.1, respectively.

5.6.2.2. Recombinant yeast estrogen screen (YES)

The YES was used to investigate the binding capacity of the compounds to the estrogen receptor alpha (ERα) and their potency to subsequently stimulate a signal-transduction cascade resulting in secretion of β-galactosidase. 17β-Estradiol served as a positive control. X and IX exhibited very weak activities, hence, the incubation times in the YES assay needed to be prolonged to five to six days in order to detect a dose response above the detection limit. Under these conditions, EC_{50} values can not be used for direct comparison of the estrogenic activities and, thus, relative potencies were determined. The results are presented in **table 5.1**.

The EC_{50} value of 214 pM for 17β-estradiol after three days of incubation demonstrates the high sensitivity of the assay. From the relative potencies observed, it can be concluded that the estrogenic activity of 8PN determined by the YES was approximately 300-fold lower than that of 17β-estradiol, but 10-, 50-, and 10,000-fold higher than the activities of DMX, 6PN, and X/IX, respectively. Furthermore, the relative induction efficiencies indicate that, when compared to the full agonist 17β-estradiol, 8PN, 6PN, and DMX produce a

maximum response and, thus, may behave as full agonists on ERα. Conversely, X and IX could not give rise to a maximum response, which may suggest that they behave as partial agonists on ERα.

Table 5.1: EC_{50} values, relative potencies (RP) and relative induction efficiencies (RIE) of hop-derived prenylflavonoids in the recombinant yeast estrogen screen (n>3 independent experiments). Materials and Methods, see: 5.3 and 5.4.2.2, respectively.

Compound	EC_{50}[a] (95% CI) (mol/L)	RP[a]	RIE[b]
17β-estradiol*	2.14E-10 (1.49E-10-3.08E-10)	1	100
8PN*	6.3E-08 (1.4E-08-2.8E-07)	3.1E-03 (9.2E-04-1.0E-02)	111.6 (7.8)
6PN*	3.6E-06 (1.3E-06-1.0E-05)	6.6E-05 (3.3E-05-1.3E-04)	106.6 (15.5)
DMX*	6.9E-07 (2.5E-07-1.9E-06)	3.0E-04 (2.1E-04-4.4E-04)	108.7 (14.3)
X**	7.6E-05 (2.3E-05-2.5E-04)	3.5E-07 (6.9E-08-1.7E-06)	64.8 (36.7)
IX**	7.2E-05 (3.0E-05-1.7E-04)	3.7E-07 (1.1E-07-1.2E-06)	63.3 (18.6)

Abbreviations: CI: confidence interval; 8PN: 8-prenylnaringenin; 6PN: 6-prenylnaringenin; DMX: desmethylxanthohumol; X: xanthohumol; IX: isoxanthohumol; [a]geometric mean; [b]arithmetic mean (standard deviation); * 3 days of incubation; ** 5-6 days of incubation.

5.6.3. Determination of the cell seeding density

5.6.3.1. Cell proliferation assay

Cells had to be seeded into flat-bottomed 96-well tissue culture plates at a certain density. Not all cell lines grew at the same rate and significant differences in cell densities could have been expected after 48 h of incubation time. Since the endpoints of the WST1 and SRB assays are determined by color formation (measured at the appropriate wavelength), it was important to confirm that measurements were within the lineair absorbance ranges for all cell lines. Therefore, the seeding density for each cell line was determined from 'blank' curves by seeding the cells at various concentrations (MCF7/6, MDA-MB-231, PC-3, DU145, and HBME: 1,000 cells/well to 10,000 cells/well; LNCaP.FGC: 20,000 cells/well to 200,000 cells/well) and incubating them during the same time as proposed for the *in vitro* assays. Cell viability or protein content was measured using the WST1 assay or the SRB assay, respectively (see: 5.4.1.1 and 5.4.1.2, respectively).

For each cell line, the innoculum (seeding concentration) that produced a color intensity within the linear range with an appropriate cell density for stimulation or inhibition of proliferation was established.

Figure 5.13 illustrates the 'blank' curves for PC-3 for which color formation was measured using the WST1 assay (A) and the SRB assay (B).

The results show that the absorbances measured with the WST1 assay are higer than those for the same cell concentrations in the SRB assay. It was preferred to use an innoculum of 6,000 cells/well for the prostate cancer cell line PC-3 with the WST1 and the SRB assays. Similarly, the innocula for the other cell lines were determined to be 2,000 cells/well for MCF-7/6, 3,000 cells/well for DU145, 10,000 cells/well for MDA-MB-231, 17,000 cells/well for LNCaP.FGC, and 6,000 cells/well for HBME.

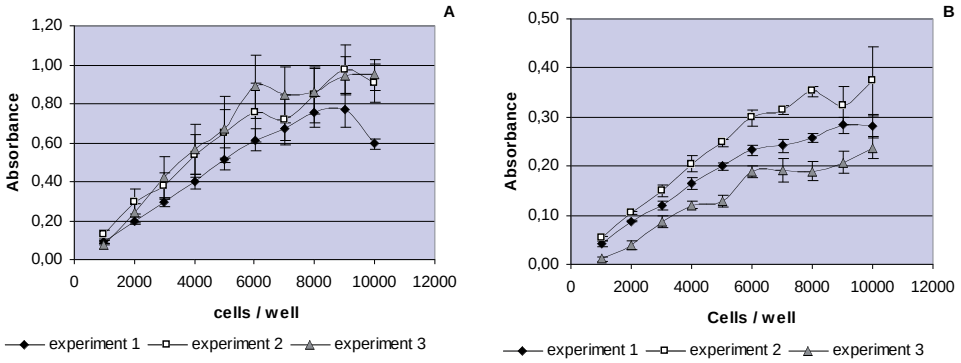


Figure 5.13: 'Blank' curves for PC-3 prostate cancer cells (A: WST1 assay, B: SRB assay). Materials and Methods, see: 5.3 and 5.4.3.1, respectively.

5.6.3.2. Growth-stimulatory assay

For the growth-stimulatory assay, 'blank' curves for MCF7/6 breast cancer cells were obtained to determine the innoculum and the concentration of steroid-free, dextran-coated charcoal-treated FBS (DCC-FBS) needed to observe proliferation stimulation. Therefore, varying concentrations of MCF7/6 breast cancer cells (from 500 cells/well to 10,000 cells/well) were incubated with experimental medium containing varying concentrations of DCC-FBS (0 %; 2 % or 5 %). The protein content was measured after an incubation time of 72 h using the SRB assay (see: 5.4.1.2). **Figure 5.14** illustrates the 'blank' curve for the MCF-7/6 breast cancer cell line.

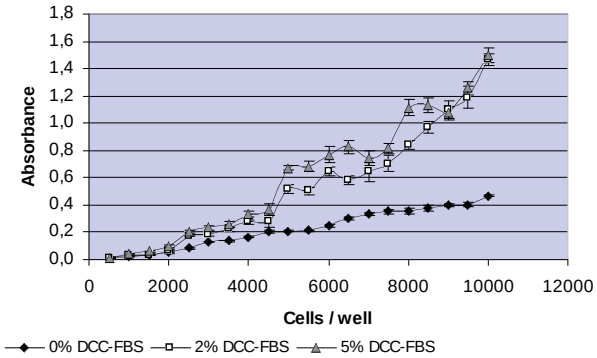


Figure 5.14: Blank curve for MCF7/6 breast cancer cells using the SRB assay. DCC-FBS: Dextran-coated charcoal-stripped foetal bovine serum. Materials and Methods, see: 5.3 and 5.4.3.2, respectively.

The dose-response curve obtained using experimental medium without DCC-FBS is linear over a broad range, but absorbances are rather low. The absorbances obtained from the experiments using experimental medium supplemented with 2% and 5% DCC-FBS cover the linear range, but the values are higher leading to a more sensitive reading. From these results, experimental medium supplemented with 0% and 2% DCC-FBS were chosen for further experiments, and the preferred innocula were determined to be 5,000 cells/well and 10,000 cells/well, respectively, giving possibilities for proliferation that can be measured within the linear area of absorption after the proposed incubation time of 72 h (see: 5.4.3.2).

5.6.4. Determination of the presence of ERα and ERβ mRNA transcripts by RT-PCR

5.6.4.1. RNA extraction

Total RNA extracts were prepared from the breast cancer cell lines MCF7/6, and MDA-MB-231, and from the prostate cancer cell lines PC-3, DU145 and LNCaP.FGC, using the RNeasy Mini Kit. Results showed that it was necessary to add the DNase-solution to the RNeasy mini column before washing and eluting the RNA. The RNA concentrations in the extracts were measured with the Beckman DU-600 UV-Vis Diode Array Spectrometer. **Table 5.2** shows the results of the RNA concentration measurements.

Table 5.2: Results of the RNA concentration measurements. Materials and Methods, see: 5.3 and 5.4.4.1, respectively.

	Absorbance (260 nm)	*Absorbance (280 nm)*	*260/280*	*Dilution factor*	*RNA concentration (µg/mL)*
Extract					
PC-3	0,7742	0,3537	2,19	10	309,66
DU 145	0,9398	0,4362	2,15	10	375,91
LNCaP	1,0299	0,4978	2,07	10	411,95
MCF7/6	0,7354	0,3346	2,20	10	294,17
MDA-MB-231	0,7081	0,3528	2,01	10	283,26

5.6.4.2. Development of selective oligonucleotide primers

For each estrogen receptor, two different sets of primers were developed using the programs 'primer 3' and 'clonewin'. The cDNA sequences of ERα and ERβ were copied from the 'NCBI' and 'Ensembl' site and 'blasting' those two sequences gave a 99% match. All four sets of primers were examined for their selectivities using the option 'blast' on the NCBI site. The sequences of the primers for both estrogen receptors are illustrated in **figure 5.15**. The positions of the primers on the cDNA sequence of ERα and ERβ are shown in **figure 5.16 and 5.17**, respectively.

	ER-alpha	ER-beta
FP1	5´- ACGGACCATGACCATGACCCT -3´	5´- TTCCCAGCAATGTCACTAACT -3´
RP1	5´- GGTGGCTGGACACATATAGT -3´	5´- CTCTTTGAACCTGGACCAGTA -3´
FP2	5´- GCTCCTTTAATTGGTGACTTGG -3´	5´- CCTATGTAGACAGCCACCATGA -3´
RP2	5´- AGGGAGGGCTTTCTTTTGTAAC -3´	5´- AAAAGGCCTTACATCCTTCACA-3´

Figure 5.15: Sequences of the primers for ERα and ERβ (FP1: Forward primer from primer set 1, RP1: Reversed primer from primer set 1, FP2: Forward primer from primer set 2, RP2: Reversed primer from primer set 2). Materials and Methods, see: 5.3 and 5.4.4.2, respectively.

