From ancient herbs to modern drugs

In search of alternatives for cancer therapy

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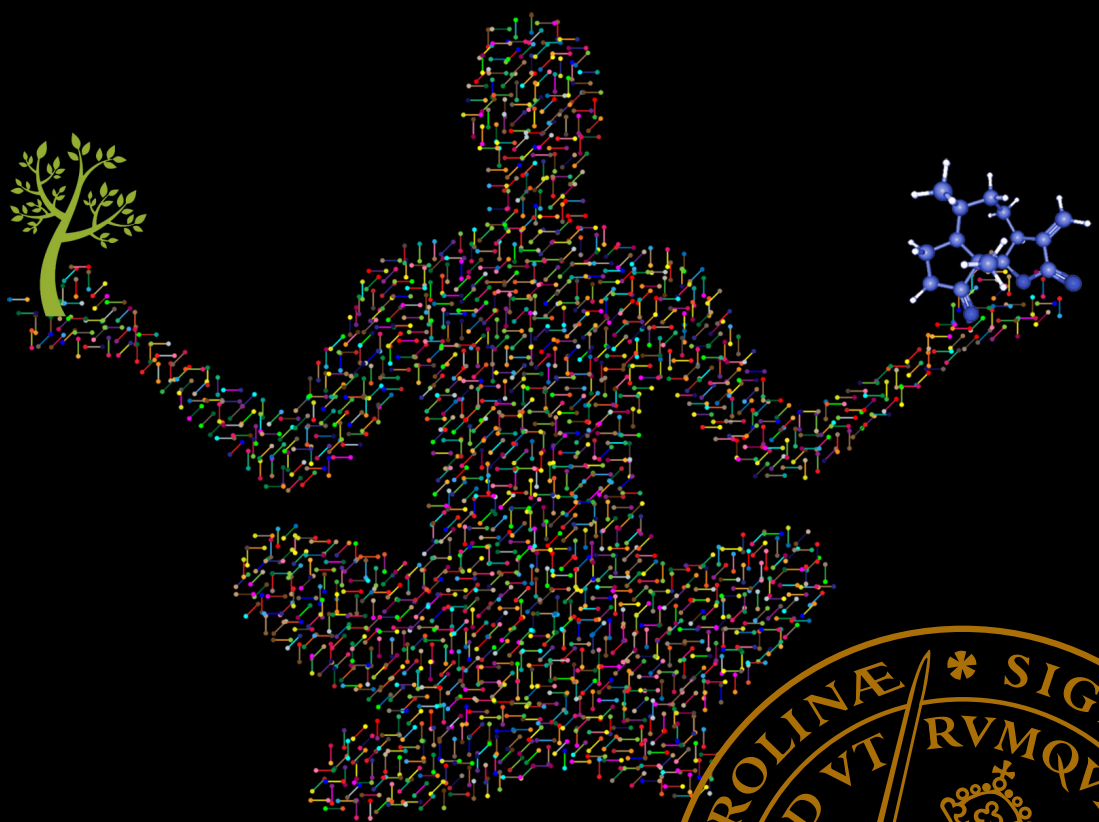

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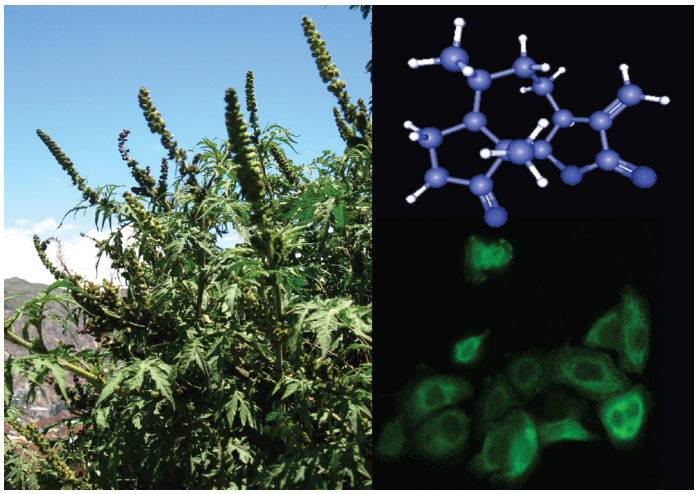

"The plant that you have in your home - have you ever truly looked at it? Have you allowed that familiar yet mysterious being we call plant to teach you its secrets? Have you noticed how deeply peaceful it is? How it is surrounded by a field of stillness? The moment you become aware of a plant's emanation of stillness and peace, that plant becomes your teacher."

Eckhart Tolle

LUND UNIVERSITY

Faculty of Science
Department of Biology

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From ancient herbs to modern drugs

From ancient herbs to modern drugs

In search of alternatives for cancer therapy

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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From ancient herbs to modern drugs, In the search of alternatives for cancer therapy

Abstract

Breast cancer is the major cause of deaths related to cancer for women worldwide. Although much is known about this malignancy, extensive research is still needed to gain complete understanding of the complexity of the disease. The inter and intra tumor heterogeneity is an important trait of breast cancer. The intratumor heterogeneity is reflected in sub-populations of cancer cells that appear to have different levels of aggressiveness. The most aggressive population termed the cancer stem cell (CSC) population is believed to play a critical role in cancer recurrence and resistance to conventional chemotherapy and CSCs seem to be the responsible for relapse and cancer death. Sesquiterpene lactones (SLs) and methoxyflavones, plant-derived molecules usually found in Asteraceae family plant extracts, have been reported to have anti-inflammatory and anti-cancer activities. This thesis describes the biological activity of natural and synthetized SLs and natural flavones on CSCs and non-CSCs in breast cancer cell lines.

Damsin is a natural SL and it was used for the chemical synthesis of damsin derivatives. Here the toxicity of damsin and the damsin derivatives have been investigated in three breast cancer cell lines and one normal-like cell line. In all, 46 compounds were evaluated in dose-response testing to obtain IC_{50} values that were used to deduce structure activity relationships. Selected SLs were studied further to gain insight into cellular and molecular mechanisms. The studied SLs inhibited cell proliferation and cell migration and remarkably also reduced the CSC population of a breast cancer cell line. The damsin derivatives were more toxic to cancer cells than to normal cells. On the molecular level, the results point to interference of the function of the transcription factor NF-κB, being the molecular initiating event. SLs are known to bind to a cysteine in the DNA binding site of NF-κB. Our data implicate that it is not only DNA binding of NF-κB that is prevented by SL treatment but also the binding of other proteins that have a role in the function of NF-κB.

The toxicity of three natural methoxyflavones was evaluated revealing that small differences in chemical structure can have a large impact on toxicity. Only one of the methoxyflavones showed anti-proliferative activity in breast cancer cell lines which may be caused by the induction of DNA strand breaks. In contrast to the SLs, treatment with the methoxyflavones did not reduce the CSC population.

Key words: Natural products, sequiterpene lactones, flavonoids, breast cancer, breast cancer stem cells

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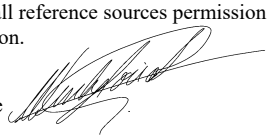
Signature  Date 2019-08-19

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UNIVERSITY

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Back cover: image of *Ambrosia arborescens* by Rodrigo Villagomez, molecular structure of damsin, and immunofluorescence image of NF-κB expression in JIMT-1 breast cancer cells by Wendy Soria Sotillo.

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Science, for me, gives a partial explanation for life. In so far as it goes, it is based on fact, experience and experiment.

Rosalin Franklin

To Daysi and all the wise women who have touched my life

Table of contents

Abstract

Breast cancer is the major cause of deaths related to cancer for women worldwide. Although much is known about this malignancy, extensive research is still needed to gain complete understanding of the complexity of the disease. The inter and intra tumor heterogeneity is an important trait of breast cancer. The intratumor heterogeneity is reflected in sub-populations of cancer cells that appear to have different levels of aggressiveness. The most aggressive population termed the cancer stem cell (CSC) population is believed to play a critical role in cancer recurrence and resistance to conventional chemotherapy and CSCs seem to be the responsible for relapse and cancer death. Sesquiterpene lactones (SLs) and methoxyflavones, plant-derived molecules usually found in Asteraceae family plant extracts, have been reported to have anti-inflammatory and anti-cancer activities. This thesis describes the biological activity of natural and synthetized SLs and natural flavones on CSCs and non-CSCs in breast cancer cell lines.

Damsin is a natural SL and it was used for the chemical synthesis of damsin derivatives. Here the toxicity of damsin and the damsin derivatives have been investigated in three breast cancer cell lines and one normal-like cell line. In all, 46 compounds were evaluated in dose-response testing to obtain IC_{50} values that were used to deduce structure activity relationships. Selected SLs were studied further to gain insight into cellular and molecular mechanisms. The studied SLs inhibited cell proliferation and cell migration and remarkably also reduced the CSC population of a breast cancer cell line. The damsin derivatives were more toxic to cancer cells than to normal cells. On the molecular level, the results point to interference of the function of the transcription factor NF-κB, being the molecular initiating event. SLs are known to bind to a cysteine in the DNA binding site of NF-κB. Our data implicate that it is not only DNA binding of NF-κB that is prevented by SL treatment but also the binding of other proteins that have a role in the function of NF-κB.

The toxicity of three natural methoxyflavones was evaluated revealing that small differences in chemical structure can have a large impact on toxicity. Only one of the methoxyflavones showed anti-proliferative activity in breast cancer cell lines which may be caused by the induction of DNA strand breaks. In contrast to the SLs, treatment with the methoxyflavones did not reduce the CSC population.

Popular science summary

Breast cancer is the most common kind of cancer and the leading cause of cancer-associated mortality in women. Substantial support for breast cancer awareness and research funding has significantly improved the diagnosis and treatment of breast cancer, causing breast cancer survival rates to increase. The challenge now is to further increase patient survival by finding more efficient patient-adapted treatments.

Breast cancer is characterized by diversity on different levels. No cancer tumors are similar, even in a single patient, and even within one tumor there are different kinds of cancer cells with varying degrees of aggressiveness. Among these, the cancer stem cells (CSCs) are known to be related to highly aggressive cancers as they are treatment resistant and have the ability to form metastases.

Different experimental systems are used to study breast cancer. One such system is cell culturing, where human breast cancer cells are grown outside of the body in controlled conditions that copy physiological conditions. When breast cancer cells grow outside the body they are called breast cancer cell lines. Here, I have used different breast cancer cell lines but also a cell line derived from normal breast as a control. These cell lines have been used for investigating unique compounds that in the future may be used in the clinic.

These compounds are used in traditional medicine practiced by a group of healers called “Kallawaya” in Bolivia. The knowledge of medicinal plants has been passed through generations and is an important part of Bolivian heritage. Bolivian researchers have documented the traditional use of the plants in an effort to understand the science behind this ancient knowledge.

Ambrosia arborescens and *Baccharis pentlandii*, two plants used as anti-inflammatories and anti-carcinogenics by the traditional healers, attracted the attention of the chemists at Universidad Mayor de San Andres in La Paz, Bolivia. In a team effort with the chemistry department at Lund University, the active molecules of the plants were extracted. The molecules are called

sesquiterpene lactones (SLs) and methoxyflavones, respectively. The chemists also synthesized a large number of analogues to damsin, one of the SLs. My work has been to investigate the toxicity of the natural product damsin, the synthesized damsin analogues, and the methoxyflavones on breast cancer cell lines.

Some of the synthesized analogues were more efficient than damsin *i.e.* they were toxic at lower concentrations. Interestingly, the normal-like breast cell line was less sensitive than the cancer cell lines. This is important from a clinical view point, since the ideal cancer treatment should spare normal cells while cancer cells should die. Even more interesting is the fact that the SLs were efficient against the most aggressive cells in the breast cancer cell lines *i.e.* the CSCs. One of the methoxyflavones, sideritoflavone, affected all cancer cells *i.e.* both the CSCs and the non-CSCs.

The main cause for breast cancer death is that cancer cells spread from the primary location to other parts in the body *i.e.* they metastasize. We discovered that, in general, treatment with the compounds reduced cell movement and thus the ability to metastasize.

When different effects are observed after treating cells with a compound, *e.g.* reduced cell growth and decreased cell movement, the question arises as to what is actually happening in the cell. The cell comprises millions of molecules defined into different chemical groups which are used in different pathways to govern the behavior of the cell. In this work, I have started to investigate which molecular pathways are affected by the compounds and what makes cells more or less sensitive.

Maybe an improved cancer cure can be achieved by a combination of SLs and methoxyflavones. For traditional healers, it may imply that combining extracts of *Ambrosia arborescens* and *Baccharis pentlandii* will be better for the patients. Although much work is still needed, this work points to a possibility of treating even very aggressive breast cancer forms with SLs. The future will tell us.

Populärvetenskap

Bröstcancer är den vanligaste cancerformen och den cancerform som orsakar högst dödlighet bland kvinnor över hela världen. Ökade resurser till forskning och till tidig upptäckt av bröstcancer har gjort att både diagnos och behandlingsresultat förbättrats oerhört. Nu är utmaningen att ytterligare minska dödligheten genom att hitta nya effektiva patientanpassade behandlingar.

Bröstcancer är inte en homogen sjukdom. Tvärtom så är alla tumörer olika, t.o.m. när de sitter i samma patient. Dessutom innehåller varje tumör flera olika typer av celler. Bland alla dessa celler är cancerstamcellerna (CSCs) mest kända för att bidraga till tumörers aggressivitet då de kan bli okänsliga för cancerbehandling och har förmåga att bilda metastaser.

För att kunna studera bröstcancer tar man ut celler ur patienterna och odlar dem i en kontrollerad omgivning som ska efterlikna omgivningen i kroppen. När cancercellerna odlas utanför kroppen kallas de cancercellinjer. I det här arbetet har jag använt ett batteri av bröstcancercellinjer men också en cellinje som kommer från ett friskt bröst. Jag har använt cellinjerna för att undersöka ett antal unika substanser som kanske kan användas kliniskt i framtiden.

Dessa unika substanser används traditionellt av "helare" i Bolivia som heter Kallawaya. Deras kunskap om traditionell örtmedicin har gått i generationer och är en viktig del av den bolivianska kulturen. Deras kunskaper har dokumenterats av bolivianska forskare som försöker förstå den medicinska kemin som ligger bakom traditionen.

Ambrosia arborescens och *Baccharis pentlandii* är två växter som traditionellt används för att behandla inflammationer och cancer. Kemister vid Universidad Mayor de San Andres i La Paz, Bolivia, blev intresserade av dessa traditionella behandlingarna. Tillsammans med kemister från Lunds Universitet lyckades de extrahera de aktiva ämnena från växterna. Ämnena heter "sesquiterpene lactones" (SLs) och "methoxyflavones" på engelska. Ett av SL-ämnena heter damsin, och kemisterna har framställt många olika varianter av damsin. Jag

har undersökt hur damsin, de olika varianterna av damsin och methoxyflavonerna påverkar bröstcancercellinjer.

Några av damsinvarianterna var mer effektiva än damsin, d.v.s. det behövdes lägre koncentrationer för att påverka bröstcancercellinjerna. I sammanhanget är det väldigt intressant att den normala bröstcellinjen påverkades mindre av behandlingen med de olika ämnena än bröstcancercellinjerna. Detta är viktigt för behandlingen av cancer eftesom den idealiska cancerbehandlingen bara påverkar cancercellerna, medan friska celler är opåverkade. Det är också mycket intressant att SL-molekylerna påverkade även CSC som är de mest aggressiva cellerna i bröstcancercellinjerna, och som normalt är väldigt svåra att slå ut. En av methoxyflavonerna som heter sideritoflavone hämmade alla cancercellerna, från de minst aggressiva till de mest aggressiva.

Den största orsaken till att cancer är dödlig är att den sprider sig i kroppen, d.v.s. att den metastaserar. Jag upptäckte att de olika ämnena som undersöktes påverkade cellernas rörlighet och därmed deras förmåga att metastasera.

Celler består av miljontals olika molekyler som arbetar tillsammans i grupper. Dessa grupper styr cellernas beteende. Jag har börjat undersöka hur damsin, varianterna av damsin och methoxyflavonerna påverkar dessa olika grupper och vad det är som gör att cellerna är olika känsliga för behandling.

