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Studies of Cytotoxic Compounds of Natural Origin and their Mechanisms of Action

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2011

ISSN 1651-6192
ISBN 978-91-554-8023-3
urn:nbn:se:uu:diva-148114

Dissertation presented at Uppsala University to be publicly examined in B21, BMC, Husargatan 3, Uppsala, Friday, April 15, 2011 at 13:15 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in English.

Abstract

Felth, J. 2011. Studies of Cytotoxic Compounds of Natural Origin and their Mechanisms of Action. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy* 141. 56 pp. Uppsala. ISBN 978-91-554-8023-3.

Cancer incidence is increasing and novel anticancer drugs with new mechanisms of action are essential for future chemotherapeutic treatment. Natural products have historically played an important role in the development of anti-cancer drugs and have potential to do so also in the future. In this thesis two classes of natural products are identified as possible drug lead candidates, and the mechanisms of their action are elucidated.

Initially, in a screening of a compound library for cytotoxic effects in colon cancer cells, natural products with potent activity were identified. Based on their potency, and on previously reported activities in cancer cells, two main groups of compounds, cardiac glycosides (CGs) and gambogic acid (GA) analogues, were selected for further in-depth studies.

The concentration-dependent cytotoxicity was confirmed in cell lines of different origin. Cardiac glycosides were mainly evaluated for their activity in colon cancer cells and in leukemic cells, whereas the GA analogues were studied using a resistance-based panel of ten human cancer cell lines. Using activity profiles and the ChemGPS-NP model, the compounds were compared, structurally and mechanistically, to standard chemotherapeutic drugs. The results from these analyses suggested that the CGs and the GA analogues act by mechanisms different from those of antimetabolites, alkylating agents, topoisomerase I and II inhibitors, or tubulin-active agents. By analysis of drug-induced gene expression, one GA analogue, dihydro GA, was identified as a possible inhibitor of the ubiquitin-proteasome system (UPS), and the CGs showed similarities to protein synthesis inhibitors.

Starting from these hypotheses, we further investigated the mechanisms of actions on a molecular level. The results showed that GA and dihydro GA act as inhibitors of the 20S proteasome chymotrypsin activity, leading to accumulation of ubiquitinated proteins. The CGs were confirmed to inhibit protein synthesis in colon cancer cell lines. However, interestingly, in leukemia cell lines, it seemed that the CGs act through a different, yet unexplored, mechanism of action. The leukemic cells (pre-B and T-ALL) were particularly susceptible to the cytotoxic effects of CGs, including at concentrations that may be achievable in the clinic.

Keywords: cytotoxic, cardiac glycoside, gambogic acid, cancer, mechanism of action

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ISSN 1651-6192
ISBN 978-91-554-8023-3
urn:nbn:se:uu:diva-148114 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-148114)

Sometimes, the more you think,
the more there is no real answer.

Winnie the Pooh

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Felth, J.**, Gullbo, J., Haglund, C., Rosén, J., Nygren, P., Larsson, R., Bohlin, L., and Rickardson, L. (2011). Screening for natural compounds with anticancer activity in colon cancer cells identifies cytotoxic gambogic acid analogues. *Manuscript.*

II **Felth, J.**, Lesiak-Mieczkowska, K., Haglund, C., Gullbo, J., Larsson, R., Linder, S., Bohlin, L., Fryknäs, M., and Rickardson, L. (2011). Gambogic acid is cytotoxic to cancer cells through inhibition of the ubiquitin-proteasome system. *Submitted for publication.*

III **Felth, J.**, Rickardson, L., Rosén, J., Wickström, M., Fryknäs, M., Lindskog, M., Bohlin, L., and Gullbo, J. (2009). Cytotoxic effects of cardiac glycosides in colon cancer cells, alone and in combination with standard chemotherapeutic drugs. *Journal of Natural Products* 72: 1969-1974.

IV Hallböök, H., **Felth, J.**, Eriksson, A., Fryknäs, M., Bohlin, L., Larsson, R. and Gullbo, J. (2011). *Ex vivo* activity of cardiac glycosides in acute leukaemia. *Plos ONE* 6: e15718.

Additional Papers Not Included in this Thesis

Ruhaak L.R., **Felth, J.**, Karlsson, P.C., Rafter, J.J., Verpoorte, R., and Bohlin, L. (2011). Evaluation of the cyclooxygenase inhibiting effects of six major cannabinoids isolated from *Cannabis sativa*. *Biological and Pharmaceutical Bulletin*, In Press.

Roggen, H., Bohlin, L., Burman, R., Charnock, C., **Felth, J.**, Görbitz, C.H., Larsson, R., Tamm, T., and Gundersen, L.L. (2011). 2-Substituted agelasine analogs: synthesis and biological activity, and structure and reactivity of synthetic intermediates. *Pure and Applied Chemistry,* 83: 645-653.

Roggen, H., Charnock, C., Burman, R., **Felth, J.**, Larsson, R., Bohlin, L., and Gundersen, L.L. (2011). Antimicrobial and antineoplastic activities of agelasine analogs modified in the purine 2-position. *Archiv der Pharmazie* 344: 50-55.

Burman, R., Svedlund, E., **Felth, J.**, Hassan, S., Herrmann, A., Clark, R.J., Craik, D.J., Bohlin, L., Claeson, P., Göransson, U., and Gullbo, J. (2010). Evaluation of toxicity and anti-tumour activity of cycloviolacin O2 in mice. *Biopolymers* 94: 626-634.

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Pettersson, J., Karlsson, P.C., Göransson, U., Rafter, J.J., and Bohlin, L. (2008). The flavouring phytochemical 2-pentanone reduces prostaglandin production and COX-2 expression in colon cancer cells. *Biological and Pharmaceutical Bulletin* 31: 534-537.

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ChemGPS-NP	Chemical global positioning system including natural products
CG	Cardiac glycoside
CI	Combination index
CLL	Chronic lymphocytic leukemia
cmap	Connectivity Map
DLCs	Digitalis-like compounds
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
FDA	Fluorescein diacetate
FMCA	Fluorometric microculture cytotoxicity assay
GA	Gambogic acid
HTS	High-throughput screening
IC_{50}	Inhibitory concentration 50% (50% survival)
Na^+/K^+-ATPase	Sodium-potassium–activated ATPase
NF-κB	Nuclear factor kappa B
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
RNA	Ribonucleic acid
SI	Survival index
TNF-α	Tumor necrosis factor alpha
Ub	Ubiquitin
UPS	Ubiquitin-proteasome system
YFP	Yellow fluorescent protein

Introduction

Originally, Nature was the source of all medicinal drugs. According to folklore and empirical observations, traditional healers used crude extracts, tinctures, or powders to treat diseases. In the beginning of the 19th century the first pharmacologically active compounds were isolated.

In modern drug development such pure compounds are preferred as pharmaceutical agents, enabling precise treatment dosage and monitoring of drug distribution in the human body. However, whole plants or plant parts are also used as herbal remedies, which provides potential for naturally occurring compounds to act synergistically. In many parts of the world medicinal treatment based on crude herbal remedies still prevails.

Thanks to the development of new techniques, many compounds can today be produced by synthesis or biotechnological methods. Yet, Nature is still an important source of new drug leads.[1] The great biological diversity of terrestrial and marine organisms is outstanding and provides chemically diverse molecules with biological activities.[2,3] Plants, animals, and microbes produce various compounds that can be utilized in drug discovery for finding molecular structures with useful pharmacological effects.[2,4]

Pharmacognosy is a multidisciplinary subject integrating pharmacology, chemistry, biology and toxicology. Bohlin and Samuelsson established a definition of the subject in their textbook of pharmacognosy:[4]

> The subject of pharmacognosy deals with natural products used as drugs or for the production and discovery of drugs.

The word pharmacognosy is derived from the Greek words pharmakon (drug) and gnosis (knowledge). Thus, the term is defined as the knowledge of drugs.[4]

Several strategies have been used in pharmacognosy research, including screening of compound libraries, ethnopharmacological research, and computational approaches.[5] In recent years, research at the Division of Pharmacognosy at Uppsala University has been focused on the discovery of new drug leads from natural sources by optimization of selection methods, in-depth studies of biological activities, and identification of new molecular targets.[6]

This thesis focuses on cytotoxic natural compounds, and was performed in collaboration with the Division of Clinical Pharmacology at Uppsala University Hospital. The work started out as a screening of a compound library, followed by in-depth studies of cytotoxic activities of gambogic acid (GA) analogues and cardiac glycosides (CGs) in cancer cells, including mechanistic studies on a molecular level.

Cancer

According to the World Health Organization, cancer is a leading cause of death worldwide, and the number of deaths is projected to continuously rise in the coming years.[7] Cancer is a group of diseases with a total of 44 672 new cases in Sweden in 2009,[8] and an estimated 12.7 million new cases worldwide in 2008.[9] In Sweden, the most common types are breast cancer in women and prostate cancer in men, followed by skin cancer and colon cancer for both genders.[8] Worldwide, the most commonly diagnosed tumor types are lung, breast, and colorectal cancer.[9]

A tumor consists of cells that have lost their normal regulation of growth or cell death. This loss can be due to genetic changes caused by chemical, physical, or biological damage of the normal regulatory genes. In a normal cell, intracellular signals regulate the cell cycle and control cell division, cell growth, and cell death. The process of carcinogenesis is a series of events that results in excessive cell division and tumor formation. The growing malignant tumor affects the surrounding tissues and may also spread to other parts of the body through lymph nodes and blood vessels.

Chemotherapy

In cancer treatment it is common to use multiple therapies, usually a combination of chemotherapy, surgery, and/or radiotherapy. Cancer chemotherapy implicates the use of anticancer drugs to treat malignant disease.

The first chemotherapeutic drugs were discovered in the 1940s, starting with experiments on the cytotoxic effects of nitrogen mustards.[10] These compounds were shown to induce tumor regression and became the starting point for research on chemotherapeutic drugs.

Today, several cytotoxic compounds are available and from experience it is known which drugs are most suitable for a specific cancer type. However, most anticancer drugs lack tumor specificity and cause damage to normal tissues, leading to side effects. Chemotherapeutic drugs are usually used in combination to give a more effective result.[10] Cytotoxic agents with different

mechanisms of action can together contribute to effective tumor killing with fewer side effects, because lower doses of each drug can be used.

If possible, solid tumors are often removed by surgery or irradiation and chemotherapeutic drugs can then be used as adjuvants. For hematological cancer types such as leukemia, chemotherapy with combinations of cytotoxic drugs is used as first-line treatment.[11] Chemotherapy is usually given in repeated cycles of treatment.