GTCGGGTCGCCCGGCTTCACCGGACCCGCAGGCTCCCGGGGCAGGGCCGGGGCCAGAGCTCGCGTGTCGGCGGGACAT
GCGCTGCGTCGCCTCTAACCTCGGGCTGTGCTCTTTTTCCAGGTGGCCCGCCGGTTTCTGAGCCTTCTGCCCTGCGGGGA
CACGGTCTGCACCCTGCCCGCGGCCACGGACCATGACCATGACCCTCCACACCAAAGCATCTGGGATGGCCCTACTGC
ATCAGATCCAAGGGAACGAGCTGGAGCCCCTGAACCGTCCGCAGCTCAAGATCCCCCTGGAGCGGCCCCTGGGCGAGG
TGTACCTGGACAGCAGCAAGCCCGCCGTGTACAACTACCCCGAGGGCGCCGCCTACGAGTTCAACGCCGCGGCCGCCG
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GAAAGGTGGGATACGAAAAGACCGAAGAGGAGGGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGGAGGGC
AGGGGTGAAGTGGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGCTCTAAG
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GCCTGGCTAGAGATCCTGATGATTGGTCTCGTCTGGCGCTCCATGGAGCACCCAGGGAAGCTACTGTTTGCTCCTAACT
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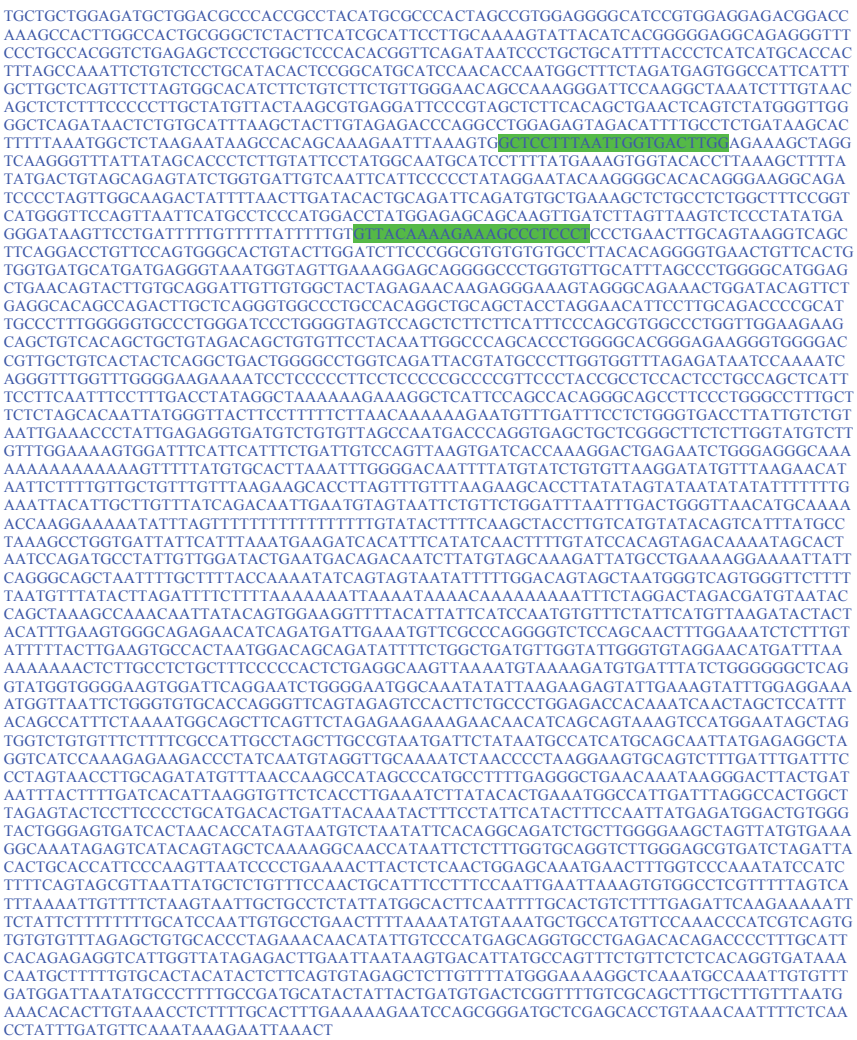
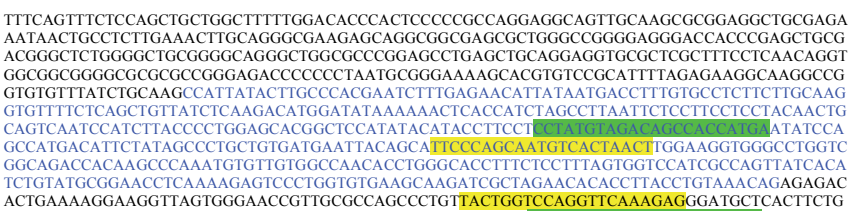


Figure 5.16: Positioning of the developed primers on the cDNA sequence of the human ERα (Primerset 1: yellow, Primerset 2: green). Transitions between the different exons are marked by transition from black to blue. Materials and Methods, see: 5.3 and 5.4.4.2, respectively.



AGCATTCAAGGACATAATGATTATATTTGTCCAGCTACAAATCAGTGTACAATCGATAAAAACCGGCGCAAGAGCTGC
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CTTGTGCGGAGACAGAGAAGTGCCGACGAGCAGCTGCACTGTGCCGGCAAGGCCAAGAGAAGTGGCGGCCACGCGCC
CCGAGTGCGGGAGCTGCTGCTGGACGCCCTGAGCCCCGAGCAGCTAGTGCTCACCCTCCTGGAGGCTGAGCCGCCCCA
TGTGCTGATCAGCCGCCCCAGTGCGCCCTTCACCGAGGCCTCCATGATGATGTCCCTGACCAAGTTGGCCGACAAGGAG
TTGGTACACATGATCAGCTGGGCCAAGAAGATTCCCGGCTTTGTGGAGCTCAGCCTGTTCGACCAAGTGCGGCTCTTGG
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TCAAGGTTTCGAGAGTTAAAACTCCAACACAAAGAATATCTCTGTGTCAAGGCCATGATCCTGCTCAATTCCAGTATGT
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GGTTTGGGTGATTGCCAAGAGCGGCATCTCCTCCCAGCAGCAATCCATGCGCCTGGCTAACCTCCTGATGCTCCTGTCC
CACGTCAGGCATGCGAGTAACAAGGGCATGGAACATCTGCTCAACATGAAGTGCAAAAATGTGGTCCCAGTGTATGAC
CTGCTGCTGGAGATGCTGAATGCCCACGTGCTTCGCGGGTGCAAGTCCTCCATCACGGGGTCCGAGTGCAGCCCGGCA
GAGGACAGTAAAAGCAAAGAGGGCTCCCAGAACCCACAGTCTCAGTGA

Figure 5.17: Positioning of the developed primers on the cDNA sequence of the human ERβ (Primerset 1: yellow, Primerset 2: green). Transitions between the different exons are marked by transition from black to blue. Materials and Methods, see: 5.3 and 5.4.4.2, respectively.

The programs 'primer 3' and 'clonewin' give additional necessary information about the exact positions of the primers on the cDNA strand and the positions of the primers regarding the different exons and introns. **Table 5.3** gives an overview of this information. From the positions of the primers on the cDNA strand, the length of the primers and the length of the introns which lie in-between the exons, the length of the amplicon that is formed in the RT-PCR reaction from the mRNA template as well as from the possibly genomic DNA present could be calculated. These programs also propose for each set of primers an optimum annealing temperature, however, these temperatures had to be optimized experimentally by using the thermogradient method.

Table 5.3 Features of the sets of primers

	ER-alpha				ER-beta			
	Primerset 1		Primerset 2		Primerset 1		Primerset 2	
	FP	RP	FP	RP	FP	RP	FP	RP
Position on cDNA (bp)	356	1015	2580	2964	591	829	522	914
Length of primer (bp)	21	20	22	22	21	21	22	22
Position on exon	1	3	8	8	2	3	2	3
Intron in between (length in bp)	1-2 (34232) 2-3 (37867)		/		2-3 (2470)		2-3 (2470)	
Annealing temperature (°C)	59		60		55		57	
Length of amplicon for mRNA template (bp)	679		406		259		414	
Length of amplicon for genomic DNA template (bp)	72777		406		2884		2329	

From the information given in **table 5.3,** it could be concluded that all proposed primers have an optimum length for stable binding and selectivity, which is around 20 bp. The amplicon formed lies within the acceptable range for RT-PCR. Primerset 2 for ERα seemed not being able to distinguish between a signal derived from the transcription of the

mRNA template and that derived from the transcription of the genomic DNA template that could be present. This primerset is, therefore, not appropriate to perform the RT-PCR reaction. The other sets of primers lie on different exons and, therefore, result in two different amplicon signals after transcription of the mRNA template and the genomic DNA template that could be present.

5.6.4.3. The RT-PCR reaction and gel electrophoresis

Two human breast cancer cell lines MCF-7/6 and MDA-MB-231 and three human prostate cancer cell lines PC-3, DU145, and LNCaP.FGC were screened for the presence of the mRNA-transcripts of the ERα and ERβ using RT-PCR. For each receptor, two sets of primers were used. The β-actin positive control for the RT-PCR reaction included in all RT-PCR reactions proved to be positive, thereby confirming that the optimized RT-PCR conditions (quantities of reagents used in the reaction mixture, temperature settings for the thermogradient method, duration of the different steps) were efficient. The control of the presence of genomic DNA for all cell extracts (mixture without MulVRTase) proved to be negative. Primer set 2 for ERα, which could not distinguish between mRNA and genomic DNA signals, was also tested along the different RT-PCR reactions performed.

To determine the optimum annealing temperature, the four sets of primers were examined on all cell extracts using the thermogradient method. The results showed that the optimum annealing temperatures for the primer sets of ERα and ERβ were 50°C and 65°C, respectively. It could be concluded that the primer set, which gave most selective and efficient results for both receptors, was primer set 1. The fixed temperature method was repeated for primer set 1 from ERα and ERβ on the breast and prostate cancer cell extracts and the results are shown in **table 5.4**.

Table 5.4: Results of the screening for the presence of the mRNA transcripts of the ERα and ERβ in the human breast cancer cell lines MCF-7/6 and MDA-MB-231, and the human prostate cancer cell lines PC-3, DU145, and LNCaP.FGC.

	MCF7/6	MDA-MB-231	PC-3	DU145	LNCaP.FGC
ER-alpha	+++	+	++	+	++
ER-beta	++	+++	+++	+	+

MCF7/6 demonstrated a very strong signal for ERα and only a weak, but positive signal for ERβ. In contrast, MDA-MB-231 showed a strong signal for ERβ and a very weak

signal for ERα. PC-3 gave a very strong signal for ERβ and only a weak, but positive signal for ERα. LNCaP.FGC showed a signal for ERα, which was stronger than for ERβ. DU145 was positive for both ERα and ERβ, but the signals were weak in both cases.