En kombination av SL och methoxyflavoner kan vara den framtida behandlingen av cancer. Dessutom borde kanske de traditionella helarna i Bolivia kombinera extrakt från *Ambrosia arborescens* och *Baccharis pentlandii* för att uppnå bättre resultat. Mycket arbete kvarstår innan det finns en färdig behandling, men jag drar slutsatsen att SL kommer att kunna användas för behandling av cancer, även för mycket aggressiv cancer. Framtiden får utvisa hur en sådan behandling ska se ut.

Resumen de divulgación científica

Entre todos los tipos de cancer, el cancer de mama es el más común y la principal causa de muerte entre mujeres a nivel mundial. El apoyo constante para la prevención del cancer de mama y su investigación han incrementado el diagnóstico temprano y su tratamiento, mejorando los porcentajes de supervivencia. El reto actual consiste en mejorar las posibilidades de supervivencia de los pacientes por medio de tratamientos personalizados.

El cancer de mama está caracterizado por su alta diversidad en diferentes niveles. Ningún tumor es similar a otro, no en un mismo paciente, y ni siquiera en un mismo tumor, existen diferentes tipos de células con diferentes grados de agresividad y malignidad. Entre estas células, las células madre cancerígenas, conocidas por sus siglas en ingles como CSCs, están relacionadas con canceres de alto nivel de agresividad, ya que son las responsables de la resistencia a los medicamentos y de generar metástasis.

Diferentes sistemas experimentales son utilizados para estudiar el cancer de mama. Uno de estos sistemas es el cultivo celular, utilizando este método, células humanas son cultivadas fuera del cuerpo simulando características fisiológicas. Cuando las células de cancer de mama crecen fuera del cuerpo, estas reciben el nombre de líneas celulares. En este trabajo, tres líneas de cancer de mama fueron utilizadas, así también una línea celular proveniente de un tejido sano. Con estas líneas celulares se desarrollaron un conjunto de experimentos que permitieron investigar nuevos compuestos que en el futuro podrían ser utilizados como tratamientos médicos.

Los compuestos estudiados fueron extraidos de algunas plantas utilizadas en la medicina tradicional boliviana por los “Kallawayas” un grupo ancestral de curanderos, cuyo conocimiento en el uso de plantas medicinales ha sido transmitido por generaciones y es parte importante de la herencia cultural boliviana. Investigadores bolivianos han logrado documentar el uso tradicional de esta colección de plantas medicinales, buscando entender de forma científica el éxito que muchas veces logra la aplicación de este conocimiento ancestral.

Ambrosia arborescens y *Baccharis pentlandii,* son plantas conocidas por sus características anti-inflamatorias y anti-carcinogénicas por los Kallawayas, estas plantas, llamaron la atención de los investigadores químicos de la Universidad Mayor de San Andrés en La Paz, Bolivia, quienes en un esfuerzo conjunto con el departamento de química de la Universidad de Lund, extrajeron varias moléculas activas de estas plantas y crearon algunas nuevas basándose en las naturales. Estas moléculas fueron identificadas como sesquiterpen lactonas y metoxiflavonas. El equipo químico sintetizo un gran número de compuestos basados en la molécula natural, damsin, una sesquiterpen lactona, con este conjunto de compuestos sintéticos, se llevaron a cabo diversos experimentos a fin de investigar la efectividad contra diferentes líneas celulares cancerígenas.

Entre los hallazgos más destacados podemos indicar que algunos de los derivados sintéticos del damsin fueron más eficientes que la molécula natural, es decir fueron efectivos a concentraciones más bajas. Un hallazgo interesante fue que la línea celular normal fue menos sensible a los compuestos comparada con las líneas celulares cancerígenas. Este hallazgo es importante desde un punto de vista médico, ya que un tratamiento ideal para el cancer no debería afectar a las células normales pero sí debería eliminar a las células cancerígenas. Un hecho aún más interesante es que las sesquiterpen lactonas redujeron la población de células madre cancerigenas, las más agresivas entre los tipos de células de cancer de mama. Una de la metoxiflavonas, la sideritoflavona, afecto a las células cancerígenas en general es decir todas las células que podrían conformar un tumor.

Es sabido que el elevado índice de muertes por cáncer, se debe a que las células cancerígenas se diseminan de su posición original a otras partes del cuerpo, a este proceso se denomina metástasis. Durante este trabajo descubrimos que cuando las células son tratadas con las moléculas estudiadas el movimiento de las células se ve reducido y esto disminuiría la probabilidad de que el paciente entre en esta mortal etapa.

Se observaron diferentes efectos al tratar las células cancerígenas con los compuestos mencionados, hubo reducción del número celular y el movimiento de las células disminuyo también. No olvidemos que una célula está conformada por millones de moléculas asociadas en diferentes grupos químicos que en coordinación controlan el funcionamiento celular. El hallazgo de un probable evento metabólico inicial, podría permitir la identificación de las posibles rutas de acción de estos compuestos y los efectos que hacen que las células cancerígenas sean más o menos sensibles a su uso.

Los resultados obtenidos son muy prometedores, pues probablemente un tratamiento efectivo puede ser establecido al combinar las sesquiterpen lactonas con las metoxiflavonas. Con este hallazgo, los antiguos remedios Kallawayas cobran nueva vigencia, pues indicarían que una combinación de los extractos de *Ambrosia arborescens* y *Baccharis pentlandii* podría tener un mejor efecto en los pacientes. Sin embargo, más estudios son necesarios para establecer una dosis y un modo de acción.

Por todo ello podemos afirmar que esté trabajo abre una nueva posibilidad al tratamiento mediante sesquiterpen lactonas y metoxiflavonas, contra las diversas formas de cancer de mama de alta agresividad y resistencia, generando una nueva línea de investigación que debera ser definida a futuro.

List of publications

This work is based on the work presented in the following papers referred to in the text by their Roman numerals:

I. Wendy Soria Sotillo, Rodrigo Villagomez , Sandra Smiljanic, Xiaoli Huang, Atena Malakpour, Sebastian Kempengren, Gloria Rodrigo, Giovanna Almanza, Olov Sterner, and Stina Oredsson. Anti-cancer stem cell activity of a sesquiterpene lactone isolated from *Ambrosia arborescens* and of a synthetic derivative. PLoS One. 2017; 1–16.

II. Maribel Lozano M, Wendy Soria Sotillo, Giovanna Almanza, Sophie Manner, Stina Oredsson, Rodrigo Villagomez, and Olov Sterner. Selective cytotoxicity of damsin derivatives in breast cancer cells. J Adv Pharm Sci Technol. 2019;2: 22.

III. Wendy Soria Sotillo, Maribel Lozano, Xiaoli Huang, Atena Malakpour, Giovanna Almanza, Olov Sterner, and Stina Oredsson. A molecular mechanisms of breast cancer cell active chemically synthesized damsin derivatives. *Manuscript*

IV. Maribel Lozano*, Wendy Soria-Sotillo*, Giovanna Almanza, Sophie Manner, Stina Oredsson, Rodrigo Villagomez, and Olov Sterner. Semisynthesis of designed derivatives and potent cytotoxic activity of novel derivatives from damsin. *Submited*

V. Wendy Soria Sotillo, Santiago Tarqui, Xiaoli Huang, Giovanna Almanza, and Stina Oredsson. Breast cancer toxicity of a flavonoid isolated from *Bacharis pentlandii*. *Manuscript*

My contributions to the papers

I. Designed, planned, and performed experiments, wrote the first draft of the paper

II. Performed dose response curve experiments and obtained IC_{50} values, wrote the biological part of the paper

III. Designed, planned, and performed experiments, wrote the first draft of the manuscript

IV. Performed dose response curve experiments, obtained IC_{50} values, and determined glutathione levels, wrote the biological part of the paper

V. Designed, planned, and performed experiments, wrote the first draft of the manuscript

Abbreviations

ALDH	aldehyde dehydrogenase
BRCA1	breast cancer gene 1
CDKs	cyclin dependent protein kinases
CFE	colony forming efficiency
CSCs	cancer stem cells
ER	estrogen receptor
γ-H2AX	gamma-H2A histone family member X
GSH	glutathione
GSTM 1	glutathione S-transferase M1 gene
HER2	human epidermal growth factor receptor 2
hTERT	human telomerase reverse transcriptase
IC_{50}	inhibitory concentration 50
IκBα	inhibitor of NF-κB alpha
Max	myc-associated factor X
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
p21	cyclin-dependent kinase inhibitor
p53	transcription factor encoded by the TP53 gene
PI	propidium iodide
PR	progesterone receptor
SLs	sesquiterpene lactones
STAT3	signal transducer and activator of transcription 3
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-alpha
Wnt	wingless-related integration site

1 The scope of the thesis in short

One in ten of all new cancers diagnosed worldwide each year is a cancer of the female breast, and it is the most common cancer in women in both developing and developed areas. Breast cancer is a heterogenous disease, both between women and within individual. It is composed of different kinds of cancer cells that have different potential to promote cancer spread and survival. Cancer cells can be divided into cancer stem cells (CSCs) and bulk cancer cells.

CSCs have become an important research target in recent years, due to strong evidence suggesting that they may be responsible for cancer relapse and cancer spread. Chemotherapy and radiotherapy have been shown to mainly target the bulk cancer cells, leaving the CSCs remaining with the ability to form new tumors.

Nature has been a source of medicinal products for millennia, with many useful drugs developed from plant sources. Traditional knowledge-based chemistry has allowed unknown molecules to be identified. These novel bioactive structures can be optimized by synthetic chemistry, thus generating new drug candidates for many diseases. Medicinal chemistry, focusing on natural products, is an attractive research field that provides the opportunity of combining chemical reactivity and molecular recognition in order to obtain an appropriate activity.

This thesis describes the biological evaluation of natural sesquiterpene lactones (SLs) obtained from *Ambrosia arborescens* and methoxyflavones from *Bacharis pentlandii,* both plants used in Bolivian traditional pharmacopeia, and chemically modified SLs with focus on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) as a target. Breast cancer cell lines were used as a model system to evaluate the biological activity of the compounds. The breast cancer cell lines contain bulk cancer cells and CSCs.

The aims :

- To determine the effect of treatment with natural and synthetized SLs on basal cellular traits like cell proliferation and cell movement
- To determine the effect of treatment with natural and synthetized SLs on the CSC population
- To determine the effect of treatment with flavonoids on basal cellular traits like cell proliferation and cell movement
- To determine the effect of treatment with flavonoids on the CSC population
- To start unravelling the molecular mechanisms of toxicity of natural and synthesized SLs and flavonoids

2 Cancer

Normal cells are constantly subjected to signals that dictate whether the cell should divide, differentiate into another cell, or die. Cancer cells develop a degree of autonomy with regard to these signals, resulting in uncontrolled proliferation and evasion of differentiation and cell death. If this proliferation is allowed to continue and cancer cells allowed to spread, it can be fatal. In fact, almost 90 % of cancer-related deaths are due to tumor spreading - a process called metastasis [1].

Initiation and progression of cancer depends on both environmental factors (*e.g.* tobacco exposure, chemicals, radiation, and infectious organisms) and factors within the cell (*e.g.* inherited mutations, mutations induced by external factors, hormones, and immune conditions). These factors can act together or in sequence, resulting in abnormal cell behaviour and excessive proliferation.

The main attribute of malignant tumors is their ability to spread beyond the site of origin. The route of spread and location of metastases varies among primary cancers. The more aggressive and malignant the cancer is, the less reminiscent it will be of the tissue structure where it originated. The growth rate of cancer depends not only on the cancer type and degree of differentiation, but is also dependent on host factors. Another characteristic of cancer is heterogeneity of the cancer cell population as a result of constantly on-going epigenetic and genetic changes [2].

2.1 History of cancer

In 2015, researchers found what is believed to be the oldest known case of cancer in humans. The cancer, leukemia, was identified in the 7000 years old skeletal remains of a woman who lived in the Neolithic period near present-day Stuttgart-Mühlhausen (Germany) [3]. The earliest record of breast cancer was found when evidence of tumors was discovered in fossilized bone in mummies in Egypt. Ancient manuscripts, the Edwin Smith Papyrus, from 3000

BC describes eight cases of tumors or ulcers of the breast that were removed by cauterization with a tool called the fire drill. However, it was not until the Greek physician Hippocrates (460-370 BC) used the term carcinos in relation to non-ulcer and ulcer-forming tumors, later the word was changed into the term cancer by Celsus (28-50 BC).

2.2 Cancer definition

Cancer is a broad term describing a group of diseases caused by deviant cells unable to form stable functional structures since the cells multiply anarchically and invade the organism. On the molecular level, cancer is a disease that involves changes or mutations in the cell genome. These changes (DNA mutations) result in the production of proteins that disrupt the delicate cellular balance between cell division and quiescence, resulting in cells that keep dividing and avoid apoptosis to form cancers [1].

Some types of cancer are characterized by rapid growth, while others grow at a slower rate. Certain forms of cancer result in visible lumps called tumors, while others, such as leukemia, do not.

Histologically, cancer is classified in six major groups based on their origin: carcinoma derived from epithelial cells, sarcoma derived from connective tissue, myeloma and leukemia are derived from bone marrow, lymphoma is derived from lymphocytes, and mixed type cancer [4].

2.3 Hallmarks of cancer

Tumorigenesis in humans is a process that involves several stages, in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Hanahan and Weinberg [5,6] suggested that although cancer cells have a high genomic variability, they still share six essential alterations in cellular physiology that ensure tumor growth and self-sufficient signalling.

In their much-cited work on cancer definition, Hanahan and Weinberg initially described six general cell biological properties that classify the cancer phenotype (Fig. 1) [5]. In 2011 [6] they included several new hallmarks *i.e.* deregulation of cellular energetics, evasion of immune destruction, tumor

promoting inflammation, and genomic instability [6]. To target these different hallmarks has been at the center of cancer research both before and after the publication of the excellent reviews by Hanahan and Weinberg. Their compilation of cancer traits has proved seminal for our understanding of cancer behavior and for rational drug design.

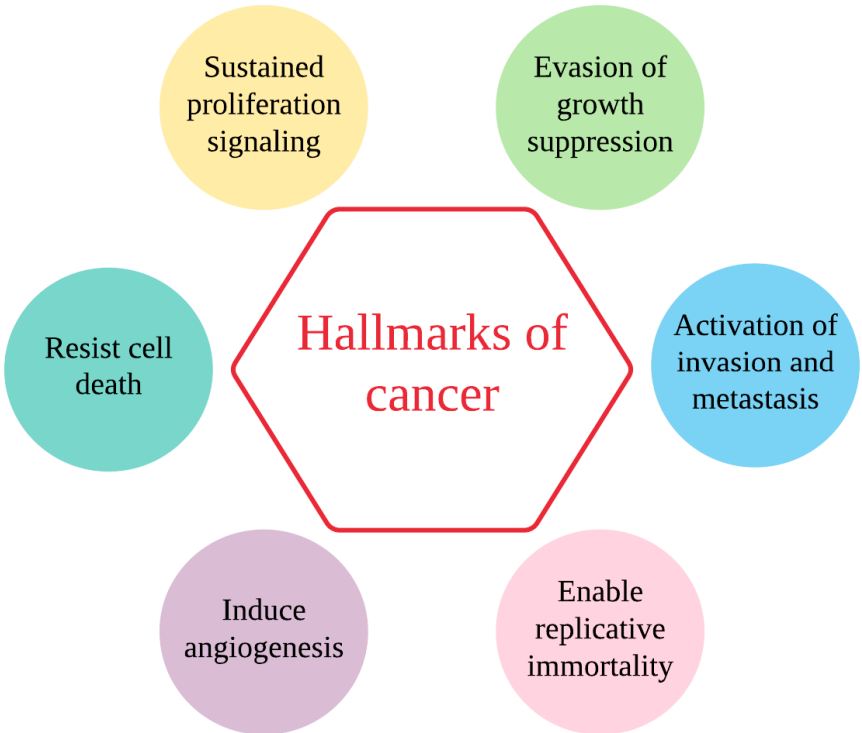


Figure 1. The "Hallmarks of Cancer" shows properties that cancer cells have in common [5,6]. Sustained proliferation signaling implies that cancer cells are self-sufficient regarding growth signaling. Evasion of growth signaling implies that cancer cells are insensitive to anti-growth signals. Resistant to cell death implies that cancer cells survive many threats that kill normal cells. Enable replicative immortality implies that the cells can go on multiplying forever. Induce angiogenesis implies that cancer cells stimulate ingrowth of blood vessels into a tumor. Activation of invasion and metastasis implies that cancer cells invade adjacent tissues and travel to distant sites where they may form new tumors.