Classical Anticancer Agents and their Mechanisms of Action

The classical anticancer agents are the alkylating agents (including the platinum compounds), antimetabolites, topoisomerase inhibitors, and tubulin-acting agents. The alkylating agents act by generating reactive molecules that form covalent bonds with deoxyribonucleic acid (DNA) bases, resulting in DNA cross-linking and strand breaks. Melphalan, cyclophosphamide, and the platinum-based alkylating agents cisplatin, carboplatin, and oxaliplatin are examples of drugs that act by causing such DNA damage. Methotrexate and 5-fluorouracil are examples of commonly used antimetabolites, and these compounds act by blocking normal nucleic acid synthesis. Also, the topoisomerase inhibitors act on DNA level, by interfering with DNA replication, and can be exemplified by the anticancer drugs doxorubicin and irinotecan. Tubulin-acting agents interfere with the dynamics of the mitotic spindle resulting in inhibition of mitosis. The *Vinca* alkaloids (including vincristine, vinblastine, and the derivative vinorelbine), and the taxanes (paclitaxel and docetaxel) are two groups of compounds that act on tubulin.

These anticancer drugs are cell-cycle dependent and act on proliferating cells. Consequently, all cells that are rapidly dividing will be affected, causing side effects, and hence, limitations of drug dosage.

The classic chemotherapeutic agents are effective in many cases, but in view of the fact that many tumors develop resistance to the drugs, there is a need for novel, effective anticancer drugs with new mechanisms of action. More targeted treatment options are also highly warranted to enable specific eradication of tumor cells without affecting normal tissues, thus reducing the side effects.

New Targets for Chemotherapy

In recent years, the understanding of intracellular pathways in cancer cells has increased rapidly, contributing to the development of drugs with more specific targets, such as growth factors, signaling molecules, cell-cycle proteins, modulators of apoptosis, and molecules that promote angiogenesis.[10,12]

The antibody bevacizumab (Avastin®), prevents angiogenesis through inhibition of the vascular endothelial growth factor receptor (VEGFR), and is used to treat metastatic cancers.[13] Other drugs are more specifically directed towards particular cancer types, such as the monoclonal antibody rituximab (Mabthera®), targeting CD 20 in lymphoma cells.[14] The tyrosine kinase inhibitors imatinib (Gleevec®) and gefitinib (IRESSA®), acting on Bcr/Abl and epithelial growth factor receptor (EGFR) respectively, and the proteasome inhibitor bortezomib (Velcade®) are other examples of recently approved anticancer drugs.

Cellular regulation is a complex network and comprises several interconnected signaling molecules. A few intracellular pathways of special interest for this thesis are described below, including the ubiquitin-proteasome system (UPS), calcium signaling, and the NF-κB pathway.

The Ubiquitin-Proteasome System

The proteasome is the major proteolytic complex in mammalian cells, responsible for degradation of many cellular proteins. The UPS has an essential role in several important cellular events, including signal transduction and cell death.[15] Prior to protein degradation, ubiquitin (Ub) molecules are attached to the target protein forming ubiquitin-protein complexes that later can be recognized by the 26S proteasome.[16]

The 26S proteasome itself is a complex of about 2.5 megadaltons, which is present in the cytoplasm and in the nucleus.[16] It consists of two subcomplexes, the 20S core particle, containing proteolytic active sites, and the 19S regulatory particle.[17] The 19S subunits are located at both ends, acting as two "cap" regions where ubiquitinated proteins are bound, de-ubiquitinated, and then introduced into the centrally located 20S cylindrical complex, in which they are processed by proteolytic degradation into oligopeptides or single amino acid residues (**Figure 1**).[15,17] The 20S subunit is the catalytic core characterized by three proteolytic actions; chymotryptic-like, tryptic-like and caspase-like enzymatic activities.[15]

In tumor cells, the level of the 26S proteasome is upregulated,[18] and it has been shown that cancer cells are more susceptible to proteasome inhibition than normal cells.[16] Inhibition of proteasomal activity is a new, important mechanism of action for anticancer drugs and the enzymatic functions of the UPS have emerged as novel therapeutic targets.[15]

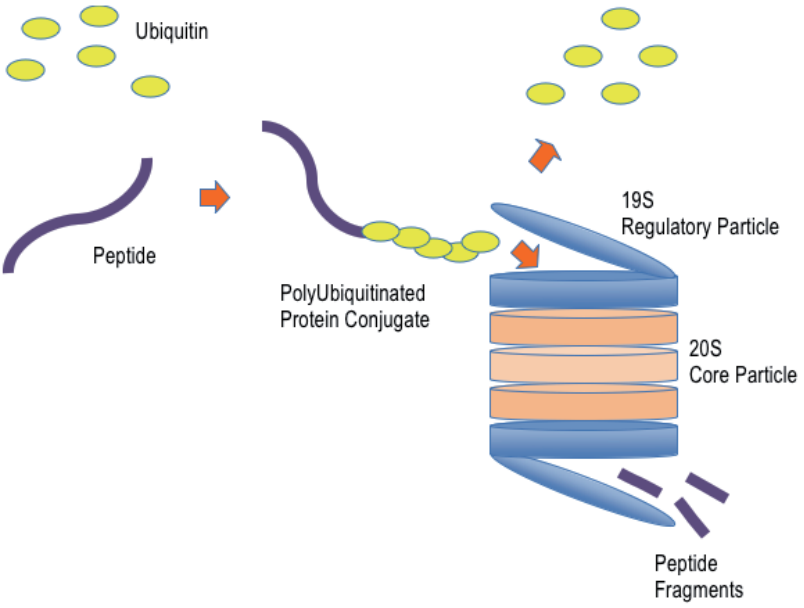

Figure 1. A schematic view shows how poly-ubiquitinated proteins are degraded by the proteasome.

Intracellular Calcium Signaling

Calcium is an ubiquitous second messenger and is involved in several cellular functions.[19] Hundreds of cellular proteins bind Ca^{2+}, and in some instances, this binding triggers cellular processes that are important for the survival of the cell.[19] Intracellular calcium oscillations influence the activation of transcription factors, such as NF-κB,[20] and calcium-mediated signaling pathways is of importance for carcinogenesis.[21] Also, many anticancer drugs elicit strong and sustained increases in cytosolic Ca^{2+} concentration, causing activation of complex signaling events. Thus, calcium plays a key role in induction of apoptosis in cancer cells.[19] The membrane-bound sodium-potassium–activated ATPase (Na^+/K^+-ATPase) is one of the cellular ion transporters that regulate intracellular calcium levels. However, several Ca^{2+} channels and pumps are involved in generation of intracellular calcium oscillations.[21] In cancer cells the expression of such channels and pumps is altered, causing changes in Ca^{2+} wave characteristics, and it has been suggested that these pumps could be potential therapeutic targets for new anticancer drugs.[21]

The NF-κB Pathway

Nuclear factor kappa B (NF-κB) is a transcription factor that is normally located in the cytoplasm of the cell, bound to inhibitor of κB (IκB). Phosphorylation and subsequent ubiquitination of the IκB protein, followed by

proteasome degradation, liberates NF-κB from its inhibitor.[22] Free NF-κB translocate to the nucleus of the cell where transcription is initiated, resulting in the activation of multiple genes involved in inflammatory responses and cell proliferation. Aberrant regulation of the NF-κB pathway has been shown to be of importance for the development of inflammatory diseases and cancer.[22] Thus, inhibition of NF-κB activation has emerged as a potential cancer drug target, and interestingly, this signaling system is also closely related to the UPS.

Cancer Drug Discovery and Development

Cancer drug discovery is focused on finding better strategies for cancer treatment, with fewer side effects and without impairment by drug resistance. As described above, identification of new targets is of huge interest. Much research is also focused on finding novel drug leads, and different methods can be employed for this purpose.

During the past decade high-throughput screening (HTS) has been a common method of identifying drug leads with anticancer activity. The process is highly automatized, and thousands of compounds can be tested against a certain target or cell type in a short period of time. To facilitate the screening process, compound libraries are commercially available, which can be screened *in vitro* against selected drug targets. In cancer drug discovery compound libraries are commonly screened for cytotoxic or antiproliferative activity, but it is also possible to screen against more specific targets.[23-25] Drug leads that are identified by HTS are further investigated by elucidation of concentration-effect relationships and mechanisms of action.

Another modern approach is to use *in silico* (i.e., computerized) methods for the identification of new drug leads. Chemical global positioning system including natural products (ChemGPS-NP) is an example of a multivariate tool that can be used for selection of high-probability hits.[26] The model is also useful for prediction of molecular properties and activities, including prediction of mechanisms of action for anticancer drugs.[27]

In recent years the development of microarray technologies have provided very useful methods for studying drug actions on a genetic level. The Connectivity Map (cmap) is a database containing genome-wide transcriptional expression data from cultured human cells treated with bioactive molecules. By comparing gene-expression signatures this tool can be used to find connections among small molecules acting on the same intracellular targets. The connectivity map has previously been used to explore the mechanisms of action of cytotoxic compounds.[28-30]

Cancer cell lines have an important role in cancer drug discovery, providing the opportunity for repeatable experiments, which is usually not possible when using primary cells from cancer patients. Cell line panels with a range of different tumor types are commonly employed for studies of cytotoxic activity, as well as for investigations of the molecular mechanisms of action.[31-33] However, the use of established cell lines has a few important drawbacks, such as the fact that they have been cultured *in vitro* for years, and hence, they do not always give a representative response, as compared to patient tumor samples.

Preclinical research *in vitro* always needs to be followed up by *in vivo* experiments, toxicological experiments, and clinical trials, and the total development process of a new anticancer drug can take a very long time, usually around 10–15 years.

Natural Products as Anticancer Agents

Natural products have an important role as anticancer agents, and many of the cytotoxic drugs used clinically today are derived from plants.[2] The *Vinca* alkaloids, isolated from the plant *Vinca rosea*, and the taxanes, from the bark of the Western yew *Taxus brevifolia* are examples of cytotoxic compounds that are commonly used in cancer treatment.[2,3] In other cases Nature has provided molecular structures that are used as precursors for semisynthesis. One example is the topoisomerase inhibitor irinotecan (Campto®), which is a derivative of the quinoline alkaloid camptothecin, from the tree *Camptoteca acuminata*.[2,3]

Hence, exploiting Nature for cancer drug development is a proven concept. The molecular and mechanistic diversity of natural products makes them very useful in cancer drug discovery.[2] Natural products, produced for defense, communication, or predation, are commonly pleiotropic (i.e., they act on more than one target), a property that potentially could be used for the development of new anticancer drugs.[34,35]

In 1989 the United States National Cancer Institute (NCI) developed an anticancer drug screen for evaluation of cytotoxic activities of both natural products and synthetic compounds in a panel of 60 human tumor cell lines.[33] Over the years thousands of plant extracts have been screened for anticancer activities, and several active compounds have been isolated.[33] Such bioassay-guided isolation is an important way of finding novel anticancer compounds, but it is also a time-consuming process.

Advances in molecular biology and newly developed techniques have increased the possibilities for evaluating mechanisms of action. Already isolated natural compounds can be collected in libraries and screened for anti-

cancer activities, followed by in-depth mechanistic studies of compounds identified as hits. Thus, active compounds can be identified in a short time, and more time can be spent on further investigations on the mechanisms of action. It has been shown that natural products occupy a larger part of the chemical space than synthetic compounds, and that screening procedures including a diverse set of compounds of natural origin are therefore more likely to be successful.[5,36]

In this thesis, gambogic acid analogues and cardiac glycosides were identified as hits in a screening for cytotoxic activity against colon cancer cells and attracted our interest for further studies on their mechanisms of action.