5.7. Discussion

Polyphenols are abundant micronutrients in our diet and evidence for their role in the prevention of degenerative diseases such as cancer and cardiovascular diseases is emerging. The hop plant proved to be a rich source of particular polyphenols notably prenylflavonoids. The main dietary source of hop-derived prenylflavonoids is through consumption of beer and their content can be up to 4 mg/L. A number of anticancer-linked biological activities have been ascribed to individual prenylflavonoids, including antioxidant properties[38], inhibition of human cytochrome P450 enzymes,[39] and induction of quinone reductase[40]. Miranda et al. investigated the ability of X, DMX, and IX, as well as some minor prenylflavonoids present in hops, to inhibit the proliferation of human breast cancer (MCF-7), colon cancer (HT-29), and ovarian cancer (A2780) cell lines *in vitro*.[16] A dose-dependent growth inhibition of MCF-7 cells was observed for X and IX with IC_{50} values of 13.3 µM and 15.3 µM, respectively, after two days of exposure. X exhibited an even more pronounced growth-inhibitory effect against A2780 ovarian cancer cells (IC_{50} of 0.52 µM). On the other hand, the viability of HT29 colon cancer cells was not affected by X and IX. DMX was the least active in breast cancer MCF-7 cells. The flavanone 8PN proved to inhibit angiogenesis with an IC_{50} of between 3 and 10 µM using bovine microvascular endothelial cells derived from the adrenal cortex (BME cells) and from the thoracic aorta (BAE cells).[20]

Proliferation assays using human cancer cell lines are commonly recognized procedures to examine compounds of various sources for their potential anticarcinogenic properties. In this study, we used the cell proliferation reagent WST-1 and the SRB assay to determine the relative potencies of the hop-derived prenylflavonoids in inhibiting the growth of cancer cells and in assessing the sensitivities of the different cell lines to these compounds. More specifically, we aimed at getting detailed insights into the effects of the chalcones X and DMX, and the flavanones IX, 8PN, and 6PN on the proliferation of different types of prostate carcinoma cells (PC-3, DU145, LNCaP.FGC). In parallel, the hop-derived prenylflavonoids were exposed to the breast cancer cell lines MCF7/6 and MDA-MB-231, which served as estrogenic reference models. Each of these cell lines represents a different stage of the disease

and a different hormonal status and response, because of the varying expression profiles of the estrogen receptors alpha (ERα) and beta (ERβ).[41,42,43,44,45,46] By means of RT-PCR, we determined the presence of the ERα and ERβ mRNA transcripts in all cell lines. Furthermore, we assayed the antiproliferative properties of the hop-derived prenylflavonoids on a human bone marrow endothelial cell line (HBME) to search for potential antiangiogenic activities.

The WST1 assay is a colorimetric assay based on the cellular reduction of WST1 to a formazan product by the mitochondrial dehydrogenases of viable cells. It is adequate for the quantification of cell viability, cell proliferation or cytotoxicity. It had been shown previously that compounds with intrinsic reductive potential such as kaempferol can interact with the dye of the proliferation assay leading to false positive results.[31] The hop-derived prenylflavonoids studied, however, did not interact with the WST1 reagent. The SRB assay provides a sensitive method for measuring drug cytotoxicity in culture. The dye binds to the proteins of cells thereby providing a sensitive index of the cellular protein content, which is directly correlated to the number of cells in culture. Thus, a growth-stimulatory or growth-inhibitory effect is evident.

X proved to be the most potent compound in inhibiting the growth of all cell lines (except for LNCaP.FGC), with IC_{50} values of 3.2 ± 1.2 μM for HBME, 12.3 ± 1.1 μM for DU145, 12.4 ± 1.1 μM for MCF-7/6, 13.2 ± 1.1 μm for PC-3, and 36.3 ± 1.1 μM for MDA-MB-231. In particular, X was highly potent in suppressing the growth of the endothelial cells (IC_{50} of 3.2 ± 1.2 μM) suggesting that X may be an efficient antiangiogenic agent. 6PN had the most pronounced growth inhibitory effect on PC-3 (IC_{50} of 18.4 ± 1.2 μM), followed by DU145, MCF-7/6, HBME, and MDA-MB-231 (IC_{50} of 29.1 ± 1.1 μM, 32.6 ± 1.0 μM, 39.8 ± 1.0 μM, and 65.1 ± 1.0 μM, respectively). 8PN showed pronounced antiproliferative effects on PC-3, LNCaP.FGC, and DU145 (IC_{50} of 33.5 ± 1.0, 42.8 ± 1.2, and IC_{50} of 43.1 ± 1.2 μM, respectively). IX was the second most active growth inhibitor for HBME with an IC_{50} of 29.4 ± 1.1 μM. The compound showed activities on prostate cancer cells comparable to those of 8PN (IC_{50} of 45.2 ± 1.1 μM on PC-3 and 47.4 ± 1.1 μM on DU145) but, was more efficient on MCF-7/6 breast cancer cells (IC_{50} of 32.9 ± 1.1 μM) and proved to be the most potent inhibitor of LNCaP.FGC prostate cancer cells with an IC_{50} of 34.4 ± 1.0 μM. DMX was the least active compound in all cell lines (data are missing for the LNCaP.FGC cell line).

All prenylflavonoids showed a biphasic dose-response behavior on MCF-7/6 breast cancer cells, stimulating growth at low concentrations and inhibiting growth at higher

concentrations. However, a stronger growth-stimulatory effect for 8PN on this estrogen-responsive breast cancer cell line was expected, as 8PN has been characterized as a highly potent phytoestrogen.[3] Therefore, growth-stimulatory experiments were performed on the MCF7/6 cells. The calculated EC_{50} values for growth stimulation of 17β-estradiol and 8PN were 9.3 ± 0.5 pM and 4.2 ± 1.3 nM, respectively, indicating that 8PN is about 500 times weaker in stimulating growth as compared to 17β-estradiol. However, the comparison is not reliable, because of the different conditions used in both assays. At a concentration of 100 nM, maximum growth stimulation was achieved and the growth-stimulatory effect diminished at higher concentrations to finally result in a growth-inhibitory effect from a concentration of 10 μM. The well-described potent cancer chemopreventive activities of X inspired us to investigate whether X could inhibit the strong growth-stimulatory effect of 8PN on MCF7/6 breast cancer cells. X did inhibit the growth-stimulatory effect of 8PN at concentrations between 1 μM and 5 μM. Concentrations above 5 μM exhibited a growth-inhibitory effect as compared to the control (100%). At a concentration of 0.1 μM, X had an additional growth-stimulatory effect leading to an increased growth stimulation as was observed for 8PN. On LNCaP.FGC prostate cancer cells, X and 6PN showed a biphasic dose-response behavior stimulating growth at low concentrations and inhibiting growth at higher concentrations. IX and 8PN, which are the most potent inhibitors for the growth of this cell line, inhibited growth at concentrations that led to stimulation by X and 6PN.

As the inhibitory effects of the prenylflavonoids on cancer cells may be mediated through hormonal - in particular estrogenic - mechanisms, it was important to additionally assess the estrogenic activity using an estrogen-inducible yeast screening assay (yeast estrogen screen; YES)[18] and to assess the hormonal status of the cell lines used. In the YES, X and IX were almost inactive, while 6PN behaved as a weak estrogen and DMX and 8PN revealed potent estrogenic activities. However, it should be noted that the high activity of DMX could contribute to its instability and subsequent conversion to a mixture of 8PN and 6PN (60% conversion after 48 h incubation at 37°C, data not shown). Therefore, activities found for DMX should be interpreted with great caution. Milligan et al. found similar dose-response curves with the YES, but results obtained with the Ishikawa Var I assay gave a different ranking of the estrogenic activity (from high to low): 8PN >> DMX > IX > 6PN > X.[3,47] The difference in activity for IX in the YES (almost no activity) compared to the Ishikawa Var I assay (weak estrogenic activity) could be explained by a possible metabolic conversion of IX to 8PN in the endometrial cells used in the latter assay. In the cell lines

applied in the present study, IX may also have been metabolized to 8PN and this feature should also be taken into account when interpreting the results.

The inhibitory effects of the hop-derived prenylflavonoids on cancer cells may be mediated through hormonal mechanisms, as seems to be evidenced by the antiproliferative activities on the breast cancer cells MCF7/6 and MDA-MB-231 that are inversely correlated to increasing estrogenic activities. Indeed, considering the potential metabolic conversion of IX into 8PN and the instability of DMX, the estrogenic activities in the YES (from high to low) were: 8PN >> DMX > 6PN > IX > X, while the antiproliferative effects on MCF-7/6 and on MDA-MB-231 cells were (from high to low): X > 6PN > IX > 8PN > DMX. The pattern of responses observed with the two prostate cancer cell lines PC-3 and DU145 was (from high to low): X > 6PN > 8PN > IX > DMX showing on their turn an inverse correlation to increasing estrogenic activities. This could be explained by the preferential binding of X and 6PN to the ERβ which is suggested to be predominantly antiproliferative. However, the pattern of response observed with LNCaP.FGC cells (IX > 8PN > X > 6PN) demonstrated no correlation with the estrogenic activity. Nevertheless, no quantitative relation between the estrogenic potency as measured in the YES and the antiproliferative effect could be established.

The observed variations in sensitivities between the different types of cancer cells may be attributed to the varying expression profiles of the ERα and ERβ. Although there are contradictory literature reports in this respect,[42,48,49,50,43] it is accepted that MDA-MB-231 cells express only low levels of ER-β and MCF-7/6 cells express high levels of ERα and low levels of ERβ.[41,42,43] Moreover, MCF-7/6 and MDA-MB-231 cells express qualitatively the same ERβ-isoforms.[44] The expression profiles of both estrogen receptors remain controversial in prostate cancer cells. Maruyama et al.[45] demonstrated the expression of ERα and ERβ in PC-3 prostate cancer cells. Lau et al.[46] confirmed these results and showed exclusive expression of ERβ mRNA in DU145 and LNCaP prostate cancer cells. However, Ito et al.[51] reported also an exclusive expression of ERβ mRNA for PC-3 prostate cancer cells. Because of the controversy regarding the presence of the human estrogen receptors in the commonly used cancer cell lines, we examined the presence of the mRNA transcripts of the ERα and ERβ in the human breast cancer cell lines MCF-7/6 and MDA-MB-231, and in the human prostate cancer cell lines PC-3, DU145, and LNCaP.FGC, by RT-PCR.

We found that all cell lines demonstrated positive signals for the ERα and ERβ transcripts, with the strongest signal for ERα in the MCF7/6 breast cancer cell line and the strongest signal for ERβ in the PC-3 and MDA-MB-231 cells. The results must be interpreted with caution, because the RT-PCR technique was not quantitative. The intensities of the signals can be compared, but only when submitted to the same RT-PCR reaction and even then, there are still events that make comparison of signals questionable. The experiments were repeated three times in exactly identical conditions and the order of cell lines submitted to a particular RT-PCR reaction were always respected. It can safely be concluded that ERα is most abundant in MCF7/6 breast cancer cells and ERβ is most abundant in PC-3 prostate cancer and MDA-MB-231 breast cancer cells.

These results are in accordance with the literature regarding the presence of ERα and ERβ for MCF7/6 and ERβ for MDA-MB-231. The presence of an ERα transcript for MDA-MB-231 was demonstrated in this study, albeit expressed lower than ERβ. For prostate cancer cells, our results are in accordance with the findings of Maruyama et al. and Lau et al. for PC-3 prostate cancer cells. However, for DU145 and LNCaP.FGC, we demonstrated that these prostate cancer cell lines express both receptor subtypes. The explanation of this difference can be found in the difference between the variants of these cell lines that are not always identified within the materials of a study. Moreover, depending on the culture conditions and media provided to the growth of the culture, the characteristics of cells can change while adapting themselves to the particular conditions.

