Preclinical Characterization in vivo and in vitro of Novel Agents for Cancer Chemotherapy

Studies on Benomyl, Carbendazim, Cryptolepine and Acriflavine

DANIEL LARYEA
Dissertation presented at Uppsala University to be publicly examined in Enghoffsalen, Entrance 50, Ground Floor, Uppsala University Hospital, Uppsala, Friday, October 15, 2010 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Preclinical methods for the identification and characterization of molecules for development into new cancer drugs were investigated. Based on repurposing, i.e. the exploration of currently prescribed drugs for new indications, and as a result of a new high throughput screening (HTS) approach, the benzimidazoles benomyl and carbendazim, the alkaloid cryptolepine and the acridine acriflavine were found interesting to characterize using these methods.

In mice the benzimidazoles inhibited $^3$H-thymidine incorporation in tissues with high cell renewal, with benomyl being more active than carbendazim. They were rapidly absorbed with highest amounts seen in the liver, kidneys and gastro-intestinal lumen as evidenced from distribution of $^{14}$C-labeled drugs. In human tumour cell lines, the benzimidazoles showed a similar activity pattern but benomyl was more potent. This was true also in tumour cells from patients but carbendazim was relatively more active against solid tumours. Analyses of drug activity cross-resistance patterns and of drug activity – gene expression correlations in a cell line panel suggested multiple mechanisms of action for the benzimidazoles.

Cryptolepine was widely distributed to tissues in vivo in the mice. It was more potent than the benzimidazoles in tumour cells, with highest activity in haematological malignancies but some patient samples of breast, colon and non small-cell lung cancer were sensitive. Cross-resistance analysis indicated cryptolepine to be a topoisomerase II inhibitor whereas drug activity – gene expression correlations suggested additional mechanisms of action.

HTS on 2 000 molecules in colon cancer cell lines and normal cells identified acriflavine as a hit molecule, subsequently shown to have unprecedented activity against colorectal cancer tumour cells in patient tumour samples. Connectivity map analysis, based on drug induced gene expression perturbation patterns in a tumour cell line, indicated acriflavine to be a topoisomerase inhibitor, subsequently confirmed in a plasmid relaxation assay. In conclusion, repurposing of drugs and HTS using stringent activity criteria followed by preclinical characterization might contribute to more efficient development of new cancer drugs.

Keywords: Benzimidazoles, cryptolepine, acriflavine, cancer, high throughput screening, cytotoxic drug, gene expression

Daniel Laryea, Department of Medical Sciences, Clinical Pharmacology, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

© Daniel Laryea 2010

ISSN 1651-6206
ISBN 978-91-554-7882-7
urn:nbn:se:uu:diva-130330 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-130330)
A lot of advances in our understanding of cancer have been made. It is too naive however to think that we could completely eliminate cancer in my time, but it is very realistic to think that in the decades ahead of medical science we can and will be able to make a better prediction and control this disease much better partly with new chemotherapeutic drugs and other anti-cancer paradigms. Thus no matter how little this thesis may be, it is my contribution to that epoch when all forms of cancer will become curable.


Everyday is a new day gratefully accepted with a chance to love people and do something better with life. [DL]
LIST of PAPERS

This thesis is based on the following papers and will be referred to in the text by their respective Roman numbers:


Reprints were made with the permission of the publishers.
Other related published articles involving the author of the thesis.

- Hellman B, **Laryea D** Inhibitory effects of benomyl and carbendazim on the 3H-thymidine incorporation in various organs of the mouse - evidence for a more pronounced action of benomyl. *Toxicology 1990;61:161-169.*