2.4 Breast cancer statistics

According to the World Health Organization, an estimate of 18.1 million new cancer cases and 9.6 million cancer deaths were expected worldwide at the end of 2018. Among all cancer types, breast cancer is the second most common cancer in the world and by far the most frequent cancer in women with an estimate of 2 million new cancer cases diagnosed at the end of 2018, which represents 11.6 % of all cancers [7].

In the female population, the cancer incidence reached 8.6 million new cases in 2018. Among these 8.6 million new cancer cases, 24.2 % are breast cancer, with a mortality of 15 %, thus approximately 630000 deaths in 2018 [7]. The range of incidence in the world varies according to the human development index. Countries that are more economically developed present a higher incidence than countries that are less economically developed or in countries having transition economies. However, the mortality reflects the economic impact on disease control. The incidence rate in North Europe was 90.1 new cases and 14.1 deaths per 100000 persons per year in 2018. Even though the incidence is lower in South America with 56.8 new cases per year, the mortality rate follows closely the mortality rate in North Europe with 13.4 deaths per 100000 persons in 2018 [7].

According to the Bolivian health minister, 110000 cancer cases are diagnosed per year in Bolivia. Around 68 % of these cases are women and 17 % of those correspond to breast cancer. In Sweden, breast cancer corresponded to 30.3 % of all cancers in females in 2011 [8].

Comparisons of low-risk populations migrating to areas with high-risk populations have revealed that the breast cancer incidence rates rise in successive generations [9,10]. Regardless of low incidence, the low survival rates in less developed countries can be explained mainly by the lack of early detection programs, resulting in a high proportion of women with late-stage disease, as well as by the lack of adequate diagnosis and treatment facilities [11].

Generally, elevated incidence rates are attributed to a higher prevalence of known risk factors related to menstruation (early age at menarche, later age at menopause), reproduction (nulliparity, late age at first birth, and fewer children), exogenous hormone intake (oral contraceptive use and hormone replacement therapy), nutrition (alcohol intake), and anthropometry (greater weight, weight gain during adult-hood, and body fat distribution). Breast feeding and physical activity are known protective factors [12].

2.5 Breast cancer

Breast cancers are classified according either to histological features or to molecular characteristics of the tumor. Each of them influences the outcome and response to treatment.

Histological classification is usually used by physicians to give the broad panorama of breast cancer (Fig. 2). Most breast carcinomas are derived from the epithelial lining of the ducts or lobules; accordingly, they are classified as *in situ* carcinoma or invasive-infiltrating carcinoma. Infiltrating ductal carcinoma is, by far, the most common subtype accounting for 70–80 % of all invasive lesions [13].

Molecular classification defines the major subtypes of breast cancers grouped by evaluating the expression of three tumor markers: the estrogen receptor (ER), the progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). The expression of these receptors is evaluated routinely because of their utility in guiding treatment strategies and for prognosis evaluation. Based on these three receptors and some other markers, breast cancer has been classified into 6 major molecular sub-types [13–15] (Fig. 2).

Recent findings indicate that immunohistochemical protein expression profiles are surrogates for intrinsic gene-derived expression profiles defining molecular breast cancer subtypes [14].

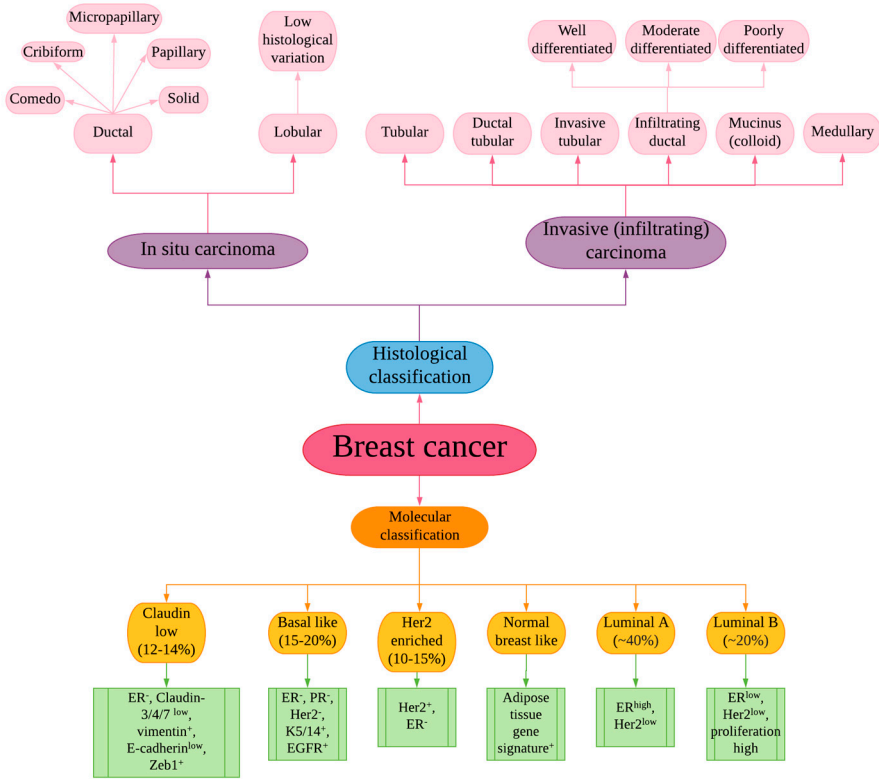


Figure 2. Histological and molecular classification of breast cancer. Histological classification is based on the current categories used by clinicians using architectural features and growth patterns. The molecular classification is based on differences in cancers identified by protein and mRNA expression analysis of patient tumor specimen [13,15].

2.6 Cancer heterogeneity

Besides inter-tumor heterogeneity, breast cancer is also characterized by a high degree of intra-tumor heterogeneity revealed by genetic and non-genetic variations across different regions of a tumor and throughout different stages of tumor progression [16]. Tumor heterogeneity is clinically important, as therapeutic responses are largely determined by the evolution of resistant subpopulations and changes in cellular phenotypes [17]. In this context, the CSCs have a specific role as they are suggested to contribute to treatment resistance and cancer recurrence [18].

One framework that has received much attention recently attempts to understand cancers as perturbed versions of the normal tissue in which they originated, with retention of many tissue-specific developmental features. The CSC hypotheses is a derivative of this framework. It states that a cancer cell population has a hierarchical developmental structure in which only a fraction of the cells, the CSCs, can proliferate indefinitely [16,17,19].

2.7 Cell lines as a model to study breast cancer

The use of appropriate *in vitro* models in cancer research is crucial for the investigation of genetic and epigenetic changes, and molecular pathways involved in the study of the deregulated cell proliferation and apoptosis, and of cancer progression. The goal is to define potential molecular markers for screening and characterization of cancer therapeutics. For breast cancer, there is a large array of different cell lines established from the tumors of women [14,20].

Populations of cancer cells in a tumor and in their models, the cancer cell lines, display many biochemical and biological features of which some are common to most cancers, and some are particular to distinct tumor types. Breast cancer cell lines are divided into molecular subtypes according to the expression of ER, PR, and HER2, and other marker proteins as are the breast tumors from which they were isolated. Thus, breast cancer cell lines can be of sub-types, claudin low, triple negative (basal), HER2 enriched, normal-like, luminal A, and luminal B (Fig. 2) [21].

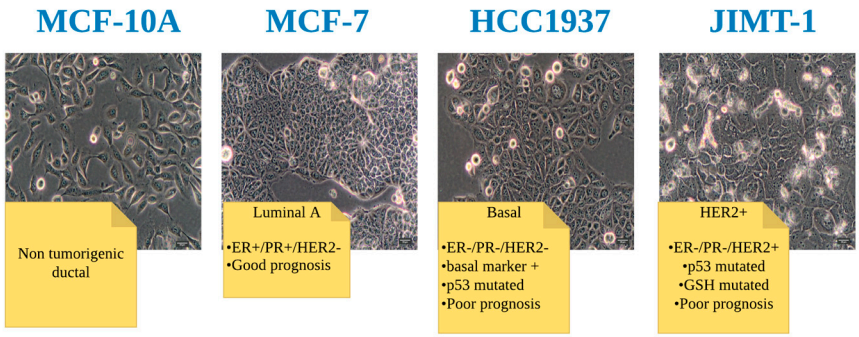


Figure 3. Phase contrast microscopy images of breast-derived cell lines used in this study. MCF-10A is a non-tumorigenic normal-like breast epithelial cell line established from a fibrocystic growth of the breast while MCF-7, HCC1937, and JIMT-1 are breast cancer cell lines established from breast tumors. The photos of the cell lines were taken with a phase contrast microscope. Bars = 20 µm.

The MCF-7 cell line (Fig. 3), histologically identified as invasive breast ductal carcinoma, was derived from a pleural effusion of a 69-year-old Caucasian woman. It was established for *in vitro* breast cancer studies by Dr. Herbert Soule [22]. This cancer cell line has retained several characteristics of the mammary epithelium. One of those is the dependence on estrogen for cell proliferation [23,24]. The MCF-7 cell line is thus ER positive. It also has wild-type p53 protein resulting in many normal reactions to insult. However, it lacks caspase-3 and is not prone to apoptosis. MCF-7 belongs to the breast cancer molecular sub-type luminal A.

The HCC1937 cell line (Fig. 3), was derived from a 24-year-old woman with a family history of breast cancer and a germ line mutation in the breast cancer gene 1 (BRCA1). This cell line belongs to the triple negative sub-group and is thus negative for HER2, ER, and PR and it has mutated p53 [25].

The JIMT-1 cell line (Fig. 3), was established from the pleural effusion of a 62-year-old woman with ductal breast cancer [26,27]. The JIMT-1 cells carry an amplified mutated HER2 oncogene and are insensitive to the HER2 targeting humanized monoclonal anti-body trastuzumab (Herceptin) [27]. The cell line lacks ER and PR and has a point mutation in p53 [26]. The JIMT-1 cell line is sometimes defined as basal-like since it behaves as a triple negative cell line because of the trastuzumab resistance [28]. Another characteristic of JIMT-1 cells is a low expression of the glutathione S-transferase M1 gene (GSTM1). This

enzyme is the responsible for detoxifying electrophilic compounds like carcinogens, by conjugation with glutathione [27].

The MCF-10A cell line (Fig. 3), is an immortalized, non-transformed epithelial cell line derived from the breast tissue of a 36-year-old patient with fibrocystic disease that has retained many normal traits, including lack of tumorigenicity in nude mice, lack of anchorage-independent growth, and dependence on growth factors and hormones for proliferation and survival [29].

All four cell lines were used in this study for screening of toxicity of SLs and methoxyflavones (Papers I and V). For more thorough experiments on mechanisms, the JIMT-1 and MCF-10A cell lines were chosen to be able to compare effects in a non-tumorigenic cell line (MCF-10A) with those in a highly tumorigenic cell line (JIMT-1) (Papers I, III, and IV).

2.8 Cancer stem cell-targeted therapy

CSCs are a subset of cells capable of dictating invasion, metastasis, heterogeneity, and therapeutic resistance in tumors. CSCs were initially discovered in leukemia [30] but have now been identified in a wide variety of solid tumours, including breast cancer [19].

By definition, stem cells are able to perpetuate themselves through self-renewal, enabling persistence through life of an individual. As they also have multilineage potential, they can give rise to any differentiated cell type in a given tissue. Stem cells divide asymmetrically producing two different daughter cells, one being a stem cell (ensuring self-renewal) and the second one being a progenitor cell that will undergo proliferation and ultimately terminal differentiation [31]. CSCs have been coined this name as they to some extent are similar to normal stem cells [32].

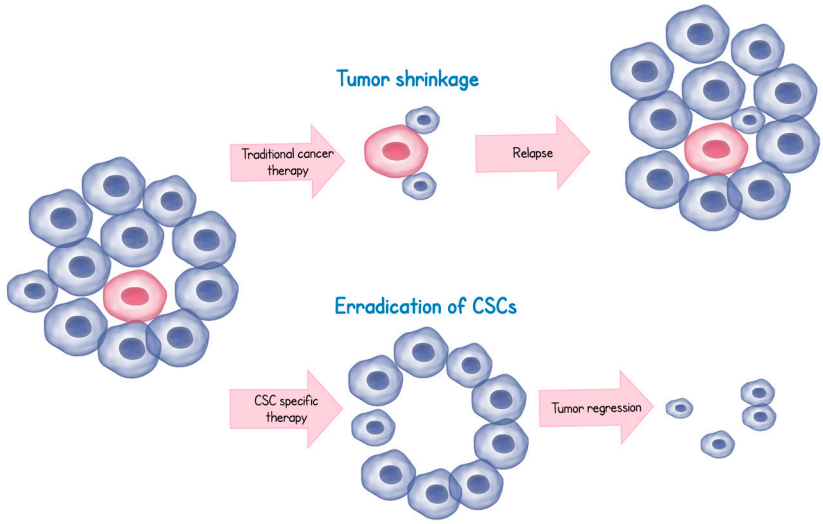


Figure 4. Schematic representation of the theory of cancer stem cell (CSC)-targeted therapy. A tumour is a heterogenous population of cells with different ability to survive insult. The CSCs (pink) are more resistent to therapy than the non-CSC cells (blue). Conventional therapy seems to target the non-CSC cells (top part of figure) which eventualy leads to tumor relapse even it is seemed to shrink initially. The CSC-targeted therapy removes the cells with the ultimate proliferative capacity *i.e.* the CSCs resulting in tumor regression.

In most breast tumours, cancer cells with more differentiated phenotype, as well as cancer cells with stem like features, *i.e.* putative CSCs, can be found [19]. The bulk cancer cells constitute the proliferating fraction of tumors while the CSCs are rare and are less proliferatively active or even dormant. The number of CSCs vary in different tumors. According to a mathematical model proposed by Johnston *et al*., CSCs could be any possible proportion of the tumor and its tumorigenesis is directly proportional to the number of CSCs [18]. The proportion of CSCs is in general larger in cancer cell lines compared to tumors [33]. A study of the proportion of CSCs in breast cancer cell lines showed a variation between 0.2 and 100 % [34].

Conventional cancer therapies are developed to target proliferating cells implying that the CSCs are less vulnerable as they may be dormant. In a clinical setting, this is reflected by the recurrence of a tumor locally or as metastases even a decade after initial successful treatment (Fig. 4). In that case, chemotherapy killed the bulk of cancer cells while the CSCs evaded the drug toxicity because of inherent properties not only involving dormancy but also *e.g.* drug resistance [31]. Since the CSCs are assumed to be the source of new

tumors, eradicating these cells by target therapy, would lead to tumor regression (Fig. 4).

Because of resistance and metastasis factors, eradication of this rare population of CSCs is necessary, to achieve successful cancer treatment. Year by year new strategies are formulated to target CSCs. However, prospective identification, characterization, and isolation of these CSCs have so far been a major challenge [31]. Lack of universal expression of surface markers on CSCs in all tumor types limits their practical management. Unfortunately, no best optimal combination of markers has yet been confirmed to universally identify CSCs capable of initiating and metastasizing tumors. As breast cancer is one of the most common cancers, many studies have focused on the identification of molecular and functional markers for potential identification and isolation of breast CSCs [35–37].

2.9 Cancer stem cell identification

A good molecular or functional marker for the detection of a CSC in a tumor, is a marker and/or function which is specifically associated with the CSC, but cannot be found in association with a normal cell or a bulk cancer cell.

2.9.1 Cell surface markers of breast cancer stem cells

Since Bonnet and Dick reported CSCs in leukemia [38], a number of cell surface markers have been discovered and correlated with solid tumors in breast, brain, prostate, pancreas, colon, melanoma, liver, and ovary.