If we correlate the findings of this study with the observed antiproliferative activities, it is difficult to explain why breast cancer MCF7/6 cells were found to be more sensitive to the hop-derived prenylflavonoids than MDA-MB-231 cells. Both cell lines express ERβ in comparable amounts, however, ERα is much more abundant in the MCF7/6 breast cancer cells, rendering the latter more sensitive to estrogenic stimulation. Biphasic effects on MCF-7/6 cells (noted here) have been observed for the soy-derived phytoestrogen genistein, which exhibited a dose-dependent growth-stimulatory effect at low concentrations, followed by a growth-inhibitory effect at higher concentrations on MCF-7 cells, while, under similar conditions, only a dose-dependent growth-inhibitory effect was observed on MDA-MB-231 cells.[52] The responses of different types of breast cancer cells to genistein were found to be cell-type specific and were attributed to a heterogeneous spectrum of mechanisms associated with growth modulation that involve more than only hormone-related effects. Indeed, in our

study, the observed sensitivity differences between MCF-7 and MDA-MB-231 cells could, other then a result of differences in ER-expression levels, be a result of a different ER-mediated response to the compounds with varying estrogenic activities. Remarkably, the observed growth-stimulatory effects on MCF-7/6 cells in this study are most pronounced for X, which is almost devoid of estrogenic activity, whereas the potent phytoestrogen 8PN was less active in growth stimulation, evidencing that other than ER-mediated effects could be involved.

No obvious differences in sensitivities between the prostate cancer cells PC-3 and DU145 were noted and our findings confirm indeed that both receptor subtypes are present in both cell lines, the difference in ER expression profiles being less obvious as compared to the breast cancer cells.

There is strong evidence that, for the prostate cancer cell lines, the presence of ERβ is significant with regard to the regulation of proliferation. Peach et al. demonstrated that 17β-estradiol inhibited transcription via ERβ, but activated transcription when signalling through ERα.[53] Additionally, studies in cell culture found that ERβ inhibited ERα-transcriptional activity at subsaturating levels of 17β-estradiol and decreased overall sensitivity of the cells to estrogens.[54] A differential response of 17β-estradiol was also observed in the immature uterus of ERβ-knockout (BERKO) mice, which exhibited an enhanced response to 17β-estradiol, compared with the wild-type, ERβ-positive mice. If transcriptional activity reflects the mitogenic effects of the ER, these studies and the finding that older ERβ-null mice develop prostatic hyperplasia[55] suggest that ERβ is predominantly antiproliferative. The corollary is that loss of ERβ, in conjunction with other unknown molecular events, may promote cell proliferation and, possibly, carcinogenesis. This hypothesis is compatible with the findings by Horvarth et al., who demonstrated that loss of ERβ expression is associated with progression from normal prostate epithelium to prostate cancer.[56] Furthermore, the growth-inhibitory effects of the pure anti-estrogen ICI on DU145 prostate cancer cells were reversed by ERβ-antisense oligonucleotides, which supports the notion that the observed antiproliferative activity on DU145 cells is mediated via an ERβ-signalling mechanism.[46] Our findings show indeed that 8PN, with a high affinity and activity on ERα, was less active in inhibiting the proliferation on PC3 and DU145 prostate cancer cells compared to X and 6PN which were proved to have more affinity for ERβ.

Interpretation of the observed results on LNCaP.FGC prostate cancer cells is complex. Even though ERα is present in larger amounts than the antiproliferative ERβ, the most potent estrogenic compounds IX and 8PN prove to be very potent proliferation inhibitors for this cell line. On the other hand, a growth-stimulatory effect is observed for X and 6PN, which have very low or low estrogenic activity, respectively. These results presume the presence of non-hormonal effects, however, this complex situation is probably also affected by the special features of the LNCaP.FGC prostate cancer cell line. The clone FGC is a LNCaP variant expressing two androgen-controlled pathways, namely, the induction of cell proliferation and the proliferative shutoff.[57] Using this clone, biphasic dose-responses are observed after treatment with androgens. Moreover, the androgen receptor (AR) of this cell line contains a mutation at amino acid 868 in the ligand-binding domain, which increases the affinity of the receptor for estrogens, progestins, and even some anti-androgens thereby leading to greatly enhanced agonistic effects on the receptor.[58,59] This explains why compounds such as the hop-derived prenylflavonoids could bind to the AR, thereby resulting in a biphasic dose-response. To fully understand the observed effects on this cell line, further specific research will need to be done.

All these findings and the facts that inhibitory properties were observed across all cell lines and that a direct correlation between estrogenic and antiproliferative activities for prostate cancer cells seemed to be absent, suggest that additional non-genomic mechanisms may be involved. So et al. examined the inhibition of proliferation of human breast cancer MCF-7/6 cells by flavonoids in the presence and in the absence of an excess of 17β-estradiol. Only the inhibition of cell proliferation by genistein was reversed by the competing estrogens, hence, the antiproliferative activities of other flavonoids seemed to involve different mechanisms.[60] Besides estrogen receptor-related actions, phytoestrogens may exert their activities by interacting with some of the key enzymes in sex steroid production such as aromatase, 17β-hydroxysteroid dehydrogenase, and 5α-reductase or by other, non-hormonal functions including induction of apoptosis, inhibition of tyrosine kinase, protein kinase C, and DNA topoisomerase II or by their antioxidant properties.[61] Further research on the effect of prenylflavonoids from hops on non-genomic mechanisms seems necessary.

The more efficient growth stimulation of X on MCF-7/6 cells as compared to 8PN could also be due to the formation of estrogenically active metabolites. Until now, very little is known on metabolism of hop-derived prenylflavonoids. It was reported that X is converted

to four metabolites by isosafrole/beta-naphthoflavone-induced rat liver microsomes and two major glucuronides were found with either rat or human liver microsomes.[62,63] 8PN is converted into at least twelve metabolites by human liver microsomes, the most abundant ones being oxidized within the prenyl group.[64] With the exception of the reported human cyclooxygenase-2 inhibitory activity for 5,7,4'-trihydroxy-8-(2-hydroxy-3-methylbut-3-enyl)flavanone, an isomer of 8PN, and the estrogenic 8-prenylapigenine, a *C*-ring dehydrogenated analog of 8PN, it remains uncertain whether these oxidation and conjugation products possess well-defined biological activities.

Previous studies on structure-activity relationships of chalcones indicated that their potencies to inhibit proliferation of colon cancer cells (CCL 220.1) was dependent on the number and the positions of hydroxyl groups in the chalcone nucleus.[65] Furthermore, the presence of an α,β-double bound, hydroxylation at the 2'- or 3'-positions of the B-ring, and the absence of a prenyl group appeared important for antiproliferative activity in ovarian cancer cells.[66] Analysis of the effects in the series used in the present study reveals that the methoxy group at the 6'-position in the A-ring of X is essential, since a hydroxy group at this position (as in DMX) abolishes the strong inhibitory capacity for all cell lines. This phenomenon has also been observed by Miranda et al. for chalcones X and DMX on breast cancer (MCF-7) cells and colon cancer (HT-29) cells, but not on ovarian cancer (A2780) cells.[16]

In the same study, Miranda et al. showed that conversion of a chalcone to a flavanone (X to IX) resulted in reduced antiproliferative activities for X on MCF-7 and A2780 cells. Our observations indicate comparable activities for X, except for the LNCaP.FGC cell line, which shows different results at all levels probably due to the sensitive AR receptor. Conversion of the chalcone DMX to the flavanones 8PN and 6PN, however, resulted in increased activities. It should be noted that DMX is unstable giving rise to 8PN and 6PN. Therefore, activities found for DMX should be interpreted with great caution and the effect of DMX is probably the sum of the effects of 8PN and 6PN separately.

5.8. Conclusions

Antiproliferative activities of xanthohumol and 6-prenylnaringenin were found to be strongest for the breast cancer cells MCF-7/6 and MDA-MB-231, and the prostate cancer cells PC-3 and DU145, while, on LNCaP.FGC, these compounds first exerted a strong

induction of cell proliferation followed by a proliferation shut-off. 8-Prenylnaringenin, a compound that has previously been identified as a potent phytoestrogen, demonstrated weaker antiproliferative effects on prostate cancer cell lines PC-3 and DU145, confirming the proposed importance of the ERβ receptor in the antiproliferative activity of prostate cancer cells. However, on LNCaP.FGC prostate cancer cells 8PN demonstrated a more potent growth inhibition. Isoxanthohumol exhibited activities on prostate cancer cells PC-3 and DU145 comparable to those of 8-prenylnaringenin, but was more effective on LNCaP.FGC and MCF-7/6 breast cancer cells. Desmethylxanthohumol was the least active compound in all cell lines. The observed stimulatory effect on MCF-7/6 breast cancer cells and LNCaP.FGC prostate cancer cells is exerted in the low micromolar range. No quantitative relation between the estrogenic potencies as measured in the YES and antiproliferative effects could be concluded. The strong antiproliferative activity of xanthohumol in endothelial cells suggests potential antiangiogenic activity compromising tumor growth and metastasis. Further research on the non-genomic effects of the hop-derived prenylflavonoids is necessary to understand the mechanisms of action and to confirm whether these compounds may be efficient in the chemoprevention of breast and prostate cancers.

6. Mechanistic insights on antiproliferation and cytotoxicity of hop-derived prenylflavonoids

Antiproliferative activities of five hop-derived prenylflavonoids, xanthohumol (X), desmethylxanthohumol (DMX), isoxanthohumol (IX), 8-prenylnaringenin (8PN), and 6-prenylnaringenin (6PN), on human prostate cancer cells (PC-3, DU145 and LNCaP.FGC), were evidenced in part of our study.[67]

In this respect, we aimed to obtain insights into the underlying mechanisms by which the compounds in view of their potential applications influence the pathogenesis of prostate cancer (PC). These investigations were performed at the laboratory of Prof. Dr. P. Vandenabeele, Ghent University-UGent, Faculty of Sciences, Flanders Interuniversity Institute for Biotechnology, Department for Molecular Biomedical Research, Molecular Signalling and Cell Death Unit, Ghent, Belgium.

6.1. Purpose

The observed antiproliferative effects of the hop-derived prenylflavonoids on the prostate cancer cells may involve different mechanisms. The observed dose-dependent decrease in cell viability as determined with the WST1 proliferation assay could be ascribed to the interaction of the hop-derived prenylflavonoids with the cell cycle resulting in a cell division delay. On the other hand, the compounds could have induced cell death in an apoptotic or necrotic way. It is also possible that viability assays such as the WST1 proliferation assay, which is based on the measurement of mitochondrial enzyme activity, could have produced false-positive results, since the mitochondrial enzymes from cells undergoing apoptosis remain active until the last phase of the cell death process. Therefore, it was necessary to perform more detailed research into the effects of the hop-derived prenylflavonoids at the cell level.

To limit the extensive manipulations due to the various compounds and cell lines, we limited our research to the effects of the compounds on cell mechanisms using the human prostate cancer cell lines PC-3 and DU145. The LNCaP.FGC cell line was not further examined, because of practical problems with this highly sensitive and difficult-to-manipulate cell culture. The chalcone DMX was not further tested because of its instability.

6.2. Introduction

Prostate cancer is the most invasive and frequently diagnosed malignancy and the second leading cause of cancer related deaths in the United States.[68] Most prostate cancers are generally slow-growing malignancies and present themselves as mixtures of androgen-dependent and androgen-independent cells during the clinical diagnosis.[69,70] Age and hormones are known factors influencing the incidence of prostate cancer and hormone-dependent cancer cells initially respond to androgen withdrawal by undergoing apoptosis. However, patients with advanced or metastatic prostate cancer develop hormone refractory status that becomes fatal because of the growth of androgen-independent tumor cells and the emergence of tumor clones. Therefore, cancer chemotherapy aimed at causing cell death in metastatic prostate cancer is necessary and urgent for clinical treatment.