Gambogic Acid Analogues

Gambogic acid (**Figure 2**) is a naturally occurring compound derived from gamboge, a brownish resin of the tree *Garcinia hanburyi* in Southeast Asia. This plant product has been used in Chinese traditional medicine for centuries.[37,38] GA has been shown to be a potent anticancer candidate with documented cytotoxic activity in several types of cancer cells.[39-41] There are also reports indicating a possible selectivity of GA towards malignant cells, as compared to normal cells,[42,43] and the α,β-unsaturated ketone has been shown to be essential for growth inhibition and apoptosis induction in cancer cell lines.[38,39] In recent years, the Chinese Food and Drug Administration has approved a phase II clinical trial of GA as an antitumor candidate.[44,45]

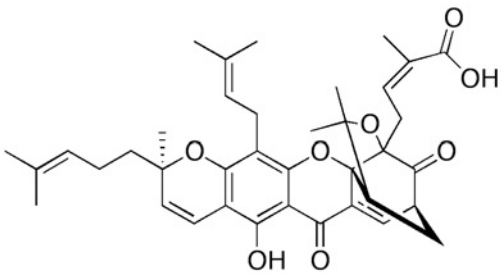

Figure 2. Chemical structure of gambogic acid.

GA affects several important cell-signaling pathways, such as the NF-κB signaling pathway,[46] and mitochondrial-dependent apoptosis pathways through suppression of anti-apoptotic Bcl-2 family proteins.[47] It has been reported that GA reduced the expression of c-MYC, accompanied by down-regulation of hTERT transcription and a subsequent reduction in telomerase activity.[48] Tubulin,[37] topoisomerase II,[45] and heat shock protein 90 (Hsp90)[49] have also been suggested as targets for the cytotoxic activity of GA. However, the mechanism for the observed anticancer activities is not yet fully

understood. Several naturally occurring GA analogues have been identified, and synthetic derivatives have also been produced.[42,50]

Cardiac Glycosides

Cardiac glycosides, also called cardenolides or cardiotonic steroids, are a group of compounds occurring in plants such as *Digitalis lanata*, *Digitalis purpurea* (**Figure 3**), *Convallaria majalis*,[4] and *Nerium oleander*.[51] Similar compounds, known as bufadienolides, have been identified in plants as well as in toads, snakes and fireflies.[52] Furthermore, endogenous digoxin-like immunoreactive factors, also termed digitalis-like compounds (DLCs) have been identified in human tissues.[53]

Figure 3. *Digitalis purpurea*, purple foxglove.
Photographed by Sara Laitinen (Eksjö, June 2010).

CGs are characterized by their ability to inhibit membrane-bound sodium-potassium–activated ATPase, causing a rise in intracellular calcium. The most well-known CGs are digoxin and digitoxin (**Figure 4**), which have clinical use in cardiology to treat congestive heart failure and atrial arrhythmias. The force of contraction of the myocardium is increased in response to elevated levels of intracellular calcium. High doses of CGs are toxic and may cause severe effects on the heart, such as bradycardia and atrioventricular block.[54]

The DLCs are believed to be synthesized in the adrenal gland, and to affect ion transport via Na^+/K^+-ATPase, thereby influencing intracellular

transduction pathways,[53] and it has been suggested that the DLCs may play a role in the development of malignancies.[55]

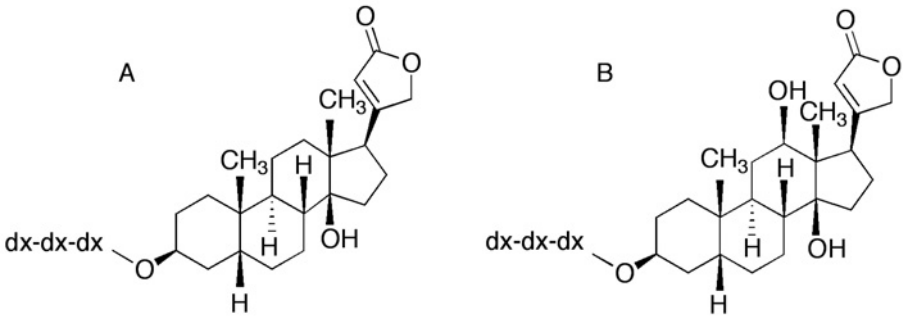

Figure 4. Chemical structures of the clinically used cardiac glycosides digitoxin (A) and digoxin (B), dx = digitoxose.

CGs have in recent years received attention as potential drugs in the treatment of various malignant diseases. Epidemiological observations have suggested that patients on digitalis medication diagnosed with breast cancer in general present with lower-proliferating tumors of smaller size, and subsequently, better prognoses than control groups. [56-59] The cardenolide derivative UNBS1450 and an aqueous extract from *Nerium oleander* (Anvirzel™) have entered clinical trials,[51,60] and studies of the addition of digoxin to combination chemotherapy and immunotherapy in patients with advanced malignant melanoma have also been initiated.[61]

In vitro studies have shown that CGs can induce cell death in several malignant cell lines of different origin,[62-64] and it has been suggested that malignant cells are more susceptible to the effects of CGs than normal cells.[65]

The mechanism of the cytotoxic activity of CGs on tumor cells has been subjected to many studies, and many different pathways have been suggested as being responsible for mediating the cytotoxic effects. Activation of caspases,[66] generation of reactive oxygen species (ROS),[67] and topoisomerase inhibition,[68] as well as inhibition of hypoxia-inducible factor 1 alpha (HIF-1α)[69] and p53 synthesis[70] have all been associated with cardiac glycosides.

Effects related to the inhibition of Na^+/K^+-ATPase have also been discussed, in view of the fact that binding to this ion pump can activate several intracellular signal transduction pathways. It has been suggested that CGs interfere with NF-κB activation through CG-induced calcium oscillations,[71] and recently, that general inhibition of protein synthesis, directly related to effects on the Na^+/K^+-ATPase pump, is the main mechanism of the anticancerous effects of CGs.[72] Interestingly, alterations in the expression of the α-isoforms of the catalytic subunit of Na^+/K^+-ATPase have been observed in tumor cells, and could potentially be a target for new anticancer drugs.[73-75]

Aims of the Thesis

Nature is an important source of new drug leads, and has played an important role in the development of novel anticancer agents.[1,2] Much natural product research has been focused on finding new bioactive molecules. In-depth studies of the pharmacological activities of these compounds are important for the understanding of their mechanisms of action. In recent years advances in molecular biology and analytical techniques have facilitated the development of new *in vitro* methods to investigate of effects on intracellular pathways. In this work we did not put any effort into isolating new compounds, instead, the main focus was to identify compounds of natural origin with potent cytotoxic activity, also in relatively resistant tumor cells, and to investigate them further. Hence, the overall aim of this study was to identify and evaluate cytotoxic compounds of natural origin, with focus on concentration-effect relationships, and mechanisms of action at a molecular level.

The specific aims were

- To identify compounds of natural origin with cytotoxic activity in human cancer cells, including in relatively more resistant tumor types, such as colon cancer cells.
- To elucidate the concentration-dependent cytotoxic effects of selected compounds, and to determine their mechanisms of action on a molecular level.
- To investigate the potential synergistic effects by combination of selected compounds and clinically used standard chemotherapeutic drugs.

Experimental Methods

This section is a summary of the experimental methods used in this thesis. For further details see the respective **Papers I–IV**.

Compounds

The Spectrum Collection™ (MicroSource Discovery Systems Inc., Gaylordsville, CT, USA) compound library was screened for cytotoxic activity in three human colon cancer cell lines (HT29, HCT116, and CC20) and two patient colorectal adenocarcinoma samples (**Paper I**). The library contains 2000 compounds, with 624 being natural products, and all compounds were supplied as 10 mM solutions in dimethyl sulphoxide (DMSO). For cytotoxicity assay the test compounds were further diluted with phosphate-buffered saline (PBS) and transferred to 384-well microplates (NUNC Brand Products, Roskilde, Denmark), using a Biomek 2000 pipetting station (Beckman Coulter Inc., Fullerton, CA, USA). All compounds were screened at a final concentration of 10 μM.

For further evaluations (**Papers II–IV**), convallatoxin, digitonin, digitoxin, digoxin, gambogic acid, ouabain, and proscillaridin A, were obtained from Sigma-Aldrich (St. Louis, MO, USA), and dihydrogambogic acid and oleandrin were from MicroSource Discovery Systems. The compounds were dissolved in DMSO and further diluted with PBS. For comparison and for combination studies, the clinically used drugs cisplatin, doxorubicin, 5-fluorouracil, irinotecan, oxaliplatin, and vinorelbine (Apoteket AB, Uppsala, Sweden) were used. The proteasome inhibitor bortezomib (LC Laboratories, Woburn, MA, USA) was used as a positive control in the UPS inhibition assays. DMSO concentration was below 0.5% (v/v) in all experiments, and solvent control was always used to exclude solvent effects.

Human Tumor Cell Lines

The colorectal adenocarcinoma cell line, HT29, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), the HCT116 cell line was kindly provided by S. Linder, Karolinska Institute, Solna, Sweden, and the CC20 cell line was a

gift from C. Sundberg (Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden). HT29 and CC20 cells were cultured in monolayer in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) and HCT116 was cultured in monolayer in McCoy's 5A medium (Sigma-Aldrich).

The resistance-based cell line panel[31] consists of the myeloma cell lines RPMI 8226, 8226/Dox40 (selected for doxorubicin resistance)[76] and 8226/LR-5 (selected for melphalan resistance);[77] the leukemia cell lines CCRF-CEM and CEM/VM-1 (selected for teniposide resistance);[78] the lymphoma cell lines U-937 and U-937-vcr (selected for vincristine resistance);[79] the small cell lung carcinoma cell lines NCI-H69 and H69AR (selected for doxorubicin resistance)[80] and the primary multidrug-resistant renal adenocarcinoma ACHN.

RPMI 8226, NCI-H69, H69AR and ACHN were from American Type Culture Collection (ATCC; Manassas, VA, USA) and the other cell lines in the panel were kind gifts from W.S. Dalton, Department of Medicine, Arizona Cancer Center, University of Arizona, Tucson, AZ, USA (8226/Dox40 and 8226/LR-5); K. Nilsson, Department of Pathology, Uppsala University, Sweden (U-937 and U-937-vcr); and W.T. Beck, Department of Pharmacology, College of Medicine, University of Tennessee, Memphis, TN, USA (CCRF-CEM and CEM/VM-1). The cells in the panel were grown in RPMI 1640.

The breast cancer cell line, MCF-7, and the cervical adenocarcinoma cell line, HeLa (ATCC), were grown in minimum essential medium Eagle (MEME; Sigma-Aldrich), extra supplemented with 1 mM sodium pyruvate.

The human melanoma cell line MelJuSo Ub^{G76V}-YFP was a kind gift from N. Dantuma, Karolinska Institute, Solna, and was grown in DMEM. MelJuSo Ub^{G76V}-YFP is a cell line expressing Ub coupled to yellow fluorescent protein (YFP)[81] and was used for the UPS experiments (**Paper II)**.