The first identification of breast CSCs was achieved by the isolation of cells with the cell surface marker profile of $CD44^+/CD24^-$ by Al Hajj *et al*. [39]. In fact, the CSC population within many tumors has been identified by the expression of the cell surface proteins CD44 and CD24 [40]. CD24 is a heat stable antigen expressed on the cell surface [41]. CD44 is a hyaluronic acid receptor and it is one of the most commonly studied cell surface markers, which is expressed by almost every tumor cell. $CD44^+$ expression is usually related to metastasis and poor prognosis in cancer [42]. Although CD44 and CD24 have been successfully used for the identification of CSCs in many tumor types, they are not universal markers for the identification of this sub-population of cancer cells [42].

The percentage of CSCs has been evaluated in the JIMT-1, HCC1937, and MCF-7 cell lines. In the JIMT-1 cell line, the percentage of cells in the $CD44^+/CD24^-$ population varied between 50 % and 70 % and in the HCC1937 cell line between 30 % and 50 % while for the MCF-7 cell line, the percentage of this population was lower than 1 % [43].

In Papers I, III, and V, the JIMT-1 cells were used to investigate the effect of treatment with SLs and methoxyflavones on the $CD44^+/CD24^-$ sub-population. Flow cytometry was used to analyse the distribution of cells in different populations based on labelling the cell population with fluorescein isothiocyanate-conjugated CD44 antibodies and phycoerythrin-conjugated CD24 antibodies. A percentage between 60 % and 80 % of the control non-treated JIMT-1 cell population was found to be $CD44^+/CD24^-$ in these studies.

2.9.2 Aldehyde dehydrogenase activity

Besides being identified by cell surface markers, CSCs are detected by the presence of the activity of the enzyme aldehyde dehydrogenase (ALDH) [37].ALDH is a cytoplasmic detoxifying enzyme that oxidizes intracellular aldehydes. This enzyme is believed to play a role in the differentiation of stem cells via the metabolism of retinal into retinoic acid. The ALDH activity can be used to isolate a subpopulation of cells that display stem cell properties from normal breast tissues and from breast tumors [36,37].

An $ALDH^+$ population between 40 % and 60 % has been reported for JIMT-1 cells but only 3 % for MCF-7 cells [44]. The $ALDH^+$ sub-population was identified using flow cytometry after incubating the cells with a substrate that is converted to a fluorescent product in cells containing ALDH. In Papers I, III, and IV, the JIMT-1 cells were used to evaluate the effect of treating with SLs and methoxyflavones on the $ALDH^+$ population. In this work, the $ALDH^+$ population was found to be between 20 % and 30 % in control non-treated JIMT-1 cells.

2.9.3 Colony forming efficiency assay

A colony forming efficiency (CFE) assay or clonogenic cell survival assay is a functional assay utilized as an *in vitro* test to investigate the potential of single cancer cells to form colonies independently of attachment to surfaces in a semisolid media containing serum [31]. The semi-solid medium reduces cell

movement and allows individual cells to develop into cell clones that are identified as single colonies. Many normal cells show the phenomenon of adherence, they grow and divide only if attached to a solid inert support, as is provided, for example, by the glass or plastic surfaces of tissue culture dishes. This requirement is frequently lost during prolonged cell culturing, resulting in the development of transformed cells. One of the phenotypically recognizable characteristics of cell transformation is the ability of cancer cells to form colonies in semisolid serum containing medium that does not provide a support. The cancer cell lines used in this thesis form colonies under these conditions while the non-tumorogenic MCF-10A cells do not.

However, to detect CSCs in a population of cancer cells, the assay must be performed in semi-solid medium without serum[43]. In this work, the CFE obtained using the serum free soft agar assay was between 50 % and 70 % for JIMT-1 cells.

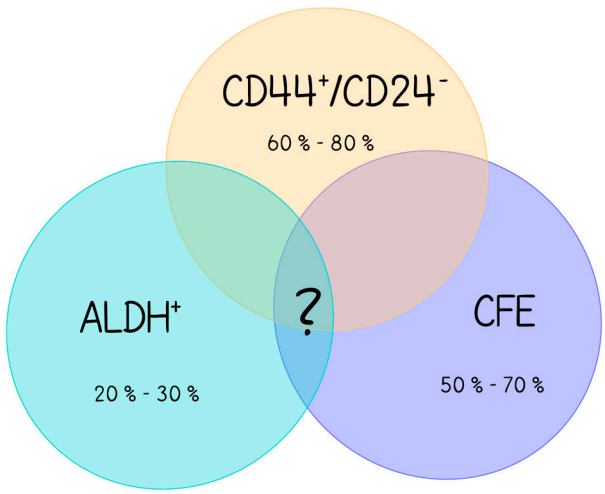


Figure 5. Schematic representation of three assays used to identify CSCs in this work. ALDH, adehyde dehydrogenase. CFE, clony forming efficiency. CD44 and CD24 are cell surface proteins. Different sizes of the CSC population is obtained using the three assays. The ? denotes a possible overlap of the three populations that maybe defines a “true” CSC population.

2.9.4 Cancer stem cell identification in this work

Here, three different assays (Fig. 5) have been used to identify the CSC sub-population of JIMT-1 cells. However, presently it is not possible to define the true CSC population and in fact the three assays show different sizes of the CSC sub-population (Fig. 5). The “true” CSCs may be a population of cells where there is an overlap of $CD44^{+}/CD24^{-}$, $ALDH^{+}$, and the CFE populations (Fig. 5). One or more interconverting stem-like populations may exist within breast tumors but a universal CSC marker may be an unrealistic prospect. So, the best way to describe the results is to talk about CSC sub-populations expressing one or more molecular markers.

3 Natural products and synthetic analogues in cancer research

Nowadays medical science reinforces the value of traditional knowledge regarding the effectiveness of medicinal plants through the identification of chemical compounds with important therapeutic properties.

Bolivia and other South American countries are recognized for having a large collection of local "herbolaria" *i.e.* a collection of plants for medicinal use [45]. Approximately 3,000 species of medicinal plants have been identified and verified in Bolivia [46], but most of these plants have not been thoroughly investigated. In the central Andes, the Kallawaya healers is the leading group in knowledge and management of medicinal plants [47] and they have made important contributions to traditional medicine. Traditional knowledge can be investigated from many perspectives, but usually it starts with empirical classification, knowledge of species, habitats, traditional harvesting techniques, and preparation for medicinal use [48]. If a plant shows medicinal potential, further toxicological investigations are usually performed in the beginning and then further tests of the drug potential.

There is still a lot to learn about the medicinal plants of Latin America. Macía, García, and Vidaurre provided the first study of medicinal plants found at Andean markets at high elevation, focusing on La Paz, Bolivia [47]. Since then, other authors have updated information related to medicinal plants in South American countries [45]. They have shown that species from the Asteraceae family are associated with broad medicinal activity, mainly related to anti-inflammatory activity.

The Asteraceae family comprises 23,600 species distributed on all continents of the world, except Antarctica. This family is usually represented by herbs and shrubs. The Asteraceae family has been reported to have a multitude of bio-active constituents that have been isolated from several plant families which usually are related to traditional medicine [49].

Ambrosia arborescens and *Bacharis pentlandii*, both plants from the Bolivian Andean "pharmacopeia" belonging to the Asteraceae family, have been reported to be sold as anti-inflammatories by vendors in traditional markets of La Paz. The plants are recommended for sprains, and muscular pain in relation to inflammatory pathologies [45].

3.1 Sesquiterpene lactones from *Ambrosia arborescens*

The Asteraceae family also contains structurally diverse SLs and furanosesquiterpenes, which are used as taxonomic characteristics [50]. More than 3000 different structures of SLs have been reported [51]. Although the quantity of SLs varies in different plants, it is common for them to be synthesized by *Ambrosia*, *Artemisia, Parthenium, Vernonia,* and other genera in concentrations from 0.01 to 8 % of the dry weight of the plant [52].

The *Ambrosia* gender is well known for containing a high content of SLs as secondary metabolites. Presumably in response to selection pressure associated with predation, the members of the *Ambrosia* gender display a propensity to shift their biosynthesis resulting in different amounts of terpenoids to which the SLs belong.

SLs have attracted considerable attention because of their vast array of biological activities. Many of them exhibit cancer cell cytotoxicity and are promising anti-cancer agents through multiple mechanisms of action. SLs are alkylating agents that form covalent adducts and inhibit enzymes and key proteins [53].

3.1.1 Structure

SLs are chemical molecules containing a basic fifteen carbon backbone with numerous modifications all having a γ-lactone ring containing an α-methylene group, this ring define five distinct groups of SLs: the pseudoguaianolides, the guaianolides, the germacrolides, xanthanolides, and eudesmanolides [54,55] (Fig. 6). The SLs in this work belong to the group pseudoguaianolides and the α-methylene-γ-lactone functional group is the structural requirement for their pharmacological activities (Fig. 7).

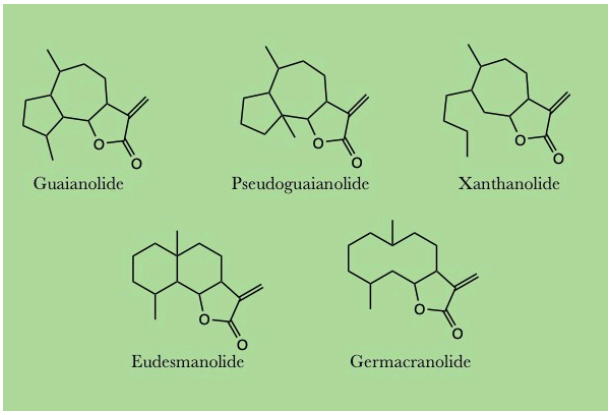


Figure 6. Basic skeletons of the main groups of sesquiterpene lactones.

The biological activity of SLs depends on three biochemical properties of these compounds: 1. The alkylating center reaction *i.e.* the α-methylene-γ-lactone functional group. 2. The side chains and lipophilicity. 3. The molecular geometry and electronic properties [54–56].

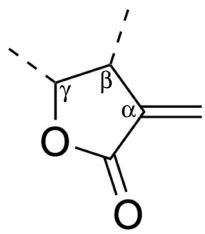


Figure 7. The α-methylene-γ-lactone ring of a sesquiterpene lactone.

3.1.2 Michael acceptor reaction

The Michael reaction (Michael addition) is the nucleophilic addition of a carbanion or another nucleophile to an α,β-unsaturated carbonyl compound [57]. The α-methylene-γ-lactone of the SLs acts as an alkylating agent in a Michael-type reaction with biological nucleophiles such as free sulfhydryl groups of cysteine residues. Cysteines are found in many macromolecules, such as enzymes, transcription factors, and structural proteins, and in the antioxidant tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) (GSH)[58–60].

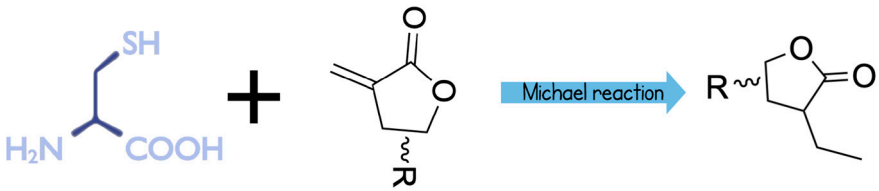


Figure 8. Michael type reaction with cysteine

Although it was initially believed that the highly reactive α-methylene-γ-lactone group might react non-specifically with any exposed thiol group, it has been shown that SLs have preference for specific cysteines [58] (Fig. 8), *e.g.* parthenolide and the thiol group of cysteine 38 residue of p65/NF-κB [59]. The SL helenalin has been shown to inhibit the transcription factor NF-κB by directly targeting cysteine 38 of p65/NF-κB [58]. Subsequently it has been shown that specifically cysteine 38 in the p65 subunit is a sensitive alkylation target for SLs, due to the ability to interact rapidly with sulfhydryl groups by Michael addition [53,60–62].

3.1.3 Damsin, ambrosin, and coronopilin

Damsin, ambrosin, and coronopilin are natural SLs and they are usually found in the Asteraceae family. The first isolation and report of cytotoxic effects was made in the '60s by Hufford and Doskotch [63,64]. Coronopilin was first reported as damsinic acid [63] and later the name was changed to coronopolin when isolated from *Ambrosia psylostachia* [65]. Ambrosin was isolated from and first reported as a cytotoxic compound found in *A. maritima L.* [66]. In *A. arborescens Mill.*, damsin was found as a majority fraction [67] together with coronopilin [68].

The natural SLs used in this study, damsin and coronopilin, were isolated from *A. arborescens* [69] (Fig. 9). Villagomez *et al.* [70] then used damsin as a starting material for the synthesis of the natural SL ambrosin and the synthetic SL dindol-01 [69]. The cytotoxicities of damsin, ambrosin, coronopilin, and dindol-01 were investigated in Paper I.

3.1.4 Compound modifications

For the further synthesis of SL analogues, Villagomes *et al.* [70] synthesized a damsin analogue with a benzaldehyde group (Fig. 9 (**5**)). DSC3 (Fig. 9 (**5**)) was then the base for the synthesis of more complex synthetic analogues with different chemical entities (Fig. 9). Different chemical modifications were made in para (R1), meta (R2), and ortho (R3) positions (Fig. 9) to initially produce compounds **6**-**25** (Table 1) (Paper II), and later on the compounds **26**-**46** shown in Tables 2-4 (Paper IV) (Table 1-4).

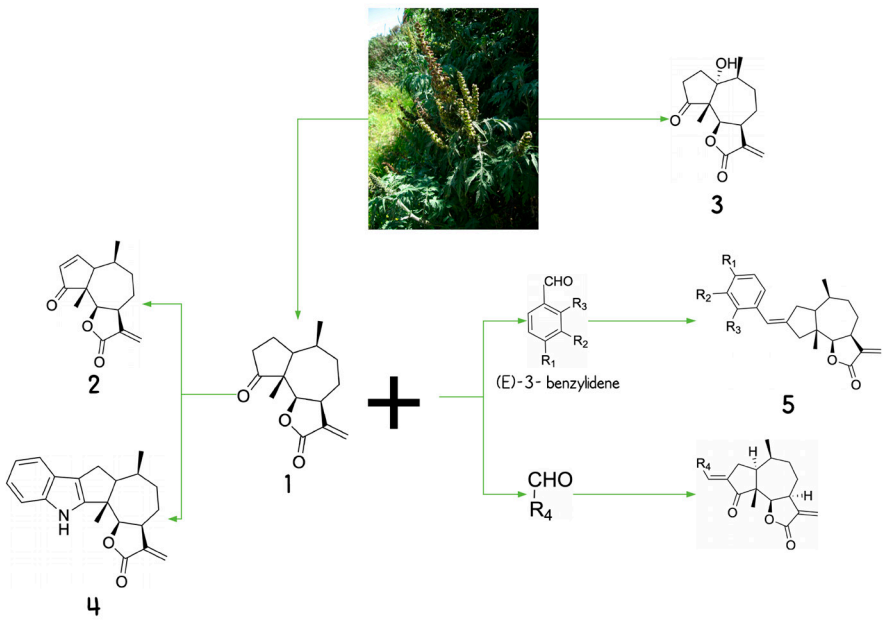


Figure 9. Damsin and coronopilin isolated from *A. arborescens*. Damsin was used for the synthesis of the natural SLs ambrosin and the synthetic SL dindol-01 and 42 analogues investigated in this study. Compounds **1**, **2**, **3** and **4** are investigated in Paper I. Compound **5** is the base for synthetized compounds **6**-**19** (Table 1) and **30**-**42** (Table 2).

Table 1. Damsin analogues (Paper II)

Number in thesis	Compound code	R1	R2	R3
5	3a (DSC3)	H	H	H
6	3b[1]	CH3	H	H
7	3c	H	CH3	H
8	3d	H	H	CH3
9	3e	CH3	H	CH3
10	3f[1]	CF3	H	H
11	3g	H	CF3	H
12	3h	H	H	CF3
13	3i[1]	OCH3	H	H
14	3j	H	OCH3	H
15	3k	H	H	OCH3
16	3l	OH	H	H
17	3m	H	OH	H
18	3n[1]	H	H	OH
19	3o	H	H	H
20	3p	R4 = (E)-cyclohexyl		
21	3q	R4 = (Z)-cyclohexyl		
22	3r	R4 = ethyl		
23	3s	R4 = propyl		
24	3t	R4 = 2-methyl propyl		
25	3u	R4 = 3-butenil		

[1]Compounds 3b, 3n, 3f, and 3i in Paper II correspond to compounds 1, 6, 7, and 10 in Paper III.