Apoptosis is a form of physiological cell death essential to normal tissue development and homeostasis.[71] After receiving an apoptotic death stimulus, cells first enter a signalling phase followed by the final degradation phase, in which apoptosis is identifiable by chromatin condensation, cell shrinkage, caspases activation, membrane lipid rearrangement, DNA fragmentation, and cell fragmentation through formation of 'apoptotic bodies'.[72] It is now well-evident that apoptosis occurs through two main pathways in cells responsive to apoptotic stimuli, namely extrinsic and intrinsic apoptosis signaling.[73,74] The extrinsic or cytoplasmic pathway is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily. The intrinsic or mitochondrial pathway leads, when stimulated, to the release of cytochrome-c from the mitochondria and activation of the death signal. Both pathways converge to a final common pathway involving activation of a cascade of caspases, starting with the activation of pro-caspase 3, leading to the morphologic manifestations of apoptosis and finally resulting in death of the cell.[75]

A wide variety of natural substances have been recognized to possess the ability to induce apoptosis in various cancerous cells.[76] Induction of apoptosis by hop bitter acids in HL-60 human leukemia cell lines has been demonstrated by Chen et al.[77] and Tobe et al.[78]

We investigated the kinetics of the time-dependent antiproliferative activities of four hop-derived prenylflavonoids and studied the treated cells morphologically for typical apoptotic features. Furthermore, the effect of the inhibition of caspases was determined and the activation of pro-caspase-3 and the caspase-3 activities were measured.

6.3. Materials

6.3.1. Hop-derived prenylflavonoids

The hop-derived prenylflavonoids X, IX, 8PN, and 6PN were isolated and purified from spent hops as described in chapter 4.

6.3.2. Chemicals

HEPES was purchased from Sigma-Aldrich (Bornem, Belgium). Solvents (HPLC grade) were from Biosolve (Valkenswaard, The Netherlands). Cell culture media, L-glutamine, non-essential amino acids (NEAA), penicillin (10,000 IU/ml), and streptomycin (10,000 µg/ml), foetal bovine serum (FBS), and sodium pyruvate were obtained from Invitrogen (Merelbeke, Belgium). The pan-caspase inhibitor was purchased from Enzyme Systems Products (Dublin, California, USA). The caspase-3-like substrate acetyl(Ac)-DEVD-AMC was obtained from the Peptide Iinstitute (Osaka, Japan). Rabbit polyclonal antibodies against caspase-3/CPP32 were purchased from BioSource (Nivelles, Belgium), the anti-rabbit secondary antibody horseradish peroxidase conjugate was derived by Amersham Biosciences (Roosendaal, Netherlands), and the 'chemiluminescence reagent plus' was purchased from Nycomed Amersham place (Buckinghamshire, United Kingdom).

6.3.3. Cells and culture media

Two established human epithelial cancer cell lines from prostatic (PC-3, DU145) origin were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were maintained in cell-specific media at 37°C in a humidified atmosphere of 5% carbon dioxide. The cells were passed at 70% confluency (i.e., two or three times a week). To reduce potential variations of cell characteristics, cells were used within 10 passages for all experiments.

Cells were cultured in a Dulbecco's Modified Eagle's Medium DMEM/F12 (Ham) 1:1 (Dubecco's Mem Nut Mix F–12 (HAM)) with L–glutamine (365,00 mg/L) and 15 mM HEPES. This medium was supplemented with 10% foetal bovine serum (FBS) and antibiotic agents (penicillin 10,000 IU/ml and streptomycin 10,000 µg/ml).

Experiments with DU145 cells were performed in DMEM without phenol red; experiments with PC-3 cells were performed in phenol red-free RPMI 1640. Both media were supplemented with 10% steroid-free, dextran-coated, charcoal-treated FBS, and 1% antibiotic agents (penicillin 10,000 IU/mL and streptomycin 10,000 µg/mL). In addition, 2 mM L-glutamine, 0.1 mM NEAA, and 1 mM sodium pyruvate were added to the DMEM medium and 1 mM sodium pyruvate and 10 mM HEPES were added to the RPMI 1640 medium.

6.3.4. Dextran-coated charcoal-stripped FBS

The preparation of dextran-coated charcoal-stripped FBS was performed as described in the methods used for the investigation of the antiproliferative, cytotoxic, antiangiogenic, and estrogenic properties of hop-derived prenylflavonoids (see: 5.3.5).

6.4. Methods

6.4.1. Time dependent cell survival assay

WST1 tetrazolium is a slightly reddish substrate, which is cleaved to its water-soluble formazan product under catalysis by succinate dehydrogenase complex II of the inner mitochondrial membrane, an enzyme involved in the oxidative phosphorylation. In death or dying cells, the damaged mitochondria are affected in their capacity to cleave significant amounts of WST1 tetrazolium to its formazan product. Therefore, this assay measures both cytostatic as well as cytotoxic effects. For the description of the WST1 tetrazolium assay we refer to 5.4.1.1. The WST1 tetrazolium assay was performed in a kinetic way to determine the incubation time necessary for the compounds to exert their effects on the human prostate cancer cell lines.

Cells were seeded into flat-bottomed 96-well tissue culture plates (Falcon, Beckton Dickinson, Grenoble, France) at a density of 6,000 cells/well for DU145 and 10,000 cells/well for PC-3 in 100 µl experimental growth medium. After 24 h, the cells (20-30% confluent) were exposed to culture media containing 100 µM or 200 µM of the hop-derived prenylflavonoids for 2, 4, 6, 8, 10, 12 and 14 h. All experiments included a solvent control. The experiments were repeated three times with three replicas per condition. At the end of each incubation period, cell viability was measured using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-*2H*-5-tetrazolio]-1,3-benzene (WST1) assay (Roche Diagnostics, Vilvoorde,

Belgium) according to the manufacturer's instructions. The average absorbance of each treated well was compared with the average absorbance of each control well to give a percentage of the control proliferation ([value/control]x100). As X and DMX absorb slightly at 450 nm, their experimental absorbance values at various concentrations were corrected for the respective blank absorbances from wells containing experimental medium and X or DMX at various concentrations, in the absence of the WST1 reagent.

6.4.2. Morphological analysis

Using a classic phase contrast and a digital modulator contrast (DIC) light microscope cells undergoing cell death after incubation with the hop-derived prenylflavonoids were examined for the specific morphological changes associated with the various cell death processes. Classic phase contrast technique gives information about cell granularity and cell content, while the DIC technique gives more information about the cell surface. The use of both techniques allows to detect all possible changes at cell level.

Cells were seeded into flat-bottomed 6-well tissue culture plates (Falcon) at a density of 200,000 cells/well. After 24 h, the cells were exposed to culture media containing 200 µM of the hop-derived prenylflavonoids. All experiments included a solvent control. After treatment for 0.5, 1, 1.5, 2, 12, and 24 h, respectively, cell changes were studied microscopically and photographs were taken using a phase-contrast microscope at 200x magnification.

6.4.3. Apoptosis inhibition with zVAD-fmk

zVAD-fmk (N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone) is a broad range (pan-)caspase inhibitor. Caspases are a family of cysteine aspartate-specific proteases. They are activated either via proximity-induced autoproteolysis or by cleavage via upstream proteases in an intracellular cascade resulting in several biochemical responses giving rise to apoptosis. If apoptosis is induced by the tested compounds, inhibition of the capsases by zVAD-fmk should result in changes in cell viability, which can be measured by the WST-1 tetrazolium assay.

Cells were seeded into flat-bottomed 96-well tissue culture plates (Falcon) at a density of 6,000 cells/well for DU145 and 10,000 cells/well for PC-3 in 100 µl experimental growth

medium. After 24 h, the cells (20-30% confluent) were exposed to culture media containing 25 µM zVAD-fmk for 2 h. After 2 h, cells were exposed for 18 h to culture media containing a serial dilution of the hop-derived prenylflavonoids prepared in ethanol from a 200 mM stock solution to a final ethanol concentration of 0.1 % in each well. All experiments included a solvent control and a zVAD-fmk control. Further manipulations were identical to those described for the time-dependent cell survival assays.

6.4.4. Western blot analysis of caspase 3-activity

Caspases are synthesized as zymogens consisting of a prodomain of variable length, followed by a p20 and p10 unit that contain the residues essential for substrate recognition and catalytic activity. The net result of these proteolytic activities is the separation of the prodomain from the p20 and p10 subunits. Proteolytic activation of procaspases leads to the appearance of typical p20 and p10 subunits, therefore, the proteolytic activation state of caspases can be examined by Western Blotting. If caspases are activated by the tested compounds, cleavage of these enzymes should be detected by the Western blot technique.

Cells were seeded into flat-bottomed 6-well tissue culture plates (Falcon) at a density of 200,000 cells/well in 1,200 µL experimental medium. After 24 h, cells were exposed to experimental medium containing 200 µM of the hop-derived prenylflavonoids for 15, 30, 60, 90, and 120 min, respectively, resulting in a final volume of 1,500 µL/well. All experiments included a solvent control. At regular time intervals (15, 30, 60, 90, 120 min), cells were collected in an eppendorf tube by scraping and centrifuged for 5 min at 1,100 rpm at 4°C. The culture medium was gently aspirated, cells were washed with 1 mL cold phosphate-buffered saline (8 g/L NaCl, 0.2 g/L KCl, 2.89 g/L $Na_2HPO_4.12H_2O$, 0.2 g/L KH_2PO_4) and centrifuged for 5 min at 1,100 rpm at 4°C. PBS was aspirated and cells were lysed in 100 µL caspase lysis buffer (1 % Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.3 mM aprotinin, 1 mM leupeptin), and left on ice for 10 min. Lysates were cleared by centrifuging at 14,000 rpm for 5 min, 1:5 volume of a 5 x Leammli buffer (312.5 mM Tris-HCl, pH 6.8, 10% SDS, 50% glycerol and 20% β-mercapto-ethanol) was added to the remaining cytosol and boiled for 5-10 min at 98°C. For Western blot analysis, equal amounts (15 µL/lane) were separated on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher & Schuell Bioscience, s'Hertogenbosch, The Netherlands). The membrane was incubated with a 1:1,500 dilution

from rabbit anti-caspase-3/CPP32 polyclonal primary antibody (BioSource, Nivelles, Belgium). Anti-rabbit secondary antibody horseradish peroxidase conjugate (1:4,000) (Amersham Biosciences, Roosendaal, the Netherlands) was used to detect and visualize with chemiluminescence Renaissance reagent (PerkinElmer Life Sciences, Vienna, Austria).

6.4.5. Analysis of caspase activity using fluorogenic substrates

The measurement of the actual activity of caspases can be examined with the use of fluorogenic substrates for caspases. These substrates contain the minimal amino acid composition corresponding with the cleavage-site of a typical substrate, coupled to 7-amino-4-methylcoumarin (AMC) or 7-amino-4-trifluoromethylcoumarin (AFC). Acetyl(Ac)-DEVD-AMC (7-amino-4-methylcoumarin, N-acetyl-L-aspartyl-Lglutamyl-L-valyl-l-aspartic acid amide) is a fluorogenic substrate for caspase-3 derived from the caspase-3 cleavage site in poly ADP ribosyl polymerase (PARP). The hydrolysis of the peptide substrate results in the release of free fluorescent AMC or AFC which can be measured with a fluorometer.