Back cover picture: Summary of the thesis in pictures and diagrams

"One Picture is Worth Ten Thousand Words," - Confucius
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Cancer chemotherapy</td>
<td></td>
</tr>
<tr>
<td>Cancer drug development strategies</td>
<td></td>
</tr>
<tr>
<td>High-throughput screening</td>
<td></td>
</tr>
<tr>
<td>Repurposing</td>
<td></td>
</tr>
<tr>
<td>Basic aspects of the molecules investigated</td>
<td></td>
</tr>
<tr>
<td>Aims of the thesis</td>
<td>14</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>15</td>
</tr>
<tr>
<td><strong>In vivo experiments</strong></td>
<td></td>
</tr>
<tr>
<td>Whole body autoradiography</td>
<td></td>
</tr>
<tr>
<td><em>In vivo</em> measurement of $^3$H-thymidine incorporation</td>
<td></td>
</tr>
<tr>
<td><em>In vivo</em> positron emission tomography (PET)</td>
<td></td>
</tr>
<tr>
<td><strong>In vitro experiments</strong></td>
<td></td>
</tr>
<tr>
<td>Patient tumour cells</td>
<td></td>
</tr>
<tr>
<td>Human tumour cell lines</td>
<td></td>
</tr>
<tr>
<td>Drugs</td>
<td></td>
</tr>
<tr>
<td>Measurement of cytotoxicity</td>
<td></td>
</tr>
<tr>
<td>Mechanistic characterization of the experimental drugs</td>
<td></td>
</tr>
<tr>
<td>Data presentation and statistics</td>
<td></td>
</tr>
<tr>
<td>Results and discussion</td>
<td>21</td>
</tr>
<tr>
<td><strong>Paper I</strong>: Effects of benomyl and carbendazim <em>in vivo</em></td>
<td></td>
</tr>
<tr>
<td>Unpublished data on the <em>in vivo</em> distribution of the benzimidazoles</td>
<td></td>
</tr>
<tr>
<td>Unpublished data on the effect of benomyl <em>in vivo</em> as assessed by PET</td>
<td></td>
</tr>
<tr>
<td><strong>Paper II</strong>: Characterization <em>in vitro</em> of the cytotoxic activity of benomyl and carbendazim</td>
<td></td>
</tr>
<tr>
<td><strong>Paper III</strong>: Characterization <em>in vitro</em> of the cytotoxic activity of cryptolepine</td>
<td></td>
</tr>
<tr>
<td>Unpublished data on the properties of cryptolepine <em>in vivo</em> and <em>in vitro</em></td>
<td></td>
</tr>
<tr>
<td><strong>Paper IV</strong>: HTS as a method to identify diagnosis specific cancer drugs</td>
<td></td>
</tr>
<tr>
<td>General discussion</td>
<td>42</td>
</tr>
<tr>
<td>Summary and conclusions</td>
<td>46</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>47</td>
</tr>
<tr>
<td>References</td>
<td>51</td>
</tr>
</tbody>
</table>
Abbreviations

ABC  ATP-binding cassette superfamily of membrane transporters
ACCID  Accession identification number
ALDH  Aldehyde dehydrogenase
ALL  Acute lymphoblastic leukemia
AML  Acute myelocytic leukemia
BBB  Blood brain barrier
76Br-BFU  Bromide-76-labeled 1-(2’-fluoro-β-D-arabinofuranosyl)-5-bromouracil
b.w.  Body weight
14C  Carbon-14 radionuclide
CLL  Chronic lymphocytic leukemia
Cmap  Connectivity map
CNS  Central nervous system
cpm  Counts per minute
CRC  Colorectal cancer
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DPM  Disintegration per minute
FDA  Fluorescein diacetate
18F-FDG  Fluoride-18-labeled 2-deoxy-2-fluoro-D-glucose
FMCA  Fluorometric microculture cytotoxicity assay
GIT  Gastrointestinal tract
3H  Tritium-labeled
i.p.  Intraperitoneal
i.v.  Intravenous
IC50  Concentration producing 50% inhibition
11C-Met  Carbon-11-labeled L-methionine
Mbq  Megabequerel
MDR  Multi-drug resistance
MRP  Multi-drug resistance related protein
MTD  Maximum tolerated dose
NHL  Non-Hodgkin’s lymphoma
NOEL  No-observed effect level
OC  Ovarian cancer
NSCLC  Non-small cell lung cancer
PBMC  Peripheral blood mononuclear cell
PBS  Phosphate buffer saline
PET  Positron emission tomography
P-gp  P-glycoprotein
p.o.  Per oral
RF  Resistance factor
RNA  Ribonucleic acid
RNS  Reactive nitrogen species
ROS  Reactive oxygen species
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI%</td>
<td>Survival index in percentage</td>
</tr>
<tr>
<td>SUV</td>
<td>Standardized uptake value</td>
</tr>
<tr>
<td>TdR</td>
<td>Thymidine</td>
</tr>
<tr>
<td>Topo</td>
<td>Topoisomerase</td>
</tr>
<tr>
<td>WBA</td>
<td>Whole-body autoradiography</td>
</tr>
</tbody>
</table>
Introduction
Cancer chemotherapy