For the synthesis of damsin derivatives based on DSC3 we highly acknowledge Maribel Lozano and Rodrigo Villagomes part of the Centre for Analysis and Synthesis in Lund University and the Bolivian Institute of Chemistry in the Universidad Mayor de San Andres, La Paz, Bolivia.

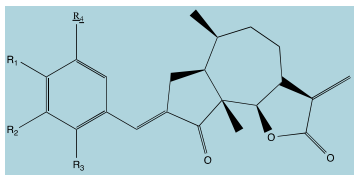


Figure 10. Structure of DSC3. Basic structure for the synthesis of compounds **6**-**19** and **30**-**42**.

Table 2. Damsin analogues based on DSC3 addition (Paper IV).

Number in thesis	Compound code	R1	R2	R3	R4
30	2a	F	H	H	
31	2b	H	F	H	
32	2c	H	H	F	
33	2d	Cl	H	H	
34	2e	Br	H	H	
35	2f	H	Br	H	
36	2g	H	H	Br	
37	2h	CH3CH2	H	H	-
38	2i	(CH3)2CH2	H	H	-
39	3a	CF3	H	F	H
40	3b	CH3	H	OH	H
41	3c	OH	CF3	H	H
42	3d	H	H	OH	CL

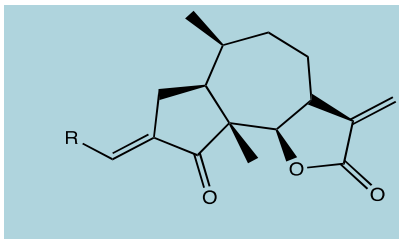


Figure 11. Basic structure for the synthesis of compounds **43** and **44**.

Table 3. Damsin analogues based on the basic structure in Figure 11 (Paper IV).

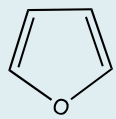
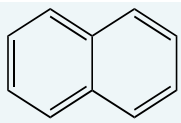
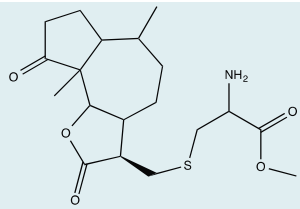
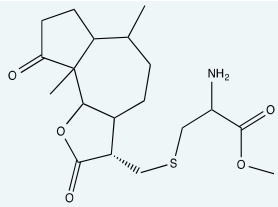
Number in thesis	Compound code	R
43	4a	
44	4b	

Table 4. SL with an open α-methylene-γ-lactone ring.

Number in thesis	Compound code	R
45	5a	
46	5b	

3.2 Flavonoids from *Baccharis pentlandii*

Bacharis pentlandii (Fig. 12) has been reported to be rich in terpenes and flavonoids, with focus on flavones, [71–73]. Flavonoids are powerful antioxidants with anti-inflammatory and immune system benefits. Anti-inflammatory agents have been correlated with a decrease in cancer incidence [74] and thus such compounds have become a new field of interest [75,76]. The flavones are classified into flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids.

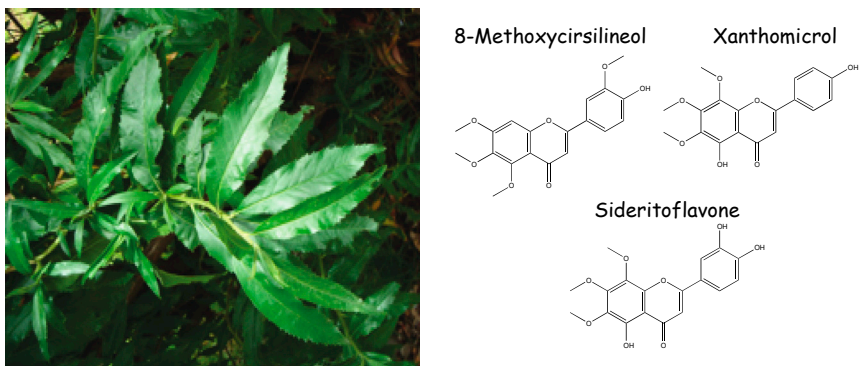


Figure 12. *Baccharis pentlandii* and the isolated methoxyflavones used in this study.

Flavones are present in fruits and vegetables which we consume inadvertently in our daily diet and they have a positive impact on our health without any major side effects. The flavones is a class of flavonoids based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one). It has a three-ring skeletons (Fig. 12), and they have three functional groups, including hydroxy, carbonyl, and a conjugated double bond; consequently they give typical reactions of all three functional groups [77].

In this Paper V, three flavones with methoxy substituents seem to play an important role for the anti-cancer effects. Walle *et al*. [78] compared methoxyflavones with highly oxygenated flavones and found that the methylation of free hydroxyl groups in the flavones results in metabolically more stable derivatives with superior membrane-penetrating properties and thus vastly improved bioavailability [78].

The natural methoxyflavones used in paper V (Fig. 12), 8-methoxycirsilineol, xanthomicrol, and sideritoflavone, were isolated from *B. pentlandii* [73] by Santiago Tarqui at the chemical research institute at Universidad Mayor de San Andres in La Paz, Bolivia.

4 Dose-response effects of sesquiterpene lactones and flavonoids

Anti-proliferative activity is related to the capacity of treatment with a compound to inhibit cell proliferation. When testing many compounds for anti-proliferative activity, a high-throughput assay is required to increase efficiency. Here, the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on reduction of the substrate in mitochondria was used to evaluate toxicity. The MTT assay is an indirect means to measure effects on the cell number after treatment with a compound at many concentrations [79]. The readout in control is compared to the readout in treated cells and the effect is expressed as % of control. It is assumed that the readout is proportional to cell number. A reduction in MTT readout after treatment implies that there are fewer cells in treated cultures compared to control, however, it does not say anything about the cause for the lower cell number in treated cultures. The data are used to construct dose-response curves from which inhibitory concentration 50 (IC_{50}) values are deduced. IC_{50} values are then compared and used as a measure of toxicity.

4.1 Treatment with sesquiterpene lactones and dose-response effects

Since there are so many natural SLs and since they have been known for very long, there are many studies of toxicity using dose-response testing. However, there is still no clear and systematic information about toxicity in different settings and much work is needed.

Several cell lines were used to screen for anti-proliferative activity in dose-response assays of natural and synthetic SLs (Tables 5 and 6). In Paper 1, damsin, ambrosin, coronopilin, and dindol-01 were studied. The IC_{50} was evaluated for three cancer cell lines (MCF-7, HCC1937, and JIMT-1) and for the normal-like MCF-10A cell line. We found that damsin and ambrosin were more toxic than coronopilin and dindol-01 as evidenced by lower IC_{50} values for the former two compounds compared to the latter two. The IC_{50} for coronopolin was in general twice as high as that for damsin. On the other hand, in a study with the monocytic leukaemia U937 cell line, coronopilin exhibited higher cytotoxicity than damsin [80]. For CaCo-2 colon cancer cells, coronopilin was also shown to exhibit higher toxicity than damsin, but the concentrations used (25–100 μM) were much higher than the ones used in paper I [81].

The results of the first four tested compounds damsin, ambrosin, coronopilin, and dindol-01, pointed towards damsin as an ideal structural base for synthetic modification. Damsin is easily isolated from *A. arborescens* and it represents a higher percentage of dry weight compared to coronopilin [67]. As a starting-point for the synthesis of damsin derivatives, a benzyl group was added to damsin, forming DSC3. In the further studies evaluating the toxicity of the synthetic SLs, JIMT-1 and MCF-10 cell lines were used to compare IC_{50} values in a breast cancer and a normal-like cell line, respectively (Papers II, III, and IV). DSC3 was more toxic to JIMT-1 cells than damsin *i.e.* the former had a lower IC_{50} than the latter, however, the IC_{50} of damsin and DSC3 were the same in MCF-10A cells (Table 5). Thus, the benzyl addition increased the selectivity of toxicity towards the cancer cells *i.e.* the ratio between IC_{50} of MCF-10A cells and JIMT-1 cells increased (Table 5).

Table 5. IC_{50} values in μM concentrations for damsin and derivatives and ratio between IC_{50} of MCF-10A cells and JIMT-1 cells.[a]

Number in thesis	Number in Papers II and IV	MCF-10A	JIMT-1	Ratio MCF-10A:JIMT-1
1	Damsin	8.1 ± 0.4	3.3 ± 0.6	2.5
2	Ambrosin	2.1 ± 0.1	1.4 ± 0.1	1.5
3	Coronopilin	15.3 ± 0.9	5.5 ± 0.8	2.8
4	Dindol-01	37 ± 9.8	16 ± 3.9	2.3
5	3a (DSC3)	8.2 ± 1.6	1.7 ± 0.4	4.8
6	3b (1)*	3.7 ± 0.4	2.1 ± 0.3	1.8
7	3c	12.6 ± 1.6	4.8 ± 0.3	2.6
8	3d	11.1 ± 1.8	4.7 ± 0.1	2.4
9	3e	5.2 ± 1.5	3.5 ± 0.7	1.5

Number in thesis	Number in Papers II and IV	MCF-10A	JIMT-1	Ratio MCF-10A:JIMT-1
10	3f (7)*	3.1 ± 0.3	1.8 ± 0.2	1.7
11	3g	11.9 ± 0.4	4.4 ± 0.7	2.7
12	3h	13.0 ± 0.8	8.1 ± 0.6	1.6
13	3i (10)*	7.9 ± 1.2	1.6 ± 0.1	4.9
14	3j	> 20	9.0 ± 1.0	na
15	3k	> 20	7.1 ± 0.2	na
16	3l	13.6 ± 0.6	2.9 ± 0.2	4.7
17	3m	10.6 ± 1.3	2.4 ± 0.1	4.4
18	3n (6)*	6.7 ± 0.9	2.1 ± 0.2	3.2
19	3o	7.1 ± 0.6	2.0 ± 0.6	3.6
20	3p	11.7 ± 1.9	8.1 ± 3.1	1.4
21	3q	> 20	12.3 ±1.3	na
22	3r	5.5 ± 1.1	1.4 ± 0.1	3.9
23	3s	12.6 ± 0.8	3.7 ± 0.1	3.4
24	3t	20.3 ± 0.3	8.1 ± 0.1	2.5
25	3u	17.5 ± 5.1	1.7 ± 0.0	10.3
26	1c	12.8 ± 1.1	6.3 ± 0.2	2.0
27	1d	> 20	> 20	na
28	1e	> 20	> 20	na
29	1f	> 20	> 20	na
30	2a	4.4 ± 1.6	2.3 ± 0.4	1.9
31	2b	2.5 ± 0.3	1.1 ± 0.1	2.3
32	2c	2.2 ± 0.4	1.1 ± 0.3	2
33	2d	3.8 ± 0.6	1.4 ± 0.3	2.6
34	2e	6.2 ± 1.3	1.8 ± 0.7	3.5
35	2f	6.1 ± 0.4	1.8 ± 0.9	3.4
36	2g	2.0 ± 0.4	1.2 ± 0.3	1.6
37	2h	2.5 ± 0.4	1.9 ± 0.8	1.3
38	2i	6.2 ± 1.4	3.8 ± 1.0	1.6
39	3a	2.3 ± 0.9	1.2 ± 0.1	2
40	3b	4.7 ± 0.7	1.9 ± 0.1	2.4
41	3c	5.4 ± 1.4	1.8 ± 0.	3
42	3d	2.1 ± 0.7	0.7 ± 0.1	2.6
43	4a	7.7 ± 2.8	2.3 ± 0.9	3.3
44	4b	3.2 ± 1.6	2.1 ± 1.0	1.5
45	5a	16.0 ± 3.5	8.8 ± 1.7	1.7
46	5b	> 20	15.9 ± 1.4	na

[a]IC_{50} values were deduced from MTT-based dose-response curves. Compounds presented in papers I, II, and III. *, the number in parenthesis corresponds to the number in Paper III. The IC_{50} values were deduced from MTT-based dose-response curves. Data represents the mean ± SD of 2-4 experiments with n=6 in each.

The toxicity of DSC3 derivatives with different additions in the three positions R_1, R_2, and R_3 (compounds **6-19**, **30-41**) and R_4 (compound **42**) was evaluated (Table 5) in Paper II. Even though the IC_{50} values are very similar for the compounds with a DSC3 base, addition of CH_3 (**6-8**), CF_3 (**10-12**), or OCH_3 (**13-15**) showed better activity when in position R_1 compared with the other two positions. On the other hand, the addition OH (**16-18**) showed to be more favourable in position R_3 compared to positions R_1 and R_2 (Table 5). Simple additions (compounds **20-24**, Table 2), did not result in compounds with better activity than compounds with the benzyl addition. Compound **25** (Table 5) showed a high selectivity towards MCF-10A cells and makes it a potential candidate for chemical improvements in the future.

In paper IV, the toxicity of compounds **30-46** were investigated (Table 5). Compounds **30-44** showed good toxicity, however, not an increased selectivity towards JIMT-1 cells compared to MCF-10A cells.

In the stereoisomeric compounds **45** and **46** (Table 4), the α-methylene-γ-lactone reaction site is occupied, implying that they cannot participate in a Michael reaction [59]. These compounds have a higher IC_{50} than the others with a free α-methylene-γ-lactone. However, these compounds are still toxic with IC_{50} concentrations not very much higher than SLs with a free α-methylene-γ-lactone. This suggests that the toxicity of SLs is not only dependent on the presence of an α-methylene-γ-lactone group but that there may be other toxic mechanisms [82,83]. Interestingly, the stereoisomers **45** and **46** have different toxicities which may be related to possible targets in the cell. Another very likely option is due to the reversibility of the Michael reaction, resulting in regeneration of damsin.

The compounds **43** and **44** with a ring addition were designed with the goal to increase the contact surface and interaction with the cysteine 38 in p65/NF-κB. These compounds have an IC_{50} that is slightly lower than that of damsin, however, the difference is not significant.

4.2 Treatment with flavonoids and dose-response curves

Anti-inflammatory activity has been reported in the context of fractions isolated from *B. pentlandii* [84]. In a comparison of four different fractions obtained from *B. pentlandii*, the dichloromethane and hexane extracts showed

higher anti-inflammatory activity in cellular systems than the ethanolic and aqueous extracts[84]. The dichloromethane and the hexane extracts were active at a concentration of 50 µg/ml and 12.5 µg/ml, respectively, whereas the ethanolic and aqueous extracts were active at a concentration of 200 µg/ml [84]. In the present work, three compounds isolated from different fractions of *B. pentlandii* (Fig. 12) [73], were evaluated for cytotoxicity in breast-derived cell lines (Table 6).

Table 6. IC_{50} values in µM concentrations for flavonoids isolated from *Baccharis pentlandii*.

	JIMT-1	MCF-7	HCC1937	MCF-10A
8-Methoxycirsilineol	> 100	na	na	na
Xanthomicrol	99.6 ± 24.1	> 100µM	> 100µM	104.1 ± 20.5
Sideritoflavone	1.9 ± 0.3	4.9 ± 1.7	4.60	6.7 ± 0.9

IC_{50} values were deduced from MTT-based dose-response curves. Data represents the mean ± SD of 1-3 experiments with n=6 in each. na, not applicable.

The methoxy flavones 8-methoxycirsilineol, xanthomicrol, and sideritoflavone have similar structures but very different toxicity profiles in our study. 8-Methoxycirsilineol did not show toxicity up to 100 µM concentration while sideritoflavone was toxic in the single digit µM range (Table 6).

Studies with xanthomicrol reported activity in six cell lines, human gastric adenocarcinoma cell lines with an IC_{50} of 13 µM in one of them while the average IC_{50} for the other 5 cell lines was 161 µM [73]. In a screening of 60 cell lines including breast cancer cell lines, sideritoflavone was reported with an IC_{50} of 4 µM [85]. Another toxicity report of 79 flavones in oral epidermoid carcinoma, found an IC_{50} of 3 µM for sideritoflavone [86].

5 NF-κB: A sesquiterpene lactone target

5.1 The NF-κB transcription factor family

A number of studies correlate the cytotoxic activity of SLs to the α-methylene-γ-lactone group, which is responsible for the reaction of the α,β-unsaturated carbonyl of the SLs sulfhydryl groups in amino acids such as cysteine in proteins [61]. These studies support the view that SLs inhibit tumour growth by selective alkylation of biological macromolecules, such as enzymes, transcription factors and structural proteins, which control different aspects of cell physiology [87,88].