Cell lysates were prepared as described for the Western blot analysis of caspase-3 activation. Glutathione was added to the lysis buffer in a final concentration of 1 mM. After lysis, cells were left for 10 min on ice. Cell debris was removed by centrifuging the lysate for 5 min at 14,000 rpm and caspase activity was measured by incubating 10 µL of the cell lysate with 50 µM of the fluorogenic substrate Ac-DEVD-AMC in 150 µL CFS-buffer (10 mM HEPES-NaOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCL, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM sodium pyruvate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). The release of fluorescent AMC was monitored for 1 h at 37°C at 2 min time intervals with a fluorometer (CytoFluor, PerSeptive Biosystems, Cambridge, Massachusetts, USA) using a filter with an excitation wavelength of 360 nm and a filter with an emission wavelength of 460 nm.

6.5. Results

6.5.1. Time-dependent cell survival assay

The time-dependent effects of the hop-derived prenylflavonoids X, IX, 6PN, and 8PN on cell viability were investigated. Human prostate cancer cell lines PC-3 and DU145 were treated with 100 µM or 200 µM X, IX, 8PN or 6PN. After varying incubation times, cell

viability was measured with the WST-1 proliferation assay. As shown in **Figure 6.1**, all compounds reduced cell viability for at least 50% after 2 h of incubation when added to the cells in a concentration of 200 µM. Treatment with 100 µM significantly reduced cell viability albeit to a smaller extent, except for 8PN and 6PN, which did not significantly reduce cell viability in DU145 prostate cancer cells. Longer incubation times (until 14 h) did not result in enhanced reduction, while treatment for 14 h with 100 µM resulted in a gradual, but small decline of the cell viability (data not shown).

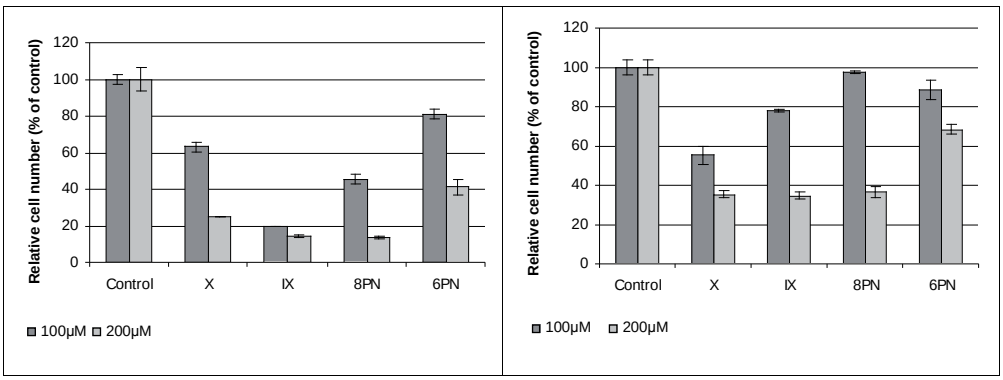

Figure 6.1. Effects of hop-derived prenylflavonoids on cell viability. PC-3 (A) and DU145 (B) cells were treated with either 100 µM or 200 µM X, IX, 8PN or 6PN for 2 h. Cell viability was measured with the WST-1 assay. Materials and Methods, see: 6.3 and 6.4.1, respectively. Data are represented as means ± SE for three determinations (X: xanthohumol, IX: isoxanthohumol, 8PN: 8-prenylnaringenin, 6PN: 6-prenylnaringenin).

6.5.2. Morphological analysis

Morphologic analysis was effected on PC-3 and DU145 prostate cancer cells treated with 200 µM X, IX, 8PN or 6PN. **Figure 6.2 and 6.3** illustrate the morphological changes observed in the cells after 12 h and 24 h of incubation. Shorter incubation times did not alter the morphology of the cells compared to the control (illustrations not shown). Complete cell death occurred after 24 h of incubation. Typical apoptotic changes could not be observed for any compound, neither in PC-3 cells, nor in DU145 cells during the complete incubation time of 24 h. However, formation of vacuoles was evident after 12 h of treatment with IX and 6PN in PC-3 cells, and with IX, 8PN, and 6PN in DU145 cells.

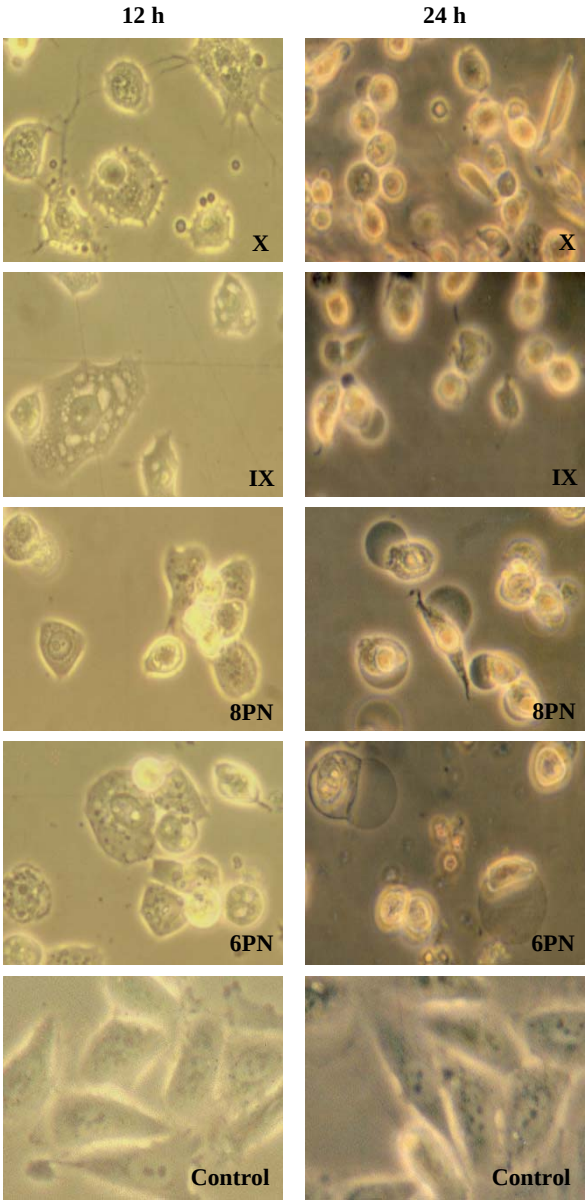

Figure 6.2 Effect of hop-derived prenylflavonoids on cell morphology in PC-3 cells. The cells were treated with 200 µM X, IX, 8PN or 6PN and pictures were taken after the indicated incubation times using a phase-contrast microscope at 200x magnification. Materials and Methods, see: 6.3 and 6.4.2, respectively. Data shown are from a representative experiment repeated three times with similar results (X: xanthohumol, IX: isoxanthohumol, 8PN: 8-prenylnaringenin, 6PN: 6-prenylnaringenin).

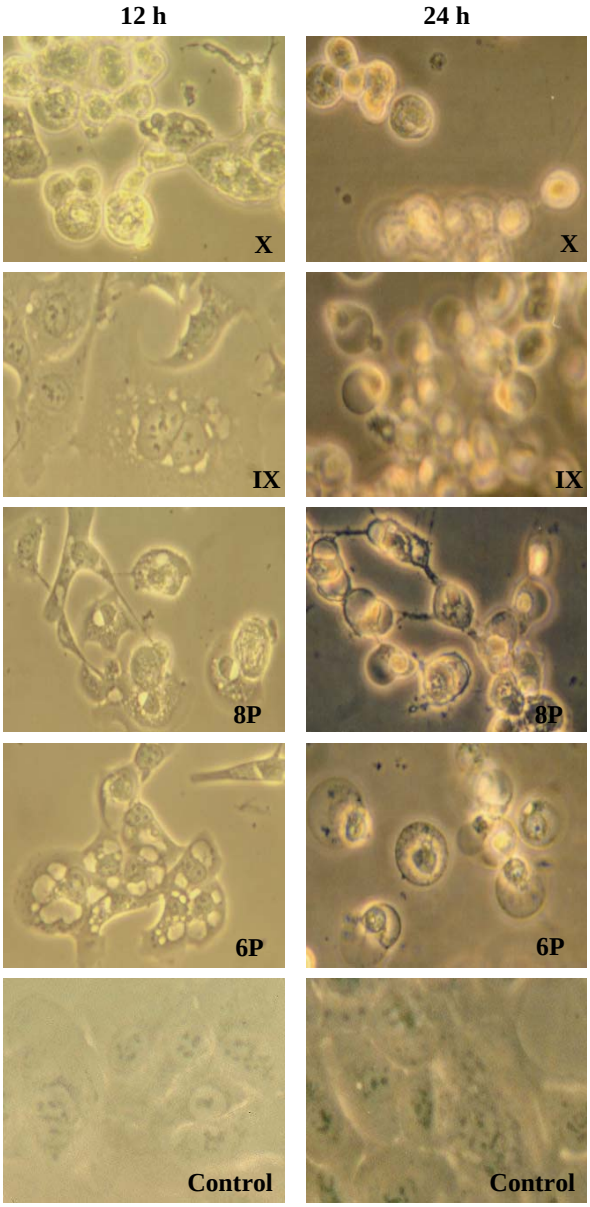

Figure 6.3 Effect of hop-derived prenylflavonoids on cell morphology in DU145 cells. The cells were treated with 200 µM X, IX, 8PN or 6PN and pictures were taken after the indicated incubation times using a phase-contrast microscope at 200x magnification. Materials and Methods, see: 6.3 and 6.4.2, respectively. Data shown are from a representative experiment repeated three times with similar results (X: xanthohumol, IX: isoxanthohumol, 8PN: 8-prenylnaringenin, 6PN: 6-prenylnaringenin).

6.5.3. Apoptosis inhibition with zVAD-fmk

We investigated the effect of the broad range pan-caspase inhibitor zVAD-fmk (*N*-benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone) on the time-dependent reduction of cell viability caused by X, IX, 8PN or 6PN. PC-3 and DU145 prostate cancer cell lines were treated with 25 µM zVAD-fmk for 2 h. After incubation, cells were exposed to varying concentrations of the hop-derived prenylflavonoids for 18 h and cell viability was measured with the WST-1 proliferation assay. As shown in **figure 6.4**, complete inhibition of caspases by zVAD-fmk did not change cell viability expressed as the amount of cell death for PC-3 or DU145 cells. However, with DU145 prostate cancer cells, we measured elevated values of cell viability as a function of the concentrations of 8PN and 6PN suggesting a partial protection from cell death. Administration of 25 µM zVAD-fmk for 2 h on PC-3 or DU145 prostate cancer cells was not toxic for the cells.

6.5.4. Analysis of caspase activity

To demonstrate whether treatment with the hop-derived prenylflavonoids could result in the cleavage of pro-caspase-3, we exposed PC-3 and DU145 cells to 200 µM X, IX, 8PN or 6PN for 15, 30, 60, 90, and 120 min, respectively, harvested cell lysates (see: 6.4.4), and determined the cleavage of pro-caspase-3 in a time-dependent manner by Western blot analysis using specific antibodies. Within the same experimental model, cell lysates were harvested (see: 6.4.5) and Ac-DEVD-AMC, a fluorogenic substrate, was used to determine the activity of caspase-3 by monitoring the release of fluorescent AMC. As shown in **figure 6.5**, no activation of pro-caspase-3 could be detected in treated PC-3 prostate cancer cells. The same observations were made for DU145 prostate cancer cells (data not shown). Consistent with these results, no activity of caspase-3 could be measured by fluorometric analysis (data not shown).