The B-precursor Philadelphia-positive cell line SUP-B15, used in **Paper IV**, was obtained from DSMZ, and was grown in McCoy's 5A medium (Sigma-Aldrich).

All media used were supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 µg/mL streptomycin, and 100 U/ml penicillin (all from Sigma-Aldrich). The cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Patient Tumor Samples

Tumor samples from patients diagnosed with colorectal adenocarcinoma (**Papers I** and **III**), and cryopreserved tumor cells from patients with B-precursor or T-acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), and chronic lymphocytic leukemia (CLL) (**Paper IV)** were used.

Peripheral blood mononuclear cells (PBMCs) from healthy donors were used as controls. Sampling for drug sensitivity testing was approved by the local Ethics Committee in Uppsala (Regionala etikprövningsnämnden i Uppsala, Sweden, approval number Dnr 21/93).

Measurement of Cytotoxic Activity

Cytotoxic activity was measured by using a fluorometric microculture cytotoxicity assay (FMCA) as previously described.[82,83] The method is based on measurement of the fluorescence derived by hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes (i.e., viable cells).

Cell suspensions were seeded into drug-prepared 96-well or 384-well microplates. Wells with medium served as blanks. The plates were incubated at 37°C for 72 hours, and then medium and drugs were aspirated. The plates were washed with PBS, FDA was added, and after 50 min of incubation, fluorescence was measured using a FLUOstar Optima microplate reader (BMG Technologies, Offenburg, Germany). The 384-well plates were analyzed using an automated HTS system controlled by an Optimized Robot for Chemical Analysis (ORCA; Beckman Coulter), programmed through SAMI software (Beckman Coulter Inc., Fullerton, CA, USA).

The fluorescence measured is proportional to the number of living cells in each well, and cell survival is presented as the survival index (SI), defined as the fluorescence value in the wells analyzed as percentage of the value in the control wells, with blank values subtracted. Quality criteria for each analysis included a signal/blank ratio of >10 and a coefficient of variation in controls and blanks of <30%. The experiments (with duplicates) were performed three times for cell lines and once for patient samples.

The cytotoxic IC_{50} values (inhibitory concentration 50%) for the drugs were determined from log concentration-effect curves in GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA), using nonlinear regression analysis. Data are presented as the means ± standard error of the mean.

Mean Graph Activity Profiles

Based on the results from FMCA analysis using the cell line panel, mean graph activity profiles for the GA analogues were constructed (**Paper I)**. The mean $\log IC_{50}$ for each compound and cell line was determined and mean graphs were created by subtracting the mean $\log IC_{50}$ from each $\log IC_{50}$. Cell lines that are more resistant than the average proceed to the right (positive values) and the cell lines that are more sensitive than the average proceed to the left (negative values).

ChemGPS-NP

The principal component analysis–based model ChemGPS-NP (http://chemgps.bmc.uu.se) is a tool for navigation in biologically relevant chemical space.[26] The model and the prediction methods are previously described in detail.[26,84] Based on their molecular properties, compounds can be compared by observation of their appearance in the chemical space. In **Papers I** and **III** ChemGPS-NP was used to compare CGs or GA analogues with anticancer agents with known mechanisms of action: alkylating agents, antimetabolites, proteasome inhibitors, topoisomerase I inhibitors, topoisomerase II inhibitors, tubulin-active agents, and tyrosin kinase inhibitors.

The model has also been demonstrated to be suitable for differentiation of biological activities, that is, anticancer drugs cluster in accordance with their mechanism of action.[27] Using activity profile data, based on cytotoxicity in the resistance-based cell line panel, the GA analogues were analyzed and compared to common anticancer agents.

Gene Expression Analysis

The drug-induced gene expression changes of dihydro GA (**Paper II**) and digoxin and digitoxin (not published) were studied using the Connectivity Map (cmap) build 02 (www.broad.mit.edu/cmap), which contains genome-wide expression data for 1309 compounds. The original protocol, as described by Lamb *et al.*,[85] was used. Breast cancer MCF-7 cells were plated and treated with 10 μM of the respective compound or vehicle control (DMSO) for 6 h. The experimental methods for cell treatment, ribonucleic acid (RNA) preparation, and gene expression analysis were recently described in more detail by Fayad *et al.*[28] A gene signature consisting of the 50 (for dihydro GA) or 100 (for digoxin and digitoxin) most up and down regulated genes was used to query the cmap database to retrieve a ranked list of compounds.

Live-cell Imaging

For the live-cell imaging experiments (**Paper II)** MelJuSo Ub^{G76V}-YFP cells[81] were plated in black optically clear bottom ViewPlates (PerkinElmer, Waltham, MA, USA) overnight and then treated with compounds in concentrations ranging from 0.08 μM to 10 μM. Bortezomib at 10 μM and 0.1 μM was used as positive control. Treatment with UPS-inhibiting compounds leads to accumulation of YFP in the cells and the generated fluorescence from the Ub construct was continuously detected and studied in an IncuCyte FLR (Essen BioScience Inc., Ann Arbor, MI, USA).

Analysis of Cellular Content of Ubiquitinated Proteins

As described in detail in **Paper II**, the cellular content of ubiquitinated proteins was studied by Western blotting. Ub^{G76V}-YFP constructs and ubiquitin-conjugated proteins were detected using anti-GFP (B-2; Santa Cruz Biotechnology, Heidelberg, Germany) and Anti-UbK48 (Apu2; Millipore, Temecula, CA, USA) antibodies respectively.

20S Proteasome Activity Assay

In **Paper II**, the enzymatic activity of chymotrypsin was examined using the 20S Proteasome Assay Kit (Boston Biochem Inc., Cambridge, MA, USA) according to instructions from the manufacturer. Chymotrypsin activity was measured by adding compounds to SDS-activated 20S enzyme in an assay buffer of 25 mM HEPES and 0.5 mM EDTA. After a 15 min incubation to allow enzyme and compound interaction, the fluorogenic peptidyl proteasome substrate Suc-LLVY-AMC was added. The increase in fluorescence was measured every third minute for 1 h at 37°C in the FLUOstar Optima using excitation and emission wavelengths of 355 nm and 460 nm. Monitoring the increase in fluorescence over time allowed quantification of the enzymatic activity. Bortezomib at 10 μM was used as positive control.

Ca^{2+} Oscillation Measurement

To investigate whether the cytotoxic activity of CGs could be associated with effects on intracellular calcium levels, Ca^{2+} oscillation in malignant cells was measured by a digital imaging fluorometry method.[86] Glass cover slips, were prepared by coating with 0.01% poly L-lysine (PLL; Sigma-Aldrich, St. Louis, MO, USA). Cell suspension (colon cancer HT29 cells, CCRF-CEM leukemia cells, or RPMI 8226 myeloma cells) was added, and the cells were incubated overnight to attach. The following day, cells were loaded for 45–60 minutes with 1.8 μM fura-2-acetoxymethyl ester (fura-2AM) in a glucose buffer (pH 7.4). Fura-2AM is a nonpolar compound that easily crosses the membrane of the cells. Inside the cell, esterases hydrolyze the compound to the negatively charged fura-2, which is calcium-ion sensitive and used for detection of intracellular Ca^{2+} oscillation.

The cover slips were placed at the bottom of an open chamber (**Figure 5B**) and positioned on the stage of an inverted Nikon Diaphot microscope equipped with an epifluorescence illuminator and a ×40 oil immersion fluorescence objective (Nikon, Kanagawa, Japan) (**Figure 5A**). The chamber holder and the objective were maintained at 37°C. A 150-W xenon arc lamp and an Optoscan monochromator (Cairn Research, Faversham, UK) pro-

vided excitation light at 340 nm and 380 nm and emission was measured at >515 nm by an intensified CCD camera (Extended ISIS-M; Photonic Science, Robertsbridge, UK) or a back-illuminated EMCCD camera (DU-887, Andor Technology, Belfast, Northern Ireland). The Metafluor software (Molecular Devices Corporation, Downingtown, PA, USA) controlled the monochromator, acquiring fluorescence images of 10 accumulated frames at 340 and 380 nm every 2 seconds. Ca^{2+} images were calculated from 340:380 nm ratio images.[86,87] Glucose buffer was superfused over the cells at a rate of 0.6 to 0.9 ml/min. CCRF-CEM, RPMI 8226 or HT29 cells (**Figure 5C**) were treated with 10 µM of digitoxin or digoxin in separate experiments. Carbachol was used as a positive control for Ca^{2+} oscillation.

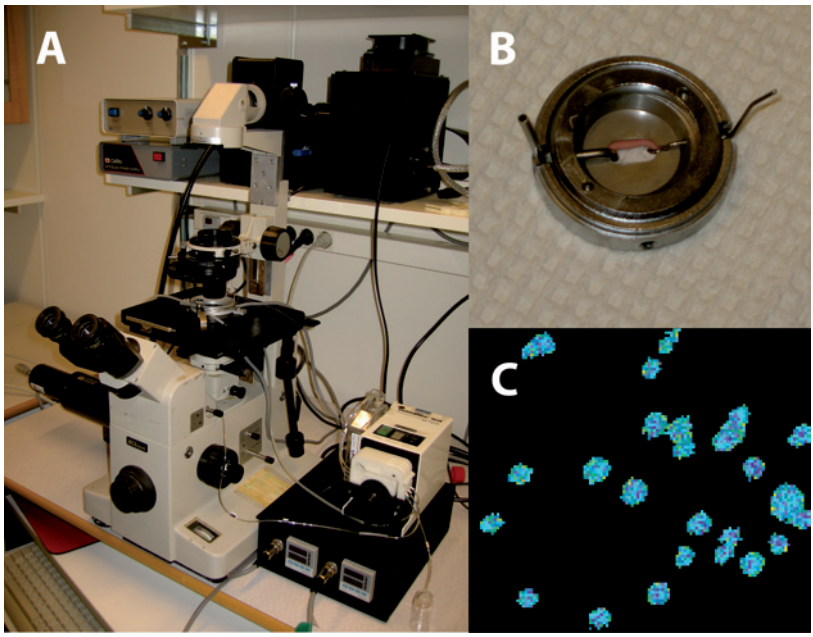

Figure 5. Digital imaging fluorometry equipment for measurement of calcium oscillation (**A**). Cells were attached to a cover slip and positioned in a chamber (**B**). Using the microscope, cells were located and selected for calcium oscillation measurement (**C**). Photographs by Sara Laitinen, 2009.

NF-κB Translocation Assay

In **Paper III**, the effects of digitoxin (0.4, 2, 10 µM), digoxin (0.4, 2, 10 µM), and oleandrin (0.2, 1, 5 µM) on NF-κB translocation in HT29, MCF-7, and HeLa cells were studied using the NF-κB Activation HCS HitKit™ (Cellomics, Pittsburgh, PA, USA). The NF-κB translocation assay was carried out according to the manufacturer's instructions.[88] Quantification of NF-κB activation was performed by measuring the spatial translocation of NF-κB

from the cell cytoplasm to the nucleus, using the ArrayScan II HCS reader (Cellomics), as previously described.[88] Tumor necrosis factor alpha (TNF-α) and bortezomib were used as controls.