The transcription factor NF-κB, discovered by Sen and Baltimore in 1986, is a dimeric unit that binds the enhancer element of the immunoglobulin kappa light-chain of activated B cells [89]. A number of signal transduction cascades can activate the NF-κB pathway resulting in the translocation of the NF-κB proteins from the cytoplasm to the nucleus where they activate the expression of specific cellular genes [90].

The NF-κB signal transduction comprises two molecular pathways: the canonical one and the non-canonical one. Studies of the activities of SLs suggest that the canonical NF-κB pathway is the most probable target [91–93]. In this work the focus was on the canonical pathway. In the canonical pathway (Fig. 13), the predominant NF-κB is the transcriptionally active p65:p50 hetero-dimer. NF-κB dimers are bound to the inhibitory IκBα protein in the cytoplasm and are released from IκBα after phosphorylation of IκBα by Iκ kinases. followed by proteasome-mediated degradation of pIκBα. After the release from pIκBα, NF-κB translocates to the nucleus and binds to DNA. The activation of the pathway occurs in response to a variety of stimuli. such as immune and inflammatory responses, developmental processes, cellular growth and apoptosis [90].

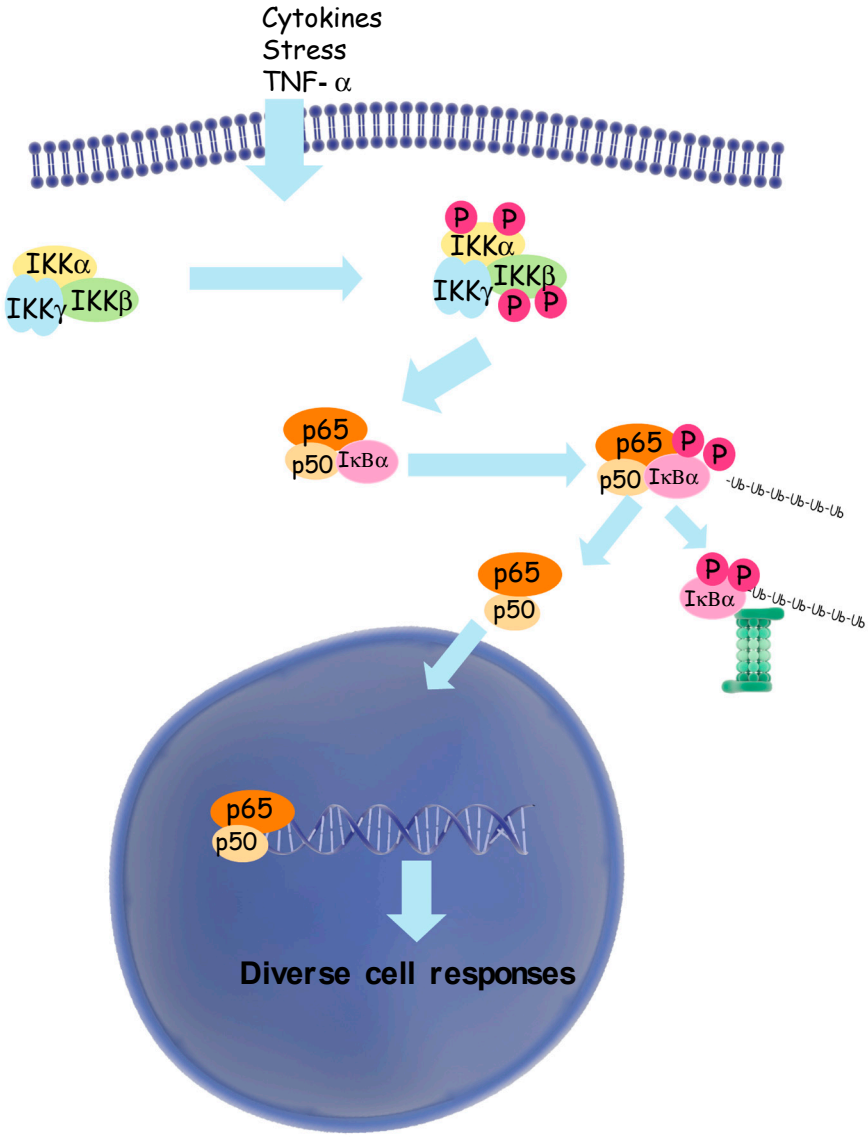


Figure 13. A schematic illustration of the NF-κB canonical pathway (description in the text).

A natural, but controversial activator of the NF-κB pathway is tumor necrosis factor-α (TNF-α). TNF-α is a major pro-inflammatory cytokine that stimulates chronic inflammation which promotes tumour development and progression. Thus, TNF-α is able to act as an endogenous tumour promoter that bridges inflammation and carcinogenesis. TNF-α stimulates proliferation, survival, migration, and angiogenesis of cancer cells. On the other hand, TNF-α also possesses anticancer properties mainly through inducing cancer cell death [94–96].

Activation of the NF-κB through stimulation by TNF-α was used in Paper I and III to demonstrate inhibition of translocation of NF-κB complex (p50:p65) from the cytoplasm to the nucleus [97].

5.2 Sesquiterpene lactones as NF-κB inhibitors

Multiple targets have been proposed for the tumour inhibiting effect of SLs. However, one of the main targets discussed and investigated is NF-κB. The SLs are assumed to bind to p65/NF-κB and inhibit its DNA binding function in the NF-κB transcription factor complex. Mechanistic studies demonstrated that the SL helenalin covalently blocked the SH group of cysteine 38 at the active site of p65/NF-κB by a reaction involving the highly reactive electrophilic α-methylene-γ-lactone ring [98–100]. As a result, the DNA binding capacity of NF-κB was blocked [101]. One means that has been used to investigate if a compound inhibits NF-κB dependent transcription is to treat cells with TNF-α and investigate if the compound inhibits the translocation of p65/NF-κB to the nucleus [97].

In Papers I and III we show evidence that treatment with a number of SLs inhibits the TNF-α-induced translocation of p65/NF-κB to the nucleus (Fig. 14). Here we have used an immunofluorescence-based assay to locate p65/NF-κB using a specific primary antibody and a secondary Alexa 488-conjugated antibody. The cells were treated with an SL at IC_{50} for 60 minutes followed by TNF-α treatment. In control, p65/NF-κB is predominantly found in the cytoplasm while after TNF-α treatment, p65/NF-κB is found in the nucleus. Pre-treatment for only 60 minutes with an SL inhibited the translocation. As far as we can judge using this method, there was no difference in the ability of the SLs **1**-**12** when used at the IC_{50} concentration in preventing the TNF-α-induced translocation of p65/NF-κB to the nucleus.

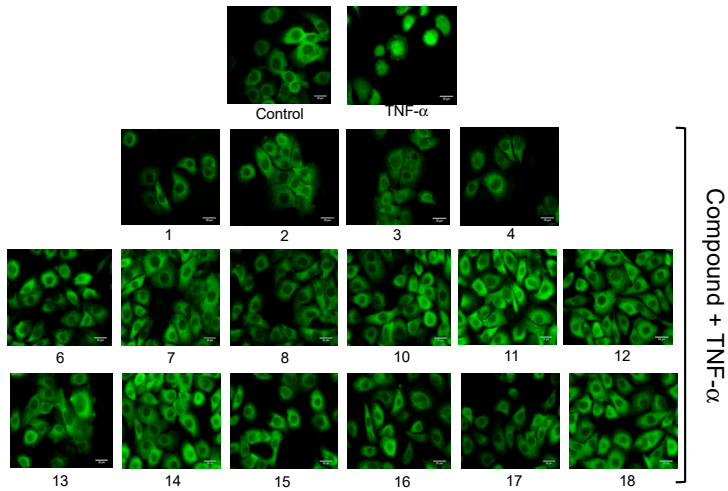


Figure 14. Sesquiterpene lactones inhibit TNF-α induced p65/NF-κB nuclear translocation. JIMT-1 cells were treated with 5 μM damsin (1), ambrosin (2), coronopilin (3), or dindol-01 (4) or, damsin derivatives (6-8,10-18) at the respective IC_{50} concentration. The cells were treated with compound vehicle (control and TNF-α) or compound for 1 hour followed by TNF-α vehicle (control) or TNF-α for 40 min before being fixed with 4% formaldehyde. p65/NF-κB was visualized by immunofluorescence microscopy after labelling with primary anti-bodies against p65/NF-κB followed by secondary Alexa 488-conjugated anti-bodies.

Our studies support the suggested anti-tumor mechanism of SLs through the suppression of the NF-κB signaling pathway. Interestingly, the SLs block the translocation of p65/NF-κB to the cell nucleus (Fig. 14), with the obvious result that there can be no DNA binding and gene activation. As mentioned above, the cysteine 38 that is alkylated in p65 is found in the DNA binding region of NF-κB. This region is also where the binding site for importin α 3 and importin α 4 which are responsible for the transport of NF-κB into the nucleus [58,102,103]. Cysteine 38 is also part of the binding region for the NF-κB co-activator coactivator ribosomal protein S3 [104]. Thus, binding of an SL to cysteine 38 of p65/NF-κB may block several molecular mechanisms involved in NF-κB signaling.

6 Cell proliferation and the cell cycle

As mentioned above, the MTT assay is an indirect means to investigate effects on the cell number after treatment with compounds. A direct means is to count the cells and construct a growth curve showing direct effects on cell proliferation. Cell proliferation is the result of several signal transduction pathways working synergistically to promote cell progression through the cell cycle. The cell cycle is divided in four phases G_1, S, G_2, and M (Fig. 15). The cell cycle is regulated by complexes that are composed of cyclins bound to cyclin-dependent protein kinases (CDKs). When activated, the CDKs phosphorylate different proteins to promote the successive progression through the cell cycle. The simple view of cell cycle regulation is that different CDKs are active during different parts of the cell cycle although they are constitutively expressed. What regulates their cell cycle phase specific activation is the cyclic expression of their respective activators, *i.e.* the cyclins. Cyclin-CDK complexes are controlled via checkpoint pathways whose role is to prevent the cell from progressing to the next stage if the conditions are not favourable [105–107].

Analysis of transformed cells and cells undergoing mitogen-stimulated growth implicate proteins of the NF-κB family in cell cycle regulation, through actions on the CDKs and CDK inhibitors system [108,109]. The best explored link between NF-κB activation and cell cycle progression involves cyclin D1, a cyclin which is expressed relatively early in the cell cycle and which is crucial to commitment to DNA replication [107–109].

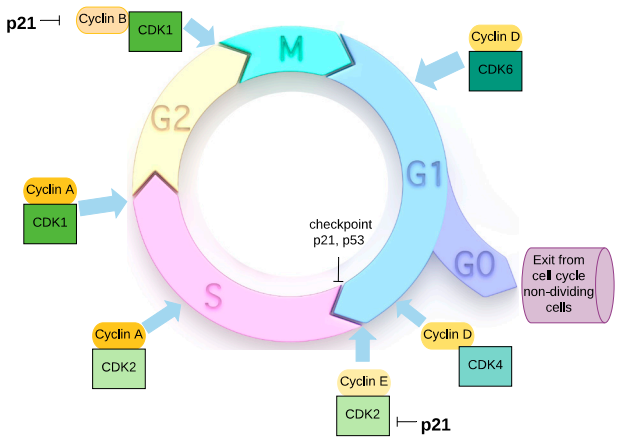


Figure 15. Schematic presentation of the eukaryotic cell cycle and its regulation. The eukaryotic cell cycle consists of two gap phases, the G_1 and the G_2 phases, the S phase, and the M (mitosis) phase. Cells can also enter a quiescent state, the G_0 phase. CDK , cyclin dependent kinase. The blue arrows point to parts of the cell cycle where the cyclin-CDK complexes are active.

The cell cycle phase distribution is determined by investigating the DNA distribution of a cell population after staining with propidium iodide (PI). PI intercalates stoichiometrically in double-stranded DNA. Using flow cytometry the PI bound to individual cells is excited at 488 nm resulting in 620 nm red fluorescence signal proportional to the DNA content [110]. In papers I and V, the cell cycle phase distribution was analysed using this method.

6.1 Sesquiterpene lactones inhibit cell proliferation and change the cell cycle phase distribution

The effect on cell proliferation of treating MCF-10A, MCF-7, HCC1937, and JIMT-1 cells with 1-5 µM damsin or ambrosin for 72 hours was investigated in Paper 1 (Figs. 16A and 16B, damsin and ambrosin, respectively, in JIMT-1 cells). Ambrosin was more efficient than damsin in inhibiting cell proliferation and negative effects on the cell number compared to control was observed for both compounds in a dose dependent manner already after 24 hours of treatment. To the best of our knowledge, we have not found any other studies investigating the effects on cell proliferation of ambrosin and damsin treatment by constructing growth curves after cell counting. It deserves mentioning that

the MTT assay only indicates if there is a change in cell number compared to control at the time of analysis but it says nothing about the nature of the change. Thus, to get an understanding of the kinetics of effects of treating with a compound, it necessary to perform growth curve experiments.

The effect on cell proliferation of treating JIMT-1 cells with different concentrations of the damsin derivative (E)-3-(2-hydroxybenzyliden)damsin (**18**) (Fig. 17C) was investigated in Paper III. It is apparent that the damsin derivative **18** results in similar growth inhibition as ambrosin (Fig. 17B).

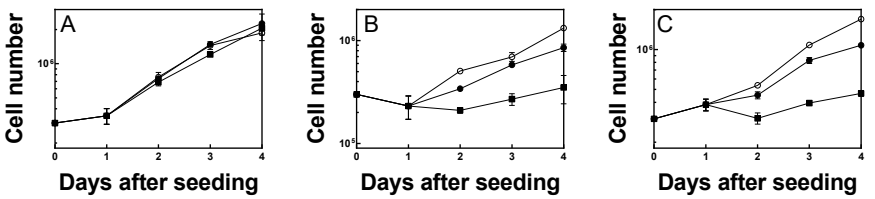


Figure 16. Sesquiterpene lactones inhibit cell proliferation of JIMT-1 breast cancer cells in a time and dose-response manner. Cells were seeded day 0 and the compounds were added 1 day after seeding. The cell number was determined by counting in a haemocytometer after cell detachment. A) Damsin. B) Ambrosin. C) (E)-3-(2-Hydroxybenzyliden)damsin (**18**). o: control. ●: 1 μM. ■: 2.5 μM.

Figures 17B and 17C show that treatment with 2.5 μM ambrosin or compound **18**, initially totally blocks JIMT-1 cell proliferation (first 24 hours of treatment *i.e.* between 1 and 2 days after seeding) compared to control. Between 48 and 72 hours of treatment, the cell number then increased again in these cells, implying a partial recovery from the initial inhibition. Presently we have no answer to this observation but it may be related to a rescue mechanism involving glutathione which will be discussed below.

In Paper III we show that even at a concentration of 0.5 μM, compound **18** has an inhibitory effect on cell proliferation during the first 24 hours of treatment. Although we do not have evidence, we believe this is because of rapid inhibition of NF-κB signalling. JIMT-1 is a HER2 over-expressing trastuzumab resistant ER negative breast cancer, and it has been suggested that NF-κB is constitutively activated in this context contributing to the resistance [111]. Thus, treatment with SLs may inhibit this constitutive signalling and this needs further investigation to exploit its utility in a clinical setting.

The cell cycle phase distribution of MCF-10A, MCF-7, HCC1937, and JIMT-1 cells was investigated after 72 hours of treatment with damsin and ambrosin.

The cell cycle phase distribution was more affected in the JIMT-1 cell line compared to the other cell lines, especially by ambrosin treatment where there was an increase in the G_2 phase (Paper I). Our data are in line with others showing a G2 accumulation of MCF-7 cells treated with pseudoguaianolides [112] and another report showing that the SL helenalin induced G_2/M cell cycle arrest [113]. Lohberger *et al.* [114] reported that treatment of three cancer cell lines with the SL dehydrocostus lactone resulted in S and G_2/M accumulation of the cells. Thus, a number of studies show a cell cycle effect of SL treatment correlating with an increase in S and G_2/M phases.