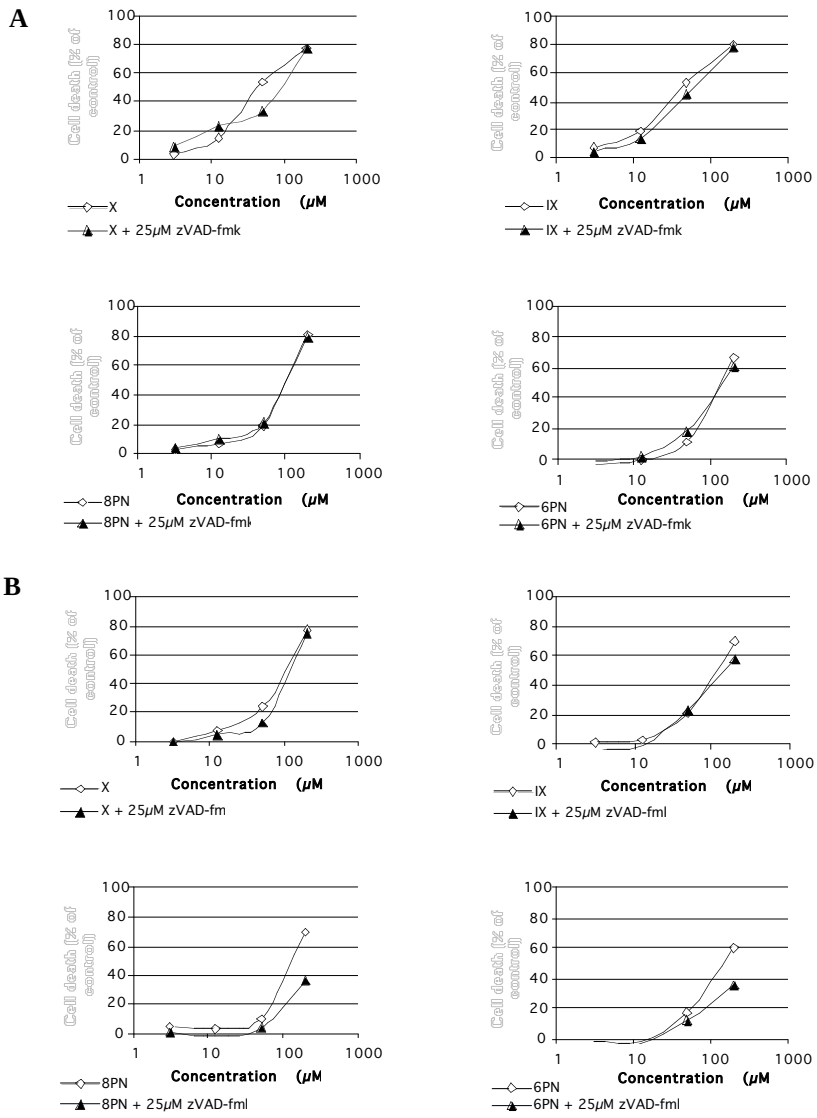

Figure 6.4: Concentration-dependent effects of zVAD-fmk on the cytotoxicity in PC-3 (A) and DU145 (B) cells. Cells were treated for 2 h with 25 μM zVAD-fmk. Then, cells were treated with varying concentrations of X, IX, 8PN or 6PN for 18 h. Cell viability was measured with the WST-1 assay. Materials and Methods, see: 6.3 and 6.4.3, respectively. Data are represented as means ± SE for three determinations (X: xanthohumol, IX: isoxanthohumol, 8PN: 8-prenylnaringenin, 6PN: 6-prenylnaringenin).

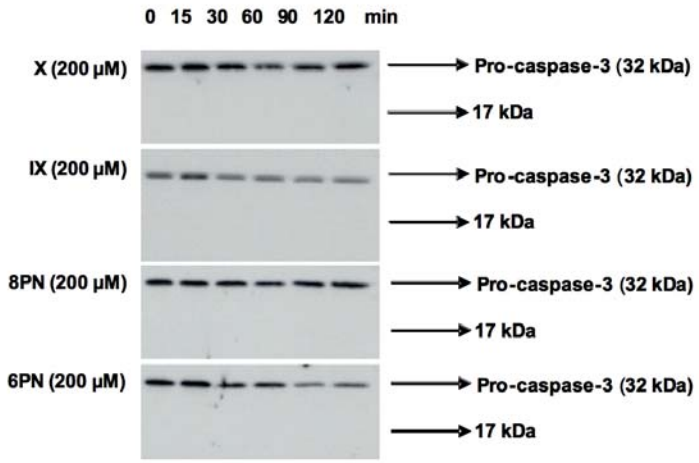

Figure 6.5: Time-dependent cleavage of pro-caspase-3 in PC-3 prostate cancer cells. Cells were treated at the indicated times with 200 µM X, IX, 8PN or 6PN and processing of pro-caspase-3 was detected by Western Blot analysis using specific antibodies. Materials and Methods, see: 6.3 and 6.4.4, respectively. (X: xanthohumol, IX: isoxanthohumol, 8PN: 8-prenylnaringenin, 6PN: 6-prenylnaringenin).

6.6. Discussion

Prostate cancer constitutes a major and escalating worldwide health problem. Androgen deprivation therapy continues to be the mainstay for metastatic disease. The aim of our research program is to examine the effects of hop-derived prenylflavonoids on the growth of prostate cancer cells and the mechanisms that are involved in these growth-modulating effects. Previous studies with X, IX, 8PN, and 6PN demonstrated dose-dependent antiproliferative activities on prostate cancer cells PC-3 and DU145.[67] In the present study, we investigated the mechanism involved by monitoring apoptotic phenomena. Apoptosis is a physiological process by which cells are removed when an agent damages their DNA.[79] Apoptosis represents a discrete manner of cell death that differs from necrotic cell death and is regarded as an efficient way to eliminate damaged cells.[80] Aberrant control of the balance of cell numbers may arise from malignant transformation and induction of apoptosis in transformed cell populations suppresses the development of cancer. Agents that can modulate apoptosis may be able to affect the steady-state cell population, which may be useful in the management and therapy of cancer.[81] Despite the acquired insights into the necrotic and autophagic cell death processes,[82,83] until now, no general biochemical features have been described for the detection of necrosis. Necrotic cell death is, therefore, usually concluded

from typical morphological features, loss of membrane integrity, and absence of any apoptotic feature.

We investigated the time-dependent cell viability with the WST1 proliferation assay and found that exposure of the PC-3 and DU145 prostate cancer cells to 200 µM of the hop-derived prenylflavonoids produced at least 50% cell death after 2 h. From these results, it could be concluded that, if apoptosis is the induced process of cell death, early apoptotic features should be detectable within 2 h of cell exposure to a concentration of 200 µM of the compounds.

Using a phase-contrast light microscope, cells undergoing cell death after incubation with the hop-derived prenylflavonoids were examined for specific morphological changes associated with the various cell death processes. None of the compounds showed specific apoptotic morphological features. Interestingly, cell death occurred only after 12 h of incubation, while, in the time-dependent cell viability assay, a reduction of cell viability of at least 50% was observed already after 2 h. This could suggest that the compounds change mitochondrial activity in an early phase leading to cell death only at a later stage. Another interesting observation was the formation of vacuoles in the DU145 prostate cancer cell line for IX and 6PN, and in the PC-3 prostate cancer cell line for IX, 6PN, and 8PN. Formation of these vacuoles could suggest the occurrence of autophagy. Autophagic cell death is a type of programmed cell death, which is characterized by the accumulation of autophagic vacuoles and the absence of caspase activation.[84,85,86] Cell death characterized by a combination of apoptotic and autophagic features is frequently observed and making a clear-cut difference between them is difficult.[87,88] Autophagy is a cell-survival mechanism during nutrient starvation[89,90] that supplies vital components until conditions improve. This process is characterized by the formation of double or multiple membrane–bound autophagosomes that engulf cytoplasmic material. After maturation, autophagosomes fuse with lysosomes to digest the sequestered material. Autophagy was recently established as a novel tumor suppression mechanism.[91] Autophagy might exert its tumor suppression function at the subcellular level by removing defective cytoplasmic components such as damaged mitochondria. In addition, it might function at the cellular level by helping the orderly removal of damaged cells. Previous studies indicated that autophagy is compromised in human breast, ovarian, and prostate cancers.[91]

Treatment with the caspase inhibitor zVAD-fmk did not rescue prostate cancer cells from the time-dependent reduction of cell viability caused by hop-derived prenylflavonoids suggesting that apoptosis is not the induced mechanism of cell death. However, in DU145 prostate cancer cells, we measured elevated values of cell viability as a function of the concentrations of 8PN and 6PN indicating partial protection from cell death. These results also suggest possible involvement of caspases in the cell-death process activated by 8PN and 6PN.

Activation of caspases, a family of cysteine proteases that specifically cleave at aspartic acid residues, is central to the execution of apoptosis.[92,93] We investigated the activation and activity of caspase-3, which is considered to play a central role in many types of stimuli-induced apoptosis.[94,95] No cleavage or activation of caspase-3 could be demonstrated for all compounds investigated.

6.7. Conclusions

There is no evidence for induction of apoptotic cell death in prostate cancer cells PC-3 and DU145 upon treatment with the hop-derived prenylflavonoids X, IX, 8PN, and 6PN. However, considering the formation of vacuoles in PC-3 prostate cancer cells when treated with IX and 6PN, and in DU145 prostate cancer cells when treated with IX, 6PN, and 8PN, it is suggested that autophagy is induced, which could consequently lead to cell death in this model. Therefore, IX, 8PN, and 6PN appear to be promising candidates for further investigation in prostate anticancer therapy.

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CONCLUSIONS

This research project has resulted in the first insights into the chemopreventive or therapeutic properties of the hop-derived prenylflavonoids xanthohumol (X), desmethylxanthohumol (DMX), isoxanthohumol (IX), 8-prenylnaringenin (8PN), and 6-prenylnaringenin (6PN) on prostate cancer.

The compounds were found to inhibit the proliferation of the human prostate cancer cells PC-3, DU145, and LNCaP.FGC in a dose-dependent manner. Antiproliferative activities of X and 6PN were found to be strongest for prostate cancer cells PC-3 and DU145, while, on LNCaP.FGC, these compounds first exerted a strong induction of cell proliferation followed by a proliferation shut-off. 8PN, a hop-derived prenylflavanone which has previously been identified as a highly potent phytoestrogen, showed weaker antiproliferative effects on prostate cancer cell lines PC-3 and DU145, confirming the proposed importance of the ERβ receptor in the antiproliferative activity of prostate cancer cells. However, on LNCaP.FGC prostate cancer cells 8PN demonstrated a more potent growth inhibition. IX exhibited activities on prostate cancer cells PC-3 and DU145 comparable to those of 8PN, but it was more effective on LNCaP.FGC cells. DMX was the least active compound in all cell lines. The observed stimulatory effect on LNCaP.FGC prostate cancer cells is exerted in the low micromolar range and could result from the stimulatory activity of X and 6PN through a mutated androgen receptor. The effect of the prenylflavonoids on early-stage prostate cancer (LNCaP.FGC) proved to be different compared to late-stage prostate cancer (PC-3 and DU145) and this should be considered when considering the possible protective effects regarding prostate cancer development and progression. No quantitative relation between the estrogenic potency as measured in the recombinant yeast estrogen screen and antiproliferative effects could be concluded. The strong antiproliferative activity of X in endothelial cells suggests potential antiangiogenic activity compromising tumor growth and metastasis.