Protein and DNA Synthesis Inhibition Assay

In **Paper IV**, the DNA and protein synthesis inhibitory activities of digoxin and digitoxin in cancer cells were analyzed. The experiments were performed in Cytostar-T® plates (Amersham International, Buckinghamshire, UK) using ^{14}C-labelled thymidine and leucine. The Cytostar-T® plates have scintillants molded into the transparent polystyrene bottom. When labeled substrate is absorbed into the intracellular compartment of the cells at the bottom of the wells, the radioisotope is brought into proximity with the scintillant, thereby generating a detectable signal. Free radio-labeled substrate in the supernatant is unable to stimulate the scintillant.[89,90]

HT29, HCT116, and CC20 colon cancer cells grow in monolayer and for protein synthesis experiments 10×10^3 cells in 200 µl medium were seeded in the plate the day before the experiment. At the day of the experiment medium was removed and medium containing ^{14}C-thymidine (111 nCi/ml; for DNA experiments) or ^{14}C-leucine (222 nCi/ml; for protein experiments) was added, yielding a final radioactivity in the wells of 20 and 40 nCi, respectively. Cell suspension (50×10^3 cells in 180 µl) was added to each well; blank wells had isotope-containing medium only.

The leukemia cells, CCRF-CEM and SUP-B15, were suspended in fresh medium containing ^{14}C-thymidine (20 nCi per well for DNA experiments) or ^{14}C-leucine (40 nCi per well for protein experiments), and 50×10^3 cells in 180 µl were added to each well.

Drugs (digoxin and digitoxin, at final concentrations of 10 µM to 1 nM) and PBS in test and control wells were added in duplicate (20 µl per well). Radioactivity was measured with a computer-controlled Wallac 1450 MicroBeta® trilux liquid scintillation counter (Wallac OY, Turku, Finland) at different time points up to 72 hours. Between measurements, the plates were stored in an incubator at 37ºC. During measurement, the plates were covered with a plate sealer to avoid microbiological contamination.

Combination Analysis *In Vitro*

The cytotoxic effects of five glycosides, convallatoxin, digitoxin, digoxin, oleandrin, and proscillaridin A, and the saponin digitonin were investigated in combination with four standard chemotherapeutic drugs used in colorectal cancer (5-fluorouracil, cisplatin, oxaliplatin, and irinotecan) in three cell lines (HT29, HCT116, and CC20) (**Paper III**). The studies were designed

using a fixed ratio of the drugs across a concentration gradient. Single drug activity in the cell lines was estimated, and microplates for combination analysis were prepared by two-fold serial dilutions in nine steps for all test compounds. Combinations were tested using a fixed concentration ratio. All compound concentrations and combinations were tested in duplicate and the experiments were repeated three times.

Data were analyzed using the median-effect method of Chou and Talalay,[91] employing the CalcuSyn, Version 2, software (Biosoft, Cambridge, UK). Each concentration-response curve (individual agents as well as combinations) was fitted onto a linear model using the median effect equation, allowing calculation of a median effect value D (corresponding to the IC_{50}) and slope. Fit was assessed using the linear correlation coefficient, r, and $r > 0.85$ was required for a successful analysis. The extent of drug interaction between the drugs was expressed using the combination index (CI) for mutually exclusive drugs: $CI = d_1/D_1 + d_2/D_2$ where D_1 and D_2 represent the concentration of drugs 1 and 2 alone, required to produce a certain effect, and d_1 and d_2 are the concentration of drugs 1 and 2 in combination required to produce the same effect. Different CI values are obtained when solving the equation for different effect levels, and the 70% effect was chosen for presentation (**Paper III**). A CI equal to 1.0 indicates additivity; a significantly lower CI value was defined as synergy, while a significantly higher value was defined as antagonism. One-sample t-tests were used to determine if CIs differed from 1.0 ($p < 0.05$).

In **Paper I**, a small combination experiment was also performed for the GA analogues, where 0.032–20 μM of each GA analogue was tested in combination with 30 μM of oxaliplatin or 55 μM of irinotecan. The cytotoxic activity in HT29 cells was measured using FMCA.

Results and Discussion

From a screening procedure cytotoxic compounds were identified and selected for further investigations of their mechanisms of action. A schematic representation of the workflow and the methods used is outlined below (**Figure 6**).

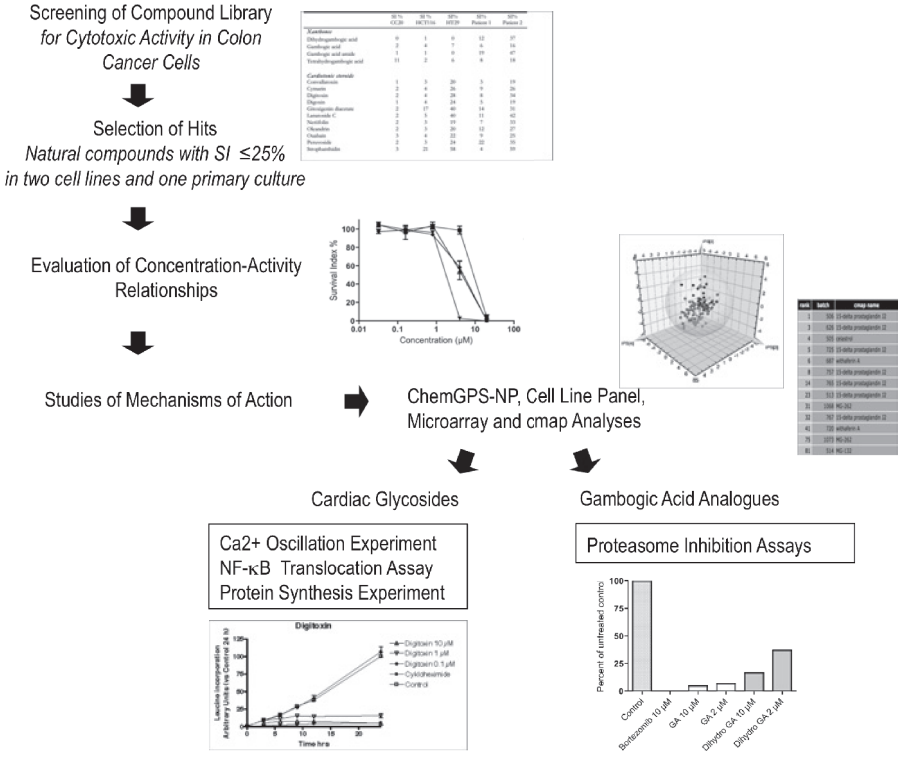

Figure 6. Schematic representation of the workflow and the methods used.

Screening Identifies Cytotoxic Natural Compounds

The Spectrum Collection™, containing 624 compounds of natural origin, was screened for cytotoxic activity in three human colon cancer cell lines (HT29, HCT116 and CC20) and two patient colorectal adenocarcinoma

samples. Natural compounds with an SI-value ≤25% in at least two of the three cell lines and in at least one of the two primary samples were selected as hits from the screen (**Paper I**).

Two major groups of compounds were identified: cardiac glycosides and gambogic acid analogues. Other promising compounds, which previously have been shown to possess anticancer activity in human cancer cell lines, such as the alkaloid tomatine,[92] and the triterpenoids pristimerin and celastrol,[93,94] were also identified as hits in this screen.

While searching the literature it was found that little or nothing had been published on the cytotoxic effects of CGs against colorectal cancer cells. Also, despite several studies, the mechanism for their cytotoxic action had not been clarified and their clinical potential remained unclear. Similar findings for the GA analogues attracted our interest for further studies of these compounds, with focus on the determination of the main mechanisms of action.

Cytotoxic Gambogic Acid Analogues

As described in **Paper I**, the concentration-dependent cytotoxic activity of the GA analogues was confirmed in the three colon cancer cell lines (CC20, HCT116, and HT29), as well as in a resistance-based cell line panel consisting of ten cell lines of various origin.[31] In these first analyses, dihydro GA possessed the most potent cytotoxic activity with a mean IC_{50} value of 2.1 μM in cells of colorectal origin (1.8 μM in HCT116 cells, 2.5 μM in HT29, and 2.1 μM in CC20), as well as in other cancer cell lines. However, while pursuing the studies on the cytotoxic activity and the mechanism of action it was clear that these results were not completely reliable. In the follow-up experiments (**Paper II**) newly obtained compounds were used, and the results showed GA to be more potent in the MelJuSo Ub^{G76V}-YFP cell line (IC_{50} 0.34 μM, as compared to 2.9 μM for dihydro GA). These results were also confirmed in the colon carcinoma cell line HCT116: IC_{50} 0.19 μM (GA) and 2.0 μM (dihydro GA). It is possible that the compounds used in the initial experiments (i.e., the Spectrum library with DMSO solutions) may have been degraded. GA might be considered as a quite reactive and unstable molecule and for future drug development, more simple and stable analogues would be preferred. Batova *et al.,* recently identified the minimum bioactive motif of the *Garcinia* xanthones and suggested the analogue cluvenone as a suitable molecule for further development as a chemotherapeutic agent.[42]

Gambogic acid has well-documented cytotoxic activity in a variety of malignant tumor cells,[39-41] with a potential selectivity towards malignant cells.[42,43] Tumor-selective compounds are of great interest for the develop-

ment of new anticancer agents. It is also important to clarify the mechanism by which the compound exerts its cytotoxic activity. GA has previously been shown to inhibit the enzyme topoisomerase II,[45] a well-known anticancer target. Tubulin has also been suggested as the target for the cytotoxic activity of GA.[37] In a recent paper, GA and its analogue cluvenone were tested on the NCI60 cell line panel and subsequently mapped to the Q-region, consisting of bioactive compounds with unknown mechanism of action.[42] That report is congruent with the analysis we had performed, using a resistance-based cell line panel consisting of ten cell lines[31] and ChemGPS-NP.[27] Our results indicated that the GA analogues act by a different mechanism than antimetabolites, alkylating agents, topoisomerase I and II inhibitors, or tubulin-active agents (**Paper I**). Thus, we set out to identify the main mechanism of action of GA and its analogue dihydro GA.

Gene Expression Analysis of GA Analogues

To generate hypotheses on the probable mechanism of action, a gene-expression microarray analysis was performed, where MCF-7 breast cancer cells were treated with dihydro GA at 10 μM for 6 h and compared to vehicle-treated MCF-7-cells. The gene signature obtained was then compared with the 1309 compounds in the Connectivity Map database. The gene-profile generated by dihydro GA treatment turned out to be similar to several compounds previously shown to inhibit the ubiquitin-proteasome system (UPS), such as celastrol,[93] withaferin A,[95] and 15-delta prostaglandin J2,[96] as well as the experimentally used proteasome inhibitors MG-132 and MG-262 (**Paper II**). Dihydro GA exposure resulted in induction of a number of genes that previously have been induced by proteasome inhibitors (i.e., lactacystin and bortezomib. This finding supported our results that the GA compounds act as inhibitors of proteasomal function.