Figure 16 (and Paper III) show both time and dose dependent effects of treatment with SLs. A study with helenalin and synthesized analogues also shows cytotoxicity dependent on treatment concentration and treatment time [115].

As mentioned above, NF-κB has been shown to participate in the regulation of cyclin D1 [109]. However, we did not find a consistent effect on cyclin D1 after 72 hours of treatment with damsin or ambrosin (Paper I). We did find the same results after treating JIMT-1 cells with damsin derivatives *i.e.* no consistent change in the cyclin D1 level compared to control after 72 hours of treatment (Paper 3, not shown).

We also investigated the level of CDK2 which is important for G_1/S transition and during S phase progression and found that CDK2 was decreased by damsin and ambrosin treatment of JIMT-1 cells (Paper I). Similar effects were found on the CDK2 level after treatment with damsin derivatives (Paper II, not shown). NF-κB has also been shown to have a role in the regulation of CDK2 through coactivators providing a mechanism for the coordination of transcriptional activation with cell cycle progression [116].

In conclusion, our data point to a very early effect after treatment initiation that may be correlated to various reported effects of the involvement of NF-κB in cell cycle regulation. This notion must of course be investigated with closer studies on early effects on cell cycle progression after addition of SLs.

6.2 Flavonoids inhibit cell proliferation and affect cell cycle distribution

Analysing cytotoxicity of sideritoflavone with the MTT assay resulted in an IC_{50} of 1.9 ± 0.3 µM (Paper IV). In growth curve experiments, JIMT-1 cells were treated with 2 and 2.5 µM sideritoflavone, two concentrations that were rather close to each other but justified by the steepness of the dose response curve.

Figure 17A shows that the JIMT-1 cells do not react to addition of sideritoflavone at IC_{50} during the first 24 hours of treatment, and sideritoflavone gradually inhibits cell proliferation between 24 and 72 hours of treatment. Different times of inhibition compared to SLs discussed above, points to Sideritoflavone and SLs inhibiting cell proliferation by different mechanisms.

Analysis of the cell cycle phase distribution after 72 hours of treatment showed an increase in the G_2 phase fraction of sideritoflavone-treated cells compared to control (Fig. 17B). An increase in cyclin B1 level was expected since it increases during S phase and peaks at the G_2/M boundary [117].

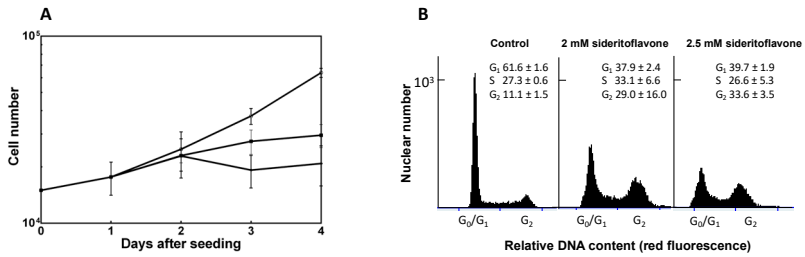


Figure 17. Sideritoflavone inhibits cell proliferation and affects the cell cycle phase distribution of JIMT-1 breast cancer cells. Cells were seeded day 0, and the compound was added 1 day after seeding to reach the final the concentrations of 2 µM (■) or 2.5 µM (▲). Control (o) was treated with 0.1% DMSO which was the same DMSO concentration as in treated cultures. A) The cell number was determined using a Holomonitor® M4. Data are presented as the mean of 6 independent samples from 3 independent experiments and bars indicate SD. B) At 72 hours of treatment, cells were sampled for analysis of cell cycle phase distribution using flow cytometry of cells labelled with propidium iodide. Numbers show the cell cycle phase distribution in % for n = 3 ± SD.

Different times of inhibition compared to SLs discussed above, points to sideritoflavone and SLs inhibiting cell proliferation by different mechanisms.

6.3 Sesquiterpene lactones and DNA damage

The p53 protein has been coined "the guardian of the genome" [118]. It is a transcription factor that has a tumor suppressor function as it prevents proliferation of cells with damaged genome [119]. This gene is a critical regulator of many cellular processes including cell signal transduction, cellular response to DNA-damage, genomic stability, cell cycle control, and apoptosis [120]. When the DNA is damaged, p53 is activated resulting in the expression of p21 which halts cell cycle progression (Fig. 15). When mutated, p53 loses its ability to bind to DNA, and as a consequence the transcription of p21 does not take place and cell cycle progression continues [26,121]. The occurrence of mutations in p53 is an early event in cancer development preventing cell cycle arrest and promoting the complex network of molecular events leading to tumor formation [26,120–122].

The NF-κB signaling pathway promotes cancer development through suppression of apoptosis [123,124]. When p53 is mutated, like in JIMT-1 cells [26], a constitutive activation of the transcription factor NF-κB is possible [125]. Hence, the search for compounds that target both NF-κB and p53 simultaneously is of special interest because of the promising therapeutic value [121].

In paper I, we show that treatment of MCF-7 and JIMT-1 cells with damsin and ambrosin results in an increased expression of p53. However, the p21 protein expression only increased in MCF-7 cells with wild-type p53. As the JIMT-1 cell line has a mutation in p53, the absence of p21 is explained by the lack of transcriptional activity of p53 (Paper I). Several α-methylene-γ-lactones have been shown to have the ability to inhibit NF-κB and activate the pro-apoptotic function of p53, therefore, these compounds have been suggested to be novel anticancer agents [121].

In Paper I, we found that treatment with damsin and ambrosin increased the number of micronuclei. Micronuclei are small, extra nuclear DNA containing bodies that are formed during mitosis. They are usually broken off from chromosomes during anaphase due to DNA double strand breaks. When cells are exposed to compounds that damage DNA, the number of micronuclei increases and the micronucleus test is recognized as one of the most reliable

for genotoxic carcinogens [126]. The presence of micronuclei in tumours has been used as a tool for cancer risk prediction, screening, and diagnosis and also for monitoring the response to therapy [127].

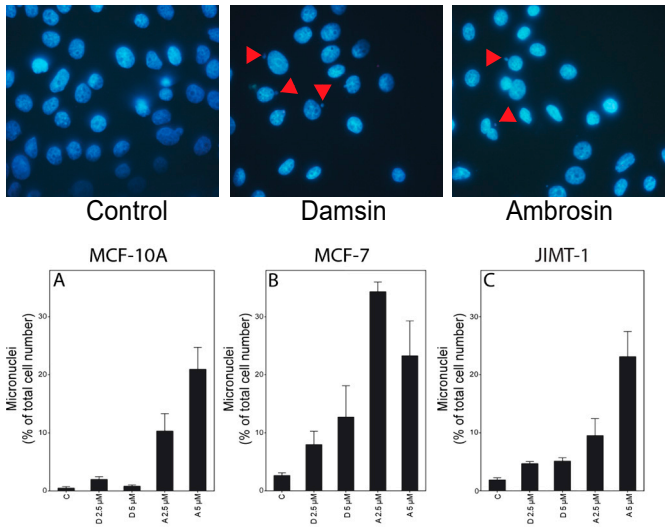


Figure 18. Treatment of (A) MCF-10A, (B) MCF-7, and (C) JIMT-1 cells with damsin (D) or ambrosin (A) increases the number of micronuclei. The cells were treated with 2.5 (D 2.5 and A 2.5) or 5 (D 5 and A 5) µM concentrations for 72 hours. The cells were then fixed and the nuclei stained with bis-benzimid. Micronuclei were counted in fluorescence microscopy images. Data are presented as mean ± SE for n = 6. On top are shown representative immunofluorescence microscopy images of JIMT-1 cells treated with 5 µM damsin or ambrosin. The cell nuclei are stained with the DNA stain bisbenzimide.

Another biomarker for DNA damage, is the histone γ-H2AX. The phosphorylation of γ-H2AX, is the first step in recruiting and localizing DNA repair proteins. DNA double strand breaks can be induced by mechanisms such as ionizing radiation or cytotoxic agents and subsequently, γ-H2AX foci quickly form [128]. The detection of γ-H2AX allows the assessment of genomic DNA damage and repair caused by cytotoxic agents, especially in the context of cancer treatment and therapy [129].

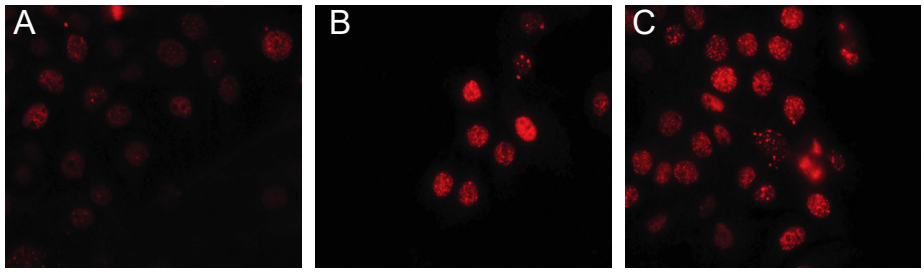


Figure 19. Treatment of JIMT-1 cells with damsin and ambrosin increases γ-H2AX labelling. The red labelling shows the presence of γ-H2AX foci in cells treated with 5 µM damsin or ambrosin at a 5 µM concentration for 72 hours. A) Control. B) Damsin. C) Ambrosin. The images were obtained using immunofluorescence microscopy.

Treatment with the SLs damsin and ambrosin resulted in an increase in the expression of γ-H2AX (Fig. 20). Thus, the evidence for DNA damage induced by damsin and ambrosin treatment is not only confirmed by changes in the levels of p53, p21, and γ-H2AX but also by the micronucleus test (Fig. 18) and γ-H2AX labelling (Figs. 19 and 20).

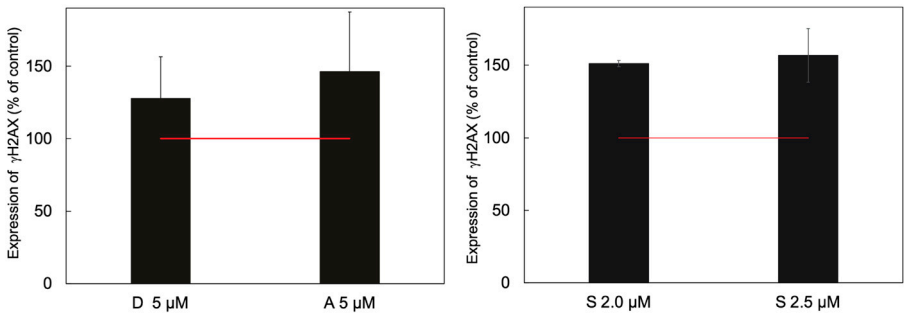


Figure 20. Increased expression of γ-H2AX in JIMT-1 cells treated with SLs or a flavone. A) Sesquiterpene lactones. (D) Damsin (A) ambrosin. B) (S) Sideritoflavone. The expression of the protein was determined by Western blot after 72 hours of treatment with the concentrations shown in the figures. The data were analyzed by densitometric scanning and expressed in % of control. n = 3 independent experiments ± SEM.

In Paper V, we found that sideritoflavone treatment increased the level of γ-H2AX. The increased level of γ-H2AX is corroborated with the accumulation of cells in the G_2 phase, which at the same time explains the increase in the expression of cyclin B1.

6.4 Sesquiterpene lactones and glutathione

GSH, a tripeptide consisting of the amino acids L-glutamyl-L-cysteinyl-glycine. It is one of the most prevalent thiol containing low molecular weight compound in cells found in high concentrations (0.5-10 mM) [92,130]. It protects cells by detoxifying electrophilic and reactive oxygen and nitrogen species. GSH can react spontaneously with various substrates but is also conjugated to xenobiotics in a reaction catalysed by glutathione S-transferase [131].

GSH reacts spontaneously with SLs at the α,β-unsaturated carbonyl by a Michael type addition [132]. In addition, glutathione S-transferase can conjugate GSH to other parts of the SL than the Michael acceptor. It has been shown that the reaction of GSH with SLs is reversible [133]. Thus, high concentrations of GSH could neutralize and thus counteract the efficiency of SLs binding to a target that causes toxicity.

We investigated if there were differences in the level of GSH in JIMT-1 cells compared to MCF-10A cells, which would partly explain the higher sensitivity of the former cells to SL treatment. Using a commercial GSH assay, we found that the concentration of the tripeptide actually differed 10-fold between the two cell lines. The intracellular GSH concentration is 0.04 mM and 0.587 mM in JIMT-1 and MCF-10A cells, respectively (Paper II). In addition, glutathione S-transferase is expressed at a low level in JIMT-1 cells [27].

We have investigated the effect of four damsin derivatives on the GSH level in JIMT-1 cells at 12 and 24 hours after addition of the SL (Fig. 21). There was no effect on the GSH level . Thus, SL treatment does not lower the GSH level compared to that in control in JIMT-1 cells. Preliminary data implicate that the GSH level increases in MCF-10A cells at 24 hours of treatment with SLs which may also explain the lower sensitivity of these cells to SL treatment compared to JIMT-1 cells. In response to SL treatment, MCF-10A cells may synthetize more GSH a notion needs testing. Depletion of intracellular GSH can be detrimental to cells as it causes different kinds of oxidative damage [130,132]. However, it appears we can exclude such damage in SL-treated cells in this study.

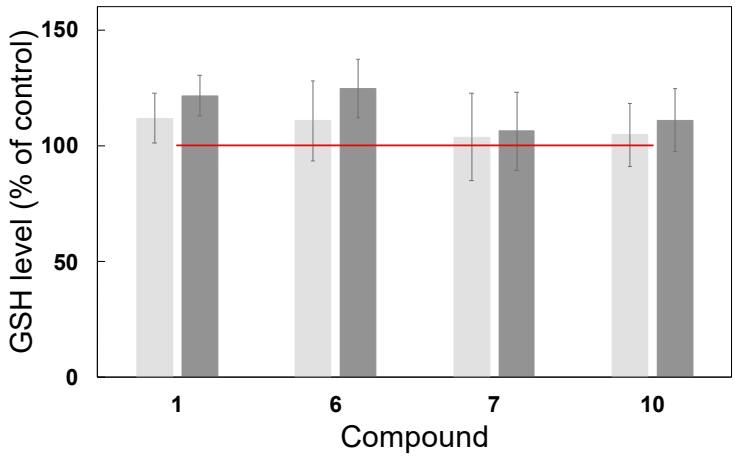


Figure 21. Treatment with compounds **6, 18, 10,** and **13** at IC50 for 12 (light grey columns) or 24 (grey columns) hours does not affect the cellular GSH level. n = 12 – 23 from 3 independent experiments. Bars, ± SD. The GSH level was determined using a commercial assay.

Our data of the difference in sensitivity of the JIMT-1 and MCF-10A cell lines to the SLs is interesting (Fig. 21) in the context of reports showing increased sensitivity of triple negative (basal) breast cancer to anti-GSH therapy [28,134,135]. JIMT-1 is not a triple negative cancer as it expresses HER-2, however, it is not sensitive to targeted therapy because of several co-existing drug resistance mechanisms that make them insensitive to HER2-targeted drugs [136]. Thus, the JIMT-1 cells have a similar behaviour as triple negative cancers and may be called a basal-like cell line [28]. Triple negative breast cancer cell lines have been shown to have up to 10 times lower GSH than normal-like cell lines (including MCF-10A). As mentioned above, the JIMT-1 cell line has 10 times lower GSH level than the MCF-10A cell line. Thus, altogether these data suggest that SLs may be successful in the treatment of triple negative and HER2 resistant breast cancer.

7 Cell movement

Movement is an important aspect of the life of a cell. Cell movement is a fundamental cellular process that occurs throughout life, starting during embryonic development and continuing until death. Increased and uncontrolled cell movement is part of many diseases such as cancer. Thus, in cancer treatment and in the development of cancer treatment strategies, it is important to integrate effects on cell movement with other effects of a compound.

7.1 Effect of treatment with sesquiterpene lactones on cell movement

Metastasis implies that cancer cells spread from the primary site of origin to a distant organ. The metastasis of cancer cells causes the majority of breast cancer-related deaths. The pre-requisite for breast cancer spread and metastasis is the ability of the cells to invade and migrate into surrounding tissues [137].