Further research into the underlying mechanisms of growth inhibition revealed that there was no evidence for induction of apoptotic cell death in prostate cancer cells PC-3 and DU145 upon treatment with the hop-derived prenylflavonoids X, IX, 8PN, and 6PN. However, considering the formation of vacuoles in PC-3 prostate cancer cells when treated

with IX and 6PN, and in DU145 prostate cancer cells when treated with IX, 6PN, and 8PN, it is suggested that autophagy is induced, which could consequently lead to cell death in this model.

It can be concluded that the hop-derived prenylflavonoids X, IX, 8PN, and 6PN appear to be promising candidates for further investigation in prostate anticancer therapy and chemoprevention, especially in the final stages.

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SUMMARY

Benign prostate hypertrophy (BPH) and prostate cancer (PC) are highly common among aging men causing bothersome symptoms or developing into life-threatening diseases. Whereas the treament and relief of BPH has noticeably progressed, the treatment of invasive prostate cancer has remained elusive. Phytoestrogens could influence hormone-dependent diseases including BPH and PC by their varying bioactivities. Hop (*Humulus lupulus* L.) contains a number of prenylflavonoids, with 5 representatives most interesting for their bioactivities: xanthohumol (X), desmethylxanthohumol (DMX), isoxanthohumol (IX), 8-prenylnaringenin (8PN), and 6-prenylnaringenin (6PN).

This research project has focused on the investigation of the chemopreventive or therapeutic properties of the hop-derived prenylflavonoids X, DMX, IX, 8PN, and 6PN on PC. We aimed to get insights in how the hop-derived prenylflavonoids influence the pathogenesis of PC and in how the underlying mechanisms operate. Therefore, a series of *in vitro* experiments was set up using the well-established human prostate cancer cell lines LNCaP.FGC, PC-3, and DU145.

The investigation of the growth-influencing properties revealed that X proved to be the most potent compound in inhibiting the growth of all cell lines (except for the LNCaP.FGC cell line) with IC_{50} values of 12.3 ± 1.1 µM for DU145, and 13.2 ± 1.1 µm for PC-3. 6PN was, for all cell lines (except for the LNCaP.FGC cell line), the second most active growth inhibitor showing most significant effects on PC-3 cells (IC_{50} of 18.4 ± 1.2 µM), followed by DU145 (IC_{50} of 29.1 ± 1.1 µM). 8PN, a compound that has been previously identified as a potent phytoestrogen, showed weaker antiproliferative effects on PC-3 and DU145 but proved the be more potent on LNCaP.FGC prostate cancer cells (IC_{50} of 33.5 ± 1.0, 43.1 ± 1.2 µM and IC_{50} of 42.8 ± 1.2, respectively). IX showed activities on prostate cancer cells comparable to those of 8PN (IC_{50} of 45.2 ± 1.1 µM on PC-3 and 47.4 ± 1.1 µM on DU145) and proved to be the most potent inhibitor of LNCaP.FGC prostate cancer cells with an IC_{50} of 34.4 ± 1.0 µM. DMX was the least active compound in all the cell lines. On LNCaP.FGC prostate cancer cells, X and 6PN showed a biphasic dose-response behavior stimulating growth at low concentration and inhibiting growth at higher concentrations. IX and 8PN, which are the most

potent inhibitors for the growth of this cell line, inhibited growth at the same concentrations where X and 6PN stimulated it.

The recombinant yeast estrogen screen (YES) was used to investigate the binding capacity of the compounds to the estrogen receptor alpha (ERα) and their potency to subsequently stimulate a signal-transduction cascade resulting in secretion of β-galactosidase. The estrogenic activity of 8PN determined by YES was approximately 300-fold lower than that of 17β-estradiol, but 10-, 50- and 10,000-fold higher than the activities of DMX, 6PN, and X/IX, respectively. Furthermore, 8PN, 6PN, and DMX behaved as full agonists, while X and IX behaved as partial agonists on ERα. Growth-stimulatory experiments using human breast cancer cells MCF7/6 confirmed the estrogenic potency of 8PN (EC_{50} value for growth stimulation of 4.2 ± 1.3 nM). No quantitative relation between the estrogenic potencies and the proliferative or antiproliferative effects could be established.

Furthermore, the prenylflavonoids X, DMX, IX, 8PN, and 6PN were examined *in vitro* for their antiangiogenic activities on a human bone marrow endothelial cell line (HBME). Chalcone X proved to be a very potent inhibtor of the growth of HBME endothelial cells with an IC_{50} value of 3.2 ± 1.2 μM. IX and 6PN were the second most active growth inhibitors with IC_{50} values of 29.4 ± 1.1 μM and 39.8 ± 1.0 μM, respectively. 8PN and DMX were the least active growth inhibitors with IC_{50} values of 77.1 ± 1.0 μM and 72.7 ± 1.0 μM, respectively. These preliminary results strongly suggest that X could be a promising antiangiogenic agent.

Further research into the mechanisms by which cell death was induced revealed that all compounds induced cell death in the absence of caspase-3 activation and typical apoptotic morphological features. The general pan-caspase inhibitor zVAD-fmk could not protect this form of cell death. In addition, we observed the formation of vacuoles in PC-3 cells treated with IX and 6PN, and in DU145 cells treated with IX, 8PN, and 6PN, which could suggest the induction of autophagy and consequent cell death.

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SAMENVATTING

Goedaardige prostaathypertrofie ('benign prostate hypertrophy", BPH) en prostaatkanker ('prostate cancer', PC) zijn veel voorkomende aandoeningen van de prostaat bij oudere mannen, gepaard gaande met onaangename symptomen en mogelijke ontwikkeling tot levensbedreigende situaties. Waar behandeling en onderdrukking van de symptomen van BPH reeds in een gevorderd stadium van ontwikkeling zijn, verkeert behandeling van invasieve PC nog in een beginfase. Fyto-oestrogenen kunnen, omwille van hun breed spectrum aan bio-activiteiten, hormoon-afhankelijke aandoeningen, zoals BPH en PC beïnvloeden. Hop (*Humulus lupulus* L.) is rijk aan prenylflavonoïden, waarvan 5 componenten zeer interessante bio-activiteiten vertonen: xanthohumol (X), desmethylxanthohumol (DMX), isoxanthohumol (IX), 8-prenylnaringenine (8PN) en 6-prenylnaringenine (6PN).

Dit onderzoeksproject omvat de studie van de chemopreventieve en/of therapeutische eigenschappen van de prenylflavonoïden X, DMX, IX, 8PN en 6PN uit hop bij PC. Onze doelstelling was inzicht te verkrijgen in de wijze, waarop de prenylflavonoïden uit hop de pathogenese van PC kunnen beïnvloeden, en in de onderliggende mechanismen, die hierbij een rol spelen. Daartoe werden een reeks *in vitro* experimenten uitgevoerd, gebruik makende van de gevestigde prostaatkankercellijnen LNCaP.FGC, PC-3 en DU145.

Het onderzoek naar de potentiële groei-beïnvloedende eigenschappen toonde aan dat X de krachtigste groeiremmer was voor alle cellijnen (behalve voor de LNCaP.FGC cellijn) met IC_{50} waarden van 12.3 ± 1.1 µM voor DU145 en 13.2 ± 1.1 µm voor PC-3. 6PN was, voor alle cellijnen (behalve voor de LNCaP.FGC cellijn), de tweede krachtigste groeiremmer en vertoonde de sterkste werking bij PC-3 cellen (IC_{50} van 18.4 ± 1.2 µM), gevolgd door DU145 (IC_{50} van 29.1 ± 1.1 µM). 8PN, een zeer sterk fyto-oestrogeen, vertoonde een zwakker antiproliferatief effect bij PC-3 and DU145, maar bleek een sterker groeiremmend effect te vertonen bij LNCaP.FGC prostaatkankercellen (IC_{50} van 33.5 ± 1.0, 43.1 ± 1.2 µM en IC_{50} van 42.8 ± 1.2, respectievelijk). IX vertoonde vergelijkbare activiteiten bij prostaatkankercellen met deze van 8PN (IC_{50} van 45.2 ± 1.1 µM bij PC-3 en 47.4 ± 1.1 µM bij DU145) en was de krachtigste groeiremmer van LNCaP.FGC prostaatkankercellen met

een IC_{50} van 34.4 ± 1.0 µM. DMX was de minst actieve verbinding bij alle onderzochte cellijnen. Bij LNCaP.FGC vertoonden X en 6PN een bifasisch dosis-responsgedrag met groeistimulatie bij lage concentraties en groeiremming bij hogere concentraties. IX en 8PN, de sterkste groeiremmers voor deze cellijn, inhibeerden de groei bij dezelfde concentraties, waarbij X en 6PN de groei stimuleerden.

De recombinante oestrogene gistassay ('yeast estrogen screen', YES) werd gebruikt om de bindingscapaciteit van de verbindingen aan de oestrogeen receptor alfa (ERα) te onderzoeken, samen met hun stimulatie van de erop volgende signaaltransductiecascade, resulterend in de afscheiding van β-galactosidase. De oestrogene activiteit van 8PN volgens YES bleek ongeveer 300-maal geringer te zijn dan deze van 17β-oestradiol, doch 10-, 50- en 10,000-maal krachtiger dan de activiteiten van, respectievelijk, DMX, 6PN en X/IX. Bovendien gedroegen 8PN, 6PN en DMX zich als volle agonisten, waar X en IX fungeerden als partiële agonisten ten opzichte van ERα. Groeistimulerende experimenten bij MCF7/6 borstkankercellen bevestigden de sterke oestrogene activiteit van 8PN (EC_{50} waarde voor groeistimulatie van 4.2 ± 1.3 nM). Er werd geen kwantitatief verband gevonden tussen de oestrogene werking en de proliferatieve of antiproliferatieve effecten.

De *in vitro* anti-angiogene eigenschappen van de prenylflavonoïden X, DMX, IX, 8PN en 6PN werden onderzocht aan de hand van een humane beenmerg endotheliale cellijn (HBME). Chalcon X bleek een zeer krachtige groeiremmer te zijn voor de HBME endotheliale cellen met een IC_{50} waarde van 3.2 ± 1.2 µM. IX en 6PN waren actieve groei-inhibitoren met IC_{50} waarden van, respectievelijk, 29.4 ± 1.1 µM en 39.8 ± 1.0 µM. 8PN en DMX waren de minst actieve groeiremmers met IC_{50} waarden van, respectievelijk, 77.1 ± 1.0 µM en 72.7 ± 1.0 µM. Deze resultaten geven reeds een sterk vermoeden dat X een veelbelovende anti-angiogene verbinding zou kunnen zijn.

Verder onderzoek naar de mechanismen van celdoodinductie toonden aan dat de prenylflavonoïden celdood induceerden in afwezigheid van caspase-3 activatie en typische apoptotische morfologische kenmerken. De algemene pan-caspase inhibitor zVAD-fmk kon de cellen niet beschermen tegen deze vorm van celdood. Bovendien hebben we de vorming van vacuolen bij de PC-3 cellen waargenomen na behandeling met IX en 6PN, en bij de DU145 cellen na behandeling met IX, 8PN en 6PN, wat inductie van autofagie gevolgd door celdood suggereert.