GA has previously been shown to affect important cellular events, such as reduction of the expression of c-MYC, accompanied by downregulation of hTERT transcription and reduction in telomerase activity,[48] and suppression of anti-apoptotic Bcl-2 family proteins.[47] Studies have shown that GA inhibits the NF-κB signaling pathway and potentiates apoptosis by interaction with the transferrin receptor.[46] Since proteasome inhibitors are known to inhibit NF-κB,[97,98] and modulation of hTERT, c-MYC, and Bcl-2 is regulated by NF-κB activation,[99] these results correspond well to GA being an inhibitor of the UPS.

GA Analogues Inhibit the Ubiquitin-Proteasome System

To confirm the findings from the gene expression analysis, UPS inhibition and cytotoxic activity of GA and dihydro GA were verified in several assays

using the melanoma cell line MelJuSo Ub^{G76V}-YFP (**Paper II**). By live-cell imaging it was observed that treatment with GA or dihydro GA resulted in an accumulation of ubiquitin conjugates in the cells, reflecting a decrease in the intracellular proteasome activity. GA was the most active compound, starting to inhibit the UPS immediately, and after 2 h at 10 μM the cells rapidly died. At lower doses, starting from 2 μM, the cells started accumulating Ub^{G76V}-YFP with peak intensity at 8–12 h. After 10 h, the cells displayed signs of apoptosis, such as blebbing of the cell membrane and formation of apoptotic bodies (**Paper II**). Dihydro GA was less potent, but showed accumulation of YFP, accompanied by signs of cell death after 4 h, at 10 μM.

Results from Western blot analysis pointed out stabilization of the Ub^{G76V}-YFP fusion protein in the presence of GA, as well as a dose-dependent effect of dihydro GA, as shown in **Paper II**. The activity of GA was most pronounced at concentrations ranging from 1 to 2 μM and higher doses were toxic to the cells, causing membrane disruption and leaking of proteins. Dihydro GA was less potent, with peak activity at 10 μM, yielding an accumulation of ubiquitinated proteins in the cells. These results correspond well to the accumulation of ubiquitin shown in the fluorescence images.

Accumulation of ubiquitin is non-specific and would be the result of most UPS inhibitors. As described in **Paper II,** we continued to examine the effect of the GA analogues on the enzymatic core of the proteasome. Both analogues inhibited the proteasome 20S chymotrypsin activity in a dose-dependent manner, again with GA as the more active compound.

The proteasome is a promising target for anticancer drugs, and, to date, bortezomib (Velcade®) is the only clinically approved 20S inhibitor. Hence, the development of new drugs acting on the UPS is of great interest. The finding that the GA analogues act on this target will add to the knowledge regarding the intracellular effects of these compounds and may have implications for future chemotherapeutic treatment.

Cytotoxic Activity of Cardiac Glycosides

In **Paper III** the cytotoxic activities of convallatoxin, digitoxin, digoxin, oleandrin, proscillaridin A, and digitonin were studied in the three colon cancer cell lines, HT29, HCT116, and CC20. The HT29 cell line was more resistant than the CC20 and HCT116 cell lines to cardiac glycosides, as well as to standard chemotherapeutic drugs. Convallatoxin, oleandrin and proscillaridin A were identified as the most potent test compounds, with IC_{50} values up to 0.55 μM. Digitoxin and digoxin, were less potent, although still active (IC_{50} 0.24–4.1 μM; **Table 1**).

Table 1. IC_{50} values (μM) for digitoxin and digoxin in the colon cancer cell lines CC20, HCT116, and HT29, and the leukemia cell lines CCRF-CEM and SUP-B15.

	IC_{50} (μM)				
	CC20	**HCT116**	**HT29**	**CCRF-CEM**	**SUP-B15**
Digitoxin	0.41	0.74	4.1	0.12	0.002
Digoxin	0.24	0.27	1.4	0.22	0.03

Results from studies in cell lines are not always representative for tumor treatment in the clinic. Therefore, to improve the relevance of the experiments, it can be valuable to include primary cells from tumor patients as a complement.[100] Such additional experiments were performed in this study, and digitoxin and digoxin were found to inhibit the survival of primary cultures of tumor cells from surgical specimens obtained from patients diagnosed with colon cancer, with IC_{50} values in the range of 0.1–1.9 μM. The IC_{50} values for digitoxin are shown in **Figure 7**.

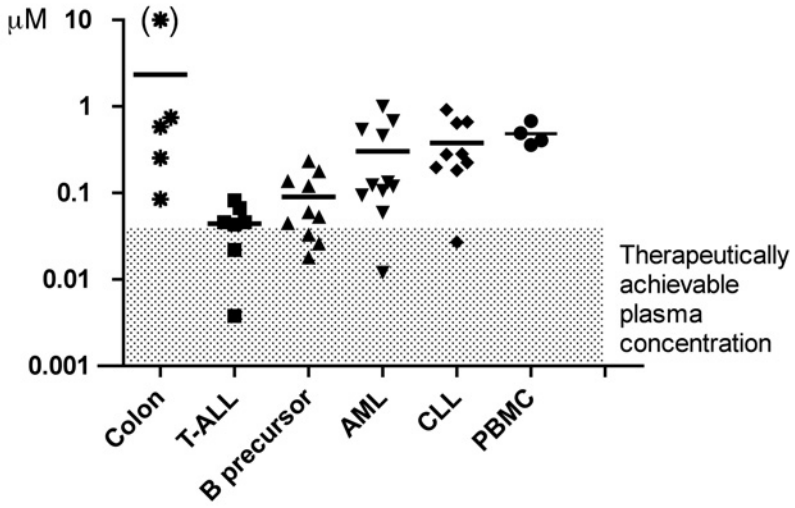

Figure 7. Cytotoxic IC_{50} values (μM) for digitoxin in primary cultures of leukemia cells (T-ALL, B-precursor ALL, AML, and CLL) and colon cancer cells (Colon), compared to PBMCs.

The IC_{50} values *in vitro* were higher than achievable plasma concentrations *in vivo*, that is, the plasma concentrations in the treatment of cardiac disease are about 1-2 nM for digoxin and 20-40 nM for digitoxin,[101] which suggests their limited therapeutic potential as anticancer agents. However, direct comparison with concentrations in plasma may be misleading, as the volume of distribution for cardiac glycosides generally is high, being up to 780 L for digoxin,[102] and furthermore, the distribution in different organs varies con-

siderably.[103] Certainly, following oral intake, high local concentrations in the intestine and portal circulation may be achieved, which may be sufficient for effects in the intestine or on early metastases in the liver. Digitoxin and its metabolites are known to be eliminated very slowly from the human body, due to biliary excretion and enterohepatic circulation, suggesting relatively high concentrations in this compartment, while digoxin is more rapidly eliminated through glomerular filtration.[101]

Studies including more sensitive cancer types, such as leukemias, have shown more promising results and may consequently be more relevant for clinical use. In **Paper IV** primary B-precursor and T-ALL cells were identified as being particularly susceptible to the cytotoxic effects of CGs. Digitoxin appeared most potent, and IC_{50} values for several patient samples were at concentrations that may be achieved in the clinic (**Figure 7**). The primary B-precursor and T-ALL cells were significantly more sensitive to digitoxin than CLL cells and PBMCs. For ouabain, the IC_{50} value was significantly lower for the T-ALL cells than for CLL cells but otherwise no significant differences were observed in the different leukemic cells or the PBMCs. With extended exposure (6 days) both T- and B-precursor ALL cells appeared sensitive at clinically achievable concentrations (**Paper IV**).

The cell line SUP-B15 was extremely sensitive to all tested CGs, and the T-lymphoblast-like cell line CCRF-CEM showed effects similar to those in the primary ALL cells. In both cell lines there was a tendency towards a lower sensitivity to digoxin than to digitoxin (**Table 1**) or ouabain.

Mechanistic Studies of CGs

ChemGPS-NP analysis (**Paper III**) indicated that the mode of action for CG cytotoxicity is mediated through another pathway than those of some other anticancer drugs. It has been suggested that the cytotoxic action is a secondary effect caused by the inhibition of the Na^+/K^+-ATPase and it was previously reported that CGs may act through induction of intracellular calcium oscillations, and by activation of NF-κB.[71] In an attempt to clarify whether these mechanisms are important for cytotoxicity in cancer cells studies on the effects on NF-κB translocation were performed (**Paper III**). Additionally, effects on the intracellular Ca^{2+} oscillation were studied (unpublished data).

Ca^{2+} Oscillation Measurement

The potential effects of CGs on calcium levels in cancer cells were studied using a digital imaging fluorometry method.[86] 10 μM of digitoxin and digoxin, respectively, was superfused over the cells (HT29 or RPMI 8226), and Ca^{2+} oscillation was measured. In these experiments no changes in Ca^{2+} oscillation were observed in colon cancer HT29 cells or in RPMI 8226 mye-

loma cells, after exposure to digitoxin (**Figure 8**) or digoxin (not shown). The leukemia CCRF-CEM cells did not attach properly to the cover slip surface, and could therefore not be analyzed.

From our results it seems that digitoxin and digoxin, at cytotoxic concentrations, do not induce any changes in calcium oscillation in the studied cancer cell lines. However, it was recently reported that CGs (i.e., digoxin and ouabain) caused a steady increase of calcium levels in HT29 cells over time (up to 24h), an event that was dependent on calcium influx via the Na^+/Ca^{2+} exchanger.[104]

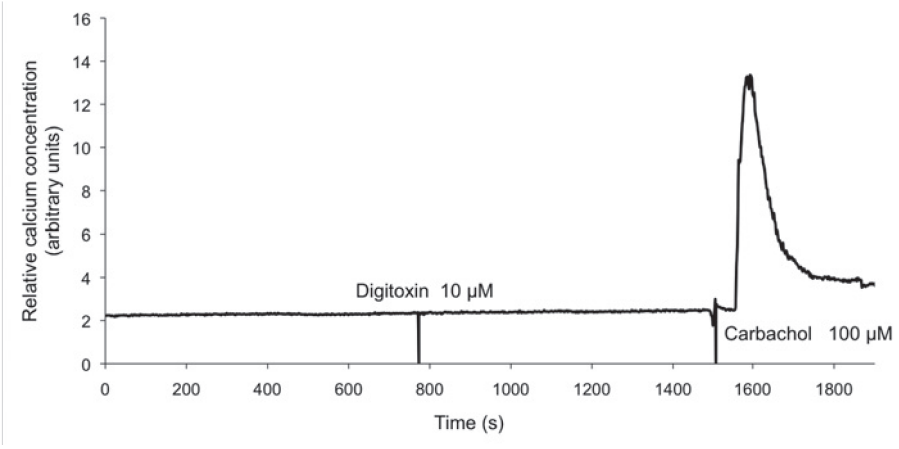

Figure 8. Relative calcium concentration in HT29 cells over time. Digitoxin (10 µM) was added after 773 seconds, and no Ca^{2+} oscillation was detected. Carbachol (100 µM) was used as positive control. Similar results were obtained for the myeloma RPMI 8226 cell line.

NF-κB Translocation

The transcription factor NF-κB has been shown to be involved in processes important for carcinogenesis and has previously been suggested as a potential target for cardiac glycosides.[105] However, in our experiments tumor necrosis factor alpha (TNF-α)–stimulated NF-κB translocation was unaffected by treatment with digitoxin, digoxin, or oleandrin in HT29 colon cancer cells, as well as in breast (MCF-7) and cervical (HeLa) cancer cells (data not shown), thus not confirming actions via this pathway.