There are different methods to investigate cell movement using cell lines. One such method is the wound healing assay, which is based on the creation of an artificial gap, a scratch, in a confluent cell monolayer. The cells on the edge of the newly created gap will migrate directionally into the opening to close the scratch until new cell-cell contacts are established again [138]. Cell migration is usually correlated with metastatic progression [139].

We have shown that SLs have the ability to decrease cancer cell proliferation by affecting *e.g.* the NF-κB and STAT3 pathways (Papers I and III). These pathways are also involved in cell migration [140]. Cooperation between NF-κB and STAT3 has also been reported to be important for cell migration of glioblastoma and ovarian carcinoma cells [141,142]. SLs have been shown to inhibit cell migration in a number of studies [143,144]. Thus, next we wanted to

investigate if treatment with SLs inhibited directed cell migration in a wound healing assay.

In paper I, significant differences in cell migration were observed when comparing JIMT-1 cells treated with damsin or ambrosin with control. Cell migration decreased for both 1 and 5 μM treatments ($p < 0.05$) with damsin and ambrosin, although the difference compared to control is higher for the 5 μM concentration.

For the damsin derivatives used at the IC_{50} concentration there is only a slight inhibition of migration compared to control (Paper III). This finding suggests that the damsin derivatives are not as efficient as damsin in inhibiting JIMT-1 breast cancer cell migration at IC_{50} concentrations. Compound **18** in Paper III (Fig. 16), reduced cell proliferation already after 24 hours of treatment with an IC_{50} concentration but apparently did not inhibit cell migration as efficiently. We did, however, find a decrease in the expression of the intermediate filament protein vimentin compared to control. Vimentin is important for cell migration by integrating mechanical input from the environment and modulating the dynamics of microtubules and the actomyosin network[145]. SLs have been shown to have a negative effect on tubulin which may affect both cell migration and cause the G_2 arrest found after treatment (Paper I) [146,147]. It has also been found that the SLs parthenolide and costunolide reduce microtentacles and thus cell attachment by selectively targeting detyrosinated tubulin independent from NF-κB inhibition [82,83]. It is possible that there is a difference in damsin and damsin derivatives in affecting this molecular mechanism, explaining differences in effects on cell migration. However, this notion needs further investigation.

7.2 Effect of treatment with sideritoflavone on cell movement

When investigating cell movement in JIMT-1 cells treated with sideritoflavone, we used two methods. The wound healing assay which as stated above detects directed cell migration, and a method using a phase holographic microscope which evaluates motility. Motility is defined as the total accumulated distance a cell has moved over the time of tracking. Motility is not *per se* a measure of how far cells are migrating from a starting point but is a measure of how the cells are moving around. Thus, a cell can have low

migratory ability but may be moving around vigorously in a small spot resulting in high motility.

Using the wound healing assay, we found that sideritoflavone treatment inhibited directed cell migration compared to control (Paper V). To the best of our knowledge, our data are the first reporting effects of sideritoflavone on cell migration, however, flavone treatment *per se* has previously been shown to inhibit cell migration and metastasis [148].

Interestingly, although sideritoflavone treatment reduced cell migration, the local motility of the cells was increased as evaluated using time-lapse phase holography for evaluation of cell movement. When treating JIMT-1 cells with the CSC inhibiting compound salinomycin we also found that motility was increased despite the fact that cell migration was decreased [44,149]. Increased cell migration and motility without a clear distinction of the terms have been associated with activation of the transforming growth factor-β (TGF-β) signaling pathway and with p65/NF-κB [150 151]. We found that sideritoflavone treatment increased the TGF-β signaling pathway activity and increased the level of p65/NF-κB.

Discussions with other researchers have revealed the observation that stressed cells seem to have increased motility but yet there are no published data on this finding. Obviously, more studies are needed to understand the connection between motility and migration and the molecular mechanisms involved.

8 Cancer stem cells

CSCs are thought to be at the top of a hierarchical cancer cell organization and killing these cells may result in a higher probability of cancer cure [32]. To investigate CSC specific effects by treating with SLs and sideritoflavone, we used three assays (Fig. 5) that reflect changes in different sub-populations. Some of the proteins and pathways we have investigated also have a role in CSC maintenance. The role of NF-κB [152] and STAT3 [153] pathways have been the subject of review articles stating that inhibition of these pathways reduces the CSC population.

8.1 Effect of treatment with sesquiterpene lactones on cancer stem cells

In Paper I, we show that treatment with damsin or ambrosin reduced the CSC population of the JIMT-1 cell line. However, treatment with 1 or 5 μM damsin or ambrosin do not give exactly the same result in the three CSC assays. Regarding the analysis of the $CD44^+/CD24^-$ population with flow cytometry, we found that only treatment with 5 μM ambrosin reduced the CSC population while treatment with damsin or 1 μM ambrosin had no effect. The flow cytometry-based method for analyzing the $ALDH^+$ population gave at hand that the CSC population was reduced by treatment with either damsin or ambrosin at 5 μM concentrations. When using the CFE assay, the CSC reducing effect was significant for 5 μM damsin and 1 and 5 μM ambrosin. Thus, the colony forming efficiency assay appears to be the most sensitive assay. However, it is also important to point out that to the best of our knowledge there are no studies that truly compare the outcome of these assays and their exact correlation with a CSC population.

In Paper III, we showed that treatment with the damsin derivatives also decreased the CSC population of JIMT-1 cells, with the CFE assay showing the highest sensitivity.

As mentioned above, the NF-κB and STAT3 pathways are important for CSCs. Our work shows that treatment with the SLs used here inhibited the translocation of NF-κB to the cell nucleus, implying that SLs bind to p38 of p65/NF-κB as shown by others. It has previously been shown that inhibition of NF-κB signaling with a synthetic inhibitor reduces the stemness characteristics in lung cancer cell lines [154] which is in line with our results. In another study it was shown that inhibition of NF-κB signaling reduced the proliferative capacity of bladder CSCs [155].

In Paper III we showed a decrease in the protein level of hTERT. STAT3 has been shown to be involved in the transcriptional regulation of hTERT [156] [157]. hTERT expression is linked to many cancer malignancies and it has been suggested as a strategic target for cancer therapy [158]. It has also been suggested that high expression of hTERT is correlated with lung and ovarian CSCs [159]. Inhibition of hTERT by a synthetic inhibitor was shown to decrease the CSC population of breast and pancreatic cell lines [160]. From our data we cannot draw the conclusion that it is a direct effect of SLs on STAT3 that reduced the level of hTERT. In fact, the promoter for hTERT also contains promoters for NF-κB and c-Myc [157]. In Paper III, we show that the Myc/Max signaling is reduced by treatment with damsin derivative **18**.

In conclusion, our data showed that SL treatment decreases the CSC subpopulation and it is widely discussed that eradication of this sub-population may result in cancer cure. Thus, pseudoguanolide damsine derivatives should be further assessed for their CSC inhibiting potential and if they can be utilized in a clinical setting. Targeting NF-κB with the germacronolide SL parthenolide has in fact been suggested as a therapeutic approach to inhibit CSCs [161].

8.2 Effect of treatment with sideritoflavone on cancer stem cells

In contrast to treatment with SLs, sideritoflavone treatment did not specifically reduce the CSC population of JIMT-1 cells (Paper V). Sideritoflavone treatment instead reduced the CSC and non-CSC populations to the same extent. This is however much more favourable than treatment with the conventional chemotherapeutic drug paclitaxel which reduces the non-CSC subpopulation more efficiently than the CSC population, leaving a higher proportion of CSCs after treatment [162]. Thus, a notion to be tested is if

combined treatment with pseudoguanolide damsine derivatives and sideritoflavone together can be more efficient in inhibiting both CSCs and non-CSCs to achieve an efficient eradication of all cancer cells.

9 Conclusions in relation to aims

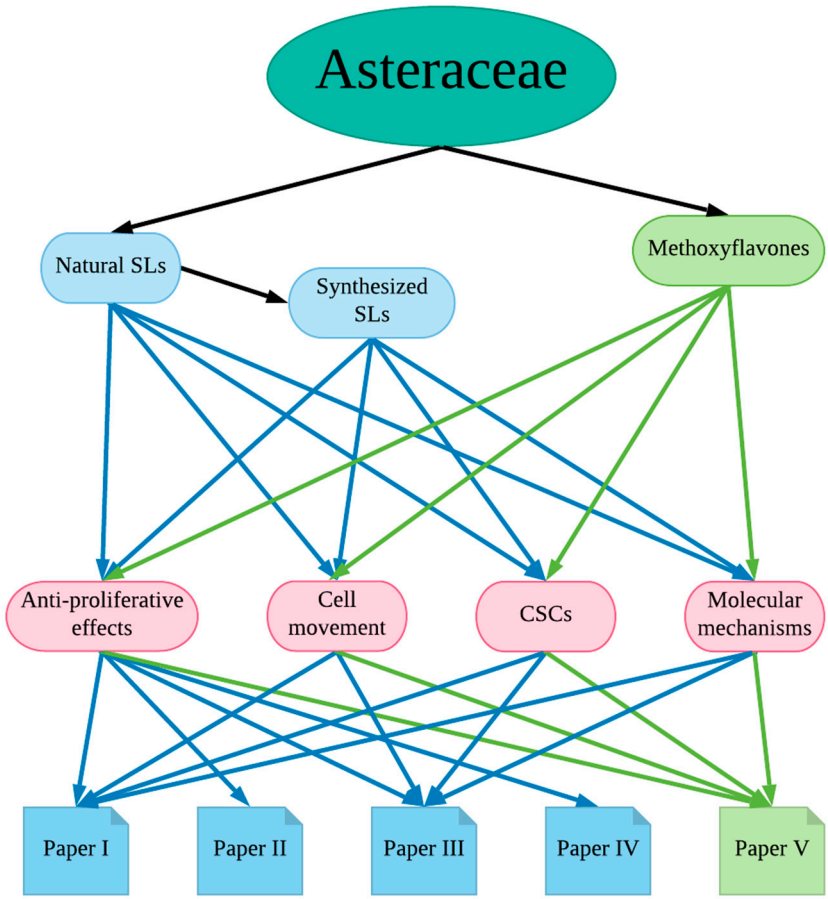


Figure 22. Schematic presentation of the compounds tested and how they were investigated to achieve the aims of the thesis. The pink ovals define cellular traits investigated. SLs, sesquiterpene lactones. CSCs, cancer stem cells. The SLs have been investigated in Papers I-IV, marked blue and methoxyflavones in Paper V, marked green.

Figure 22 shows a schematic presentation of how the different aims were met in Papers I-V. Below is a compilation of data to verify that the aims have been fulfilled in this thesis work.

- To determine the effect of treatment with natural and synthetized SLs on basal cellular traits like cell proliferation and cell movement

The anti-proliferative effects were initially investigated in MTT-based dose-response assay using IC_{50} values as read-out for toxicity. MTT is an end point assay that indirectly determines effects on the cell number. We investigated the outcome of treating three breast cancer cell lines and one normal-like breast epithelial cell line with 46 sesquiterpene lactones (SLs). As the SL damsin showed the most promising effects, it was used as the starting material for the synthesis of derivatives. A large proportion of the synthesized derivatives were more toxic than damsin. More importantly, many of the synthesized derivatives were shown to have increased selectivity against cancer cells compared to the normal cells.

We also investigated anti-proliferative effects by performing growth curve assays with a breast cancer cell line, where effects on cell proliferation are followed during treatment to get information about time and dose effects. Addition of an SL to the medium at IC_{50} concentrations resulted in reduction of cell proliferation within 24 hours of treatment.

Cell movement is a complex process that can be described in different ways. Here we have investigated effects on directed cell migration in a wound healing assay. In this assay the cells move into a cell free area. The SLs inhibit this cell migration at IC_{50} concentrations, but, interestingly, the natural SLs are more effective than the synthetic damsin derivatives. However, only a few damsin derivatives were tested and thus we cannot state that this is a general phenomenon for the synthesized damsin analogues.

- To determine the effect of treatment with natural and synthetized SLs on the CSC population

CSCs have been suggested to be the culprits of cancer metastases and cancer death due to their ability to survive chemical insult and to move to different places in the body to initiate a new tumors. Thus, compounds which target CSCs are of interest in cancer research. Here we found that treatment with both natural and synthetic SLs reduced the CSC population of a breast cancer cell line. This implies that when treatment with an SL reduces the total cancer cell

number the decrease in the percentage of CSCs of that population is greater than the decrease in the non-CSC population.

- To determine the effect of treatment with flavonoids on basal cellular traits like cell proliferation and cell movement

Using MTT, only one of the methoxyflavones was found to have an IC_{50} at a single digit μM concentration. Treatment with the IC_{50} concentration of a methoxyflavone required more than 24 hours to exert its anti-proliferative effect.

The methoxyflavone inhibited cell migration in cells treated with an IC_{50} concentration. We also investigated motility, which is another aspect of cell movement. Motility is defined as the total accumulated distance a cell has moved over the time of tracking. A cell can have low migratory ability but may be moving around vigorously in a small spot resulting in high motility. We found that treatment with a methoxyflavone increased cell motility.

- To determine the effect of treatment with flavonoids on the CSC population

Treatment with a methoxyflavone did not specifically reduce the CSC population implying an equal sensitivity to treatment of the CSC and non-CSC populations.

- To start unraveling the molecular mechanisms of toxicity of natural and synthesized SLs and flavonoids

The SLs are molecules with a reactive group called α-methylene-γ-lactone. This group is very reactive in a so-called Michael addition which occurs with sulfhydryl groups, especially sulfhydryl groups of the amino acid cysteine. The cysteine 38 in the p65 unit in the transcription factor NF-κB has been shown to be prone to alkylation of SLs. This prevents NF-κB to bind to DNA and initiate transcription. All SLs we have tested appear to inhibited NF-κB. This inhibition is very rapid, evident already 1 hour after addition of the SL to the cell culture medium. We did find many molecular effects by treatment with SLs but the binding to NF-κB can be the molecular initiating event leading to downstream effects. The higher sensitivity of breast cancer cells compared to normal cells to treatment with SLs may be a consequence of the former having a lower concentration of the tripeptide GSH which can neutralize SLs. Methoxyflavone treatment for 24 hours resulted in activation of the Myc/Max signal transduction pathway. Activation of this pathway is related to the DNA

damage response. In fact, we found that methoxyflavone treatment increased the expression of a marker for DNA double strand breaks.

10 Future perspectives

Breast cancer is the leading cause of cancer-associated mortality in females. It is also associated with the most common type of malignancy found in females worldwide. Several new treatment approaches give patients with breast cancer the chance to survive longer than before. However, even better treatments are sought for to further increase the survival rate. The results from the current studies on the effect of SL and flavonoid treatment on cancer cell lines indicate that better treatments can be found in the traditional Bolivian herbal medicine. This study has been conducted with very few cell lines and the results need to be confirmed using many more cell lines. Breast cancer is a heterogeneous disease and no general conclusions can be drawn by this thesis work.

The notion that the GSH level is important for the degree of SL cytotoxicity must be further studied. The reported data, that a number of basal breast cancer cell lines have much lower GSH levels than normal cell lines, leads to the thought that the effect of SL treatment must be compared to the GSH levels in many normal and cancer cell lines. Here we found that SLs inhibit the translocation of p65/NF-κB to the cell nucleus. Based on the results of others, we assume that this is due to the binding of the SLs to cysteine 38 of p65/NF-κB. It has been shown that this blocks the NF-κB binding to DNA. However, our data implicate that it also blocks the binding of the nuclear transport proteins of NF-κB, the importins, something that needs further investigations. It would also be interesting to investigate whether the SLs block the binding of NF-κB to STAT3. This would explain the SL-induced decrease in hTERT, *i.e.* the SLs may not have a direct effect on STAT3 but via NF-κB.

The CSC reducing effect of SLs should be further exploited. SLs should be combined with other compounds and sideritoflavone may be a favourable choice. Sideritoflavone treatment did not specifically reduce the CSC population. However, neither did sideritoflavone treatment increase the CSC population as has been found after treatment with chemotherapeutic drugs used in the clinic. Thus, the anti-proliferative effect of sideritoflavone is similar for the CSC and non-CSC populations. This may be exploited by combining *e.g.*

sideritoflavone and SL treatment with the goal to achieve an eradication of the total cancer cell population.

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