Gene Expression Analysis of Cardiac Glycosides

To generate new hypotheses about the mechanism of action for the CG compounds, we performed a gene expression analysis of digitoxin and digoxin. The results for digoxin (10 µM, 24 h) on MCF-7 cells were uploaded in cmap, and a ranked list of compounds was obtained, displaying similarities in gene expression profiles with other cardiac glycosides and also with a few protein synthesis inhibitors (**Table 2**). Thus, our results indicated that cancer

cells treated with CGs (i.e., digoxin) upregulate and downregulate the same set of genes as they do if they are treated with protein synthesis inhibitors such as cycloheximide, anisomycin, or lycorine. While we were performing further studies to explore this possible mechanism, another group published results strongly pointing in the same direction, also concluding that CGs act as inhibitors of protein synthesis.[72]

Table 2. Connectivity Map (cmap) results after treatment of MCF-7 cells with digoxin (10 μM). The results for digitoxin were similar.

rank	cmap name	mean	n	enrichment
1	helveticoside	0.910	6	0.996
2	proscillaridin	0.907	3	0.996
3	digitoxigenin	0.853	4	0.995
4	digoxin	0.878	4	0.995
5	digoxigenin	0.868	5	0.995
6	lanatoside C	0.900	6	0.995
7	ouabain	0.876	4	0.995
8	anisomycin	0.616	4	0.988

Protein Synthesis Inhibition

Our findings from the cmap analysis led us to further investigate the protein synthesis inhibition effects of CGs in sensitive leukemia cells, as well as in more resistant cell lines of colorectal origin. Both digoxin and digitoxin inhibited DNA, as well as protein synthesis, in leukemia CCRF-CEM and SUP-B15 cells. The effects were, however, only observed at relatively high concentrations, and at 100 nM, surprisingly, no significant effects were detected during the 24-hour observation period. At the highest concentration tested (1 μM), well exceeding the IC_{50} value for cytotoxicity, the effects on DNA and protein synthesis were similar in time and magnitude, indicating that the macromolecule synthesis was stopped as a consequence of toxic effects, possibly related to severe ionic imbalance (**Paper IV**).

However, in contrast to the results in the leukemia cell lines, protein synthesis in the more resistant colon cancer HCT116 cells was decreased more efficiently (i.e., at concentrations corresponding to cytotoxic activity) and at an earlier time point than DNA synthesis. As the HCT116 cell line is more tolerant to the cytotoxic effects of glycosides, slightly higher CG concentrations were used. With 1 μM digitoxin, protein synthesis in HCT116 cells was effectively inhibited at early time points (significant from 6 h), while DNA synthesis did not appear to slow down until 24 h. This concentration is comparable to the cytotoxic IC_{50} value for HCT116 cells measured in the FMCA (0.74 μM). The effects in two other colorectal adenocarcinoma cell lines (HT29 and CC20) were similar. The leukemic SUP-B15 cell line was approximately 500 times more sensitive to the cytotoxic effects of digitoxin,

with an IC_{50} value of 1.5 nM. Despite this, exposure of SUP-B15 cells to 10 nM digitoxin had no effect on protein synthesis up to 24 h, but it effectively reduced viability at 72 h. Results in the leukemic CCRF-CEM cell line were similar (**Paper IV**).

The recent report by Perne *et al*., proposed that the main mechanism of action of CGs is to inhibit general protein synthesis, mediated through effects on the Na^+/K^+-ATPase pump.[72] Our results support this finding, but only for the more resistant colon cancer cells. Protein synthesis is effectively inhibited at early time points, leading to decreased cell numbers at 72 h, indicating that the cells die due to their inability to synthesize vital proteins. However, this is not the case for the leukemia cell lines, where protein synthesis levels are unaffected at time points up to 24 h, including at concentrations that are indeed cytotoxic to these cells (measured at 72 h). Thus, for the leukemia cell lines it seems that the cells stop synthesizing proteins because they are dying, and the mechanism for the initiating mechanism for cytotoxicity is most probably mediated through a pathway other than protein synthesis inhibition.

Combination with Standard Chemotherapeutic Drugs

Interactions with other cancer drugs are important to investigate, and synergistic activities may be used in the clinic to reduce toxicity and to enhance the anticancer activity of drug treatment. In **Paper III**, the effects of cardiac glycosides were evaluated in combination with the clinically used colon cancer drugs cisplatin, 5-fluorouracil, irinotecan, and oxaliplatin. Using the median effect method of Chou and Talalay,[91] with a fixed ratio design (i.e., varying the concentration of both drugs), all compounds tested showed additive activity in combination with 5-fluorouracil, while they were synergistic (digoxin; digitonin; convallatoxin, in HT29 cells) or additive (digitoxin; convallatoxin, in HCT116) in combination with oxaliplatin. Interestingly, the combination of the clinically used digoxin with oxaliplatin retained synergistic cytoxicity also for the otherwise highly drug-resistant HT29 cell line. In the case of irinotecan, commonly used as a second-line therapy for patients with advanced colon cancer, opposing effects were found when combined with cardiac glycosides, depending on the cell line. Concurrent exposure of colon cancer cell lines to irinotecan and CGs demonstrated cell-specific activities ranging from synergistic cytotoxicity in HCT116 cells, to antagonism in HT29 cells. The cellular events underlying this intriguing observation are unknown, but may be associated with multifactorial mechanisms contributing to multidrug resistance in the HT29 cell line. Interestingly, an interaction between topoisomerase II inhibition and cardiac glycosides has been previously noted, where digitoxin significantly reduced the etoposide- and idarubicin-induced topoisomerase II cleavable complexes in K562 leukemia

cells.[106] Also, blockade of the Na^+/K^+-ATPase pump by ouabain caused doxorubicin resistance and decreased topoisomerase II–mediated DNA strand breakage in a hamster cell line,[107] as well as in three human tumor cell lines, including HT29 colon carcinoma cells.[108]

Additional experiments with fixed concentrations of convallatoxin, digitoxin, and digoxin were also performed. At clinically achievable (plasma) concentrations the effects of the interaction were marginal (**Paper III** - Supporting Information).

The preliminary combination experiment (mentioned in **Paper I**) including the GA analogues (dihydro GA, GA, GA amide, tetrahydro GA), in combination with oxaliplatin or irinotecan did not indicate any synergistic activity. However, investigations including other chemotherapeutic agents, at different concentrations and involving other tumor types, could be of interest for future studies and for the evaluation of GA as an anticancer agent.

Conclusions and Future Perspectives

Natural products are important in the development of anticancer drugs. Using modern HTS techniques, compounds collected in libraries can be screened for new biological activities. In this thesis, GA analogues and CGs were identified as hits in a screening for cytotoxic activity in colon cancer cells, and were selected for further investigation. Even though many compounds of commercially available libraries have already been previously studied, in-depth studies of the molecular mechanisms of action can add to the knowledge about important drug targets. When using compound libraries, caution about the compounds is recommended and verification or counter screens should be performed, as compounds potentially can be degraded.

Studies of drug-induced gene expression changes by cmap analysis proved to be a very useful method for generating hypotheses of possible mechanisms of action. Using other methods, we confirmed both the protein synthesis–inhibiting activity of CGs, and the proteasome-inhibitory properties of GA. However, it is important to keep in mind that different tumor types can react differently to the drugs tested. The results of the gene expression analysis may depend on the selected cell line, as well as on the selected drug concentration.

Gambogic acid is a naturally occurring compound with interesting biological activities and with potential to be used as a lead structure for development of novel anticancer agents. The cytotoxic effects of GA have been previously acknowledged, but the mechanism of action has not yet been clarified.

In the current work GA and closely related analogues showed cytotoxic activity in cancer cells of various origins. From analyses using the resistance-based cell line panel and the ChemGPS-NP model, we concluded, in line with a previous report,[42] that GA acts through a different mechanism than the classic anticancer agents. Connectivity map analysis indicated a similar gene profile, as compared to other compounds acting on the ubiquitin-proteasome system, and our studies showed that GA and dihydro GA caused accumulation of ubiquitin in MelJuSo Ub^{G76V}-YFP cells. Further investigations led us to the conclusion that the GA analogues act as inhibitors of the ubiquitin-proteasome system through inhibition of 20S chymotrypsin activity.

The proteasome inhibitor bortezomib has shown promising activity as an anticancer drug, and the UPS is indeed an important drug target for future cancer treatment. The potential of GA as an anticancer agent needs further investigation. The outcome of ongoing clinical trials will be crucial, and the UPS-inhibiting activity of GA should be clarified to deduce whether tolerable concentrations are effective in clinical use of GA as a proteasome inhibitor. It would also be valuable to perform more extensive studies on potential synergistic interactions with other chemotherapeutic agents that act through different mechanisms of action.

Cardiac glycosides have previously shown promising anticancer activities in several tumor types. The results presented in this thesis show that CGs are also cytotoxic in generally resistant human colon cancer cells, but at concentrations not achievable in human plasma. A few CGs also displayed synergistic activity when combined with standard chemotherapeutic agents, where the combination of digoxin and oxaliplatin was more effective than either of the drugs alone.

The mechanism of action of CGs has not been fully elucidated, although many different molecular effects have been described. Our analyses could not confirm any NF-κB–inhibiting activity, or any effect on intracellular Ca^{2+} oscillations. From cmap analysis and *in vitro* experiments it was shown that CGs are inhibitors of protein synthesis in colon cancer cell lines, but not in the more sensitive leukemia cell lines. The effects observed in cells of colorectal origin are supported by results recently published by another group,[72] in which they conclude that the potential of CGs as anticancer drugs is limited, due to inhibition of general protein synthesis. However, the results included in this thesis also demonstrate that CGs may have clinical potential in leukemic cells, since the cytotoxic activity appears to be mediated through another, yet unknown, mechanism of action, and at considerably lower concentrations. Thus, it seems likely that CGs affect the cells differently, depending on the tumor type.

It is suggested that future research should be directed to further investigations of the activities of CGs in hematological cancers, and to the interactions with other chemotherapeutic drugs used in these diagnoses. Previous epidemiological observations have shown potential chemopreventive activities of digitalis in breast cancer, and such studies might be of interest also for other malignancies.

In conclusion, by combining several methods and modern techniques new mechanisms of action are demonstrated for two supposedly well-characterized families of natural products. Hence, new targets can be revealed for already known compounds. This approach has potential to be very useful for expanding the knowledge about intracellular mechanisms that are of importance for future chemotherapeutic treatment.

Populärvetenskaplig Sammanfattning

Cancer är en term som används för en grupp sjukdomar som alla har gemensamt att de beror på onormal celltillväxt i ett eller flera organ i kroppen. I normala celler regleras tillväxt och spridning genom cellens normala signaleringsvägar, men i en cancercell har något gått fel och viktiga signaler för hur cellen skall styras kan vara felaktiga eller utebli helt. Därmed kan cellen ohämmat växa och dela sig så att en grupp cancerceller bildas. Detta kallas för en tumör. De vanligaste cancerformerna är prostatacancer hos män och bröstcancer hos kvinnor, tätt följda av lungcancer och koloncancer (tarmcancer). Tumörsjukdomar drabbar många människor och behandlingen behöver effektiviseras.

Naturen har alltid varit en viktig källa för att bota, lindra och förebygga sjukdom. Ett flertal av de läkemedel som idag används har ett naturligt ursprung. Många läkemedel mot cancer är framställda utifrån molekyler som forskare har funnit i växter. Till exempel finns ett vanligt läkemedel mot bröstcancer där den aktiva substansen paklitaxel ursprungligen renframställdes ur bark från idegranar.

Växter och djur tillverkar olika ämnen för att försvara sig mot fiender. Dessa ämnen är avsedda att avskräcka eller döda angripare och kan ha flera biologiska effekter Ämnenas biologiska aktiviteter kan utnyttjas farmakologiskt för att påverka sjukdomsprocesser i människokroppen. Det gäller dock att hitta en lagom dos och att undersöka molekylens effekter noggrant för att undvika biverkningar och oönskade sidoeffekter.

Den framgångsrika utvecklingen inom molekylärbiologi och kemi har lett till stora framsteg inom farmakologi och läkemedelsframställning. Molekyler kan samlas in till stora substansbibliotek och utnyttjas sedan i läkemedelsforskning. Ämnenas biologiska effekter kan studeras på molekylnivå med hjälp av olika instrument och analysmetoder.

I den här avhandlingen beskrivs ett forskningsprojekt där vi har identifierat molekyler från naturen som kan döda cancerceller. I vår studie användes ett stort substansbibliotek med 2000 olika ämnen, varav ungefär 600 är molekyler med naturligt ursprung. De övriga ämnena är syntetiskt framställda. Cancerceller behandlades med små mängder av varje ämne och vi undersökte

sedan hur många procent av cellerna som kunde dödas med respektive substans.

Utifrån denna analys kunde vi hitta molekyler som effektivt dödade cancercellerna. Vi valde ut två olika grupper av molekyler som inbördes liknar varandra, om man ser till molekylernas struktur. Den ena gruppen kallas för hjärtglykosider och innefattar molekyler såsom digitoxin och digoxin, som länge har använts i behandling av hjärtsjukdom. Ursprungligen kommer dessa substanser från fingerborgsblomman, *Digitalis purpurea.* Den andra gruppen bestod av gambogiasyra (eng. gambogic acid) och mycket snarlika molekyler (analoger).

Gambogiasyra är en substans som ursprungligen kommer från ett träd som växer i Asien. *Garcinia hanburyi* är det latinska namnet för detta träd och frukten mangostan hör till samma släkt som den här arten.

Den cancercell-dödande effekten av hjärtglykosider och gambogiasyra utvärderades mera noggrant för att se vilka doser som var effektiva. Dessutom var vi intresserade av att undersöka vilka bakomliggande mekanismer i cellen som påverkades, d.v.s. på vilket sätt de här ämnena kunde döda cancercellerna.

När en cell utsätts för ett biologiskt aktivt ämne leder det ofta till att olika signaler sätts igång i cellen. Genom att behandla en tumör med ett läkemedel får cancercellerna på något vis en signal om att dö. Att ta reda på mekanismen för hur denna signalering går till inne i cellen är komplicerat och kräver studier i flera steg. Vi började därför med att undersöka hur cellen reagerar på gen-nivå vid behandling med hjärtglykosider respektive gambogiasyra. Förändringar i uttrycket av olika gener leder till förändringar i cellens produktion av specifika, viktiga proteiner. Resultaten från gen-analysen jämfördes med analyser från välkända läkemedel och andra molekyler som är väl studerade sedan tidigare.

Jämförelsen visade att när cellerna utsattes för hjärtglykosider påverkades cellens generella proteinsyntes och vi undersökte senare om så kunde vara fallet. Det verkade stämma att proteinsyntesen påverkades i koloncancerceller, men i leukemiceller tycktes inte mekanismen vara densamma. Vår slutsats blev därför att olika cancerformer påverkas på olika sätt vid behandling med hjärtglykosider.

När celler utsattes för gambogiasyra visade gen-analysen att många gener påverkades och den gemensamma nämnaren kunde troligtvis vara proteasomen. Proteasomen finns inne i cellen och är uppbyggd av proteiner. Den har en viktig roll eftersom den bryter ned andra cellulära proteiner. Forskare har tidigare visat att man kan använda farmakologiska substanser för att påverka proteasomen så att dess aktivitet minskar. Cancerceller har ofta en onormalt hög proteasom-aktivitet och genom att blockera denna aktivitet i tumörcel-

lerna kan man hämma cancerns tillväxt. Det finns t.ex. ett läkemedel (Velcade®) som innehåller ämnet bortezomib som fungerar på detta sätt.

Även denna hypotes testades genom olika laborativa försök med cancerceller och resultaten visade att gambogiasyra hämmade proteasomens aktivitet.

Sammanfattningsvis kan man säga att denna avhandling beskriver grundforskning kring några utvalda ämnen med naturligt ursprung och deras cancercelldödande effekter. Växter och djur framställer många ämnen för att försvara sig och de ämnena kan vi utnyttja i läkemedelsutvecklingen. Vid de laborativa försök som har utförts i detta projekt har vi använt cell-linjer samt tumörceller från patienter. Huruvida hjärtglykosider eller gambogiasyra kan användas för att behandla tumörer hos människor är ännu oklart och återstår att utreda. De studier som beskrivs i den här avhandlingen har bidragit till kunskapen om cellulära effekter av hjärtglykosider och gambogiasyra och kan kanske på sikt bidra till utvecklingen av nya läkemedel.

Acknowledgments

I would like to express my sincere gratitude to everyone who, in one way or another, has supported me during the years as a PhD student:

My supervisors:

Prof. Lars Bohlin, for interesting scientific discussions, and for guidance in the pharmacognosy field of research.

Dr. Joachim Gullbo, for being encouraging and supportive, and for teaching me a lot about cancer research and science in general.

Dr. Ulf Göransson for contributing with good ideas and valuable comments.

All former and present colleagues in the Division of Pharmacognosy:

Dr. Jan G. Bruhn, *Dr. Per Claeson* and *Dr. Premila Perera* for inspiring me to work with Pharmacognosy research.

Dr. Anders Backlund, for encouraging me in developing my teaching skills and for help with computer-related issues.

Dr. Ulrika Huss Melin, for your kind support and for sharing experiences and knowledge. Thank you for all the good advice and nice chats!

Dr. Robert Burman and *Dr. Josefin Rosén*, for friendship during the years as PhD students, sharing the tough parts, as well as the good times, including enjoyable travels abroad.

My roommates, *Elisabet Vikeved,* for good company, *Dr. Erika Svedlund*, for advice and support, and *Dr. Catarina Ekenäs*, for relaxed discussions about both scientific and non-scientific subjects, and for pleasant field trips to Sri Lanka and Taiwan with the Global Pharmacy course.

Dr. Sonny Larsson, Dr. Erik Hedner, Dr. Anders Herrmann, Dr. Petra Lindholm, Dr. Sofia Ortlepp, Stefan Svan, Park Sungkyu, Teshome Leta Aboye, Mariamawit Yonathan Yeshak, Dr. Hesham El-Seedi, Dr. Cecilia Alsmark, Dr. Christina Wedén, Dr. Adam Strömstedt, Dr. Sunithi Gunasekera, Kerstin Ståhlberg, Maj Blad, Gunilla Eriksson, Eva Strömberg, Anna-Maria Andersson, Åke Strese, Dr. Wimal Pathmasiri, Dr. Martin Sjögren & Dr. Mia Dahlström: We have shared many good times over the years "working hard and playing hard!"

Magnus Jansson, for assistance in computer-related issues.

All my co-authors for fruitful collaborations:

Dr. Linda Rickardson, for sharing your expertise in working with compound libraries, cancer cells, assays, methods, equipment, and for valuable comments and discussions. It has been a pleasure to work together!!

Dr. Malin Wickström, for always being very helpful, sharing your laboratory skills and scientific knowledge, and *Caroline Haglund* and *Anna Eriksson*, for successful collaborations as well as good company in the lab and in the lunchroom.

Prof. Rolf Larsson, for good advice, and for providing nice working facilities and equipment at the Division of Clinical Pharmacology, *Dr. Mårten Fryknäs*, for good advices and for contributing with your knowledge about gene expression analyses and cmap, *Dr. Magnus Lindskog* for generating creative, open-minded ideas, *Dr. Helene Hallböök* for good collaboration in the cardiac glycoside ALL project, and *Prof. Peter Nygren* for expertise in the field of colon cancer research.

Dr. Karolina Lesiak-Mieczkowska and *Prof. Stig Linder* for good collaboration in the gambogic acid project.

Prof. Joseph Rafter and *Dr. Pernilla Karlsson*, for sharing your expertise in colon cancer research and cell culturing.

Prof. Rob Verpoorte and *Dr. Young Hae Choi*, for introducing me to the field of metabolomics. Special thanks to *Rob* for valuable discussions regarding my research.

All colleagues at the Division of Clinical Pharmacology, especially *Lena Lenhammar*, *Christina Leek,* and *Anna-Karin Lannergård* for being very helpful in the lab.

Prof. Erik Gylfe and *Dr. Anders Tengholm* for providing the equipment for measurement of intracellular calcium oscillations. Special thanks to *Helene Dansk* for helpful instructions.

My students:

Sara Laitinen, for good work with the calcium oscillation experiments, and special thanks for providing photos for my thesis!

Sandra Erlund, for your ambitious work on the literature survey of gambogic acid.

All former and present colleagues in the Division of Analytical Pharmaceutical Chemistry for company in the lunch/coffee room in A5:3.

Ann-Marie Falk, Maria Swartling, Emma Lundkvist and *Emma Boström* for interesting discussions about teaching and pharmacotherapy.

Värmlands nation, Uddeholms Aktiebolags stipendiefond, Swedish Academy of Pharmaceutical Sciences (Apotekarsocieteten) and *Uppsala University* for the scholarships and travel grants that enabled my PhD studies as well as my participation in several international conferences.

Dr. Renee Ruhaak and *Dr. Marica Fraccaroli,* for being close friends, even though you live far away, and for sharing your experiences from international research. Additional thanks to *Renee* for good collaboration while working on the cannabinoid article!

Karin Önneby and *Dr. Elina Hjertström*, for sharing experiences of life being a PhD student and a Mother, and *Karin Sandberg*, for encouraging me and showing interest in my research. *All my other Friends* outside the university walls for being just friends, sharing good times. Relaxing and having fun once in a while is important!

My parents *Kajsa* and *Per* and my siblings *Erik, Anders*, and *Sara* for being such a wonderful family. I also want to thank all my other *relatives and extended families* for being there. Especially, my grandparents, *Barbro, Torsten, Margareta,* and *Folke*, for sharing your life experiences as I was growing up, and for being honorable people to look up to.

My dearly loved family, *Per-Axel & Gunnar*, the best guys in the world — you are forever in my heart!

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