Cancer chemotherapy is the application of chemical compounds with various mechanisms of action against the growth and survival of tumour cells. It is administered with a goal to cure or to alleviate tumour-related symptoms and prolong life. Chemotherapy is one of several modalities used in the clinical management of cancer and dates back to the serendipitous discovery of mustard alkylating agents in the 1940s [1]. Cancer chemotherapy celebrated its fiftieth anniversary in 1995 [2], and still remains a major clinical strategy against many tumour types.

Chemotherapy can be given alone as the only treatment or in combination with surgery and radiotherapy [3]. Indeed, some agents such as cisplatin have had a great beneficial impact on the survival of testicular cancer patients, raising hopes that all cancers could eventually become curable by chemotherapy.

Tremendous efforts have been made to discover new chemotherapeutic drugs for treatment of the major cancer types. However, previous and current test systems have identified only a limited number of clinically active agents, often with limited efficacy and associated with pronounced toxicity. Despite this, chemotherapy as used in the clinic today plays a significant role in reducing cancer mortality and morbidity and has improved the quality of life of cancer patients.

The number of investigational cancer drugs that fail in the clinic for various reasons far outweighs those considered effective, suggesting that the selection procedure for progression of molecules into the clinic is in need of improvement [4]. Drug resistance and intolerance are the two major reasons for failure of new drugs. The need to discover and develop new cancer drugs is thus unequivocal.

Cancer drug development strategies

In principle, there are three established strategies in use for the development of new anti-cancer drugs: (i) rational drug design, (ii) drug screening and (iii) development of analogues with improved properties of drugs already available [5]. Repurposing of a molecule from a current non-anticancer application to cancer chemotherapy evaluation could be considered a fourth strategy that might accelerate cancer drug development [6,7].
Targeting is a major approach to rational drug design and is receiving increasing intention [8]. In contrast to empirical drug development, the targeting paradigm involves the accumulation of information on the causes of the tumour cell phenotype followed by development of a molecule interacting with a suitable drug target. Such targets are often part of an array of organized proteins that form functional pathways. Deregulated pathways can often be linked to genomic aberrations that can be traced by genomic profiling, and used as a basis for diagnosis and therapeutic considerations.

Target-based drug discovery begins with identifying and validating the function of a possible therapeutic target and its role in disease [9]. This can be a difficult task given the abundance of signalling pathways with extensive interactions. The ‘drugability’ of a given target is defined by how well a therapeutic small molecule or biotherapeutic can reach the target and by the effect a therapeutic agent actually has. To date, targeted therapy has mostly focused on membrane receptors bearing intracellular kinase motifs.

**High-throughput screening**

High-throughput screening (HTS) is an approach in cancer drug discovery in which a large number of potentially biologically active compounds are screened for cytotoxic activity in established tumour cell lines [10]. HTS has been crucial for the development of technologies currently in use in various drug discovery platforms. One important goal of HTS is to accelerate drug discovery. The major HTS platform for development of cancer drugs has been the US National Cancer Institute panel of 60 tumour cell lines with companion strategies for data handling and presentation [11].

**Repurposing**

The targeting approach has produced several new useful cancer drugs, yet progress in the medical treatment of the major cancer types using the new ‘targeted’ drugs has been modest [4]. Moreover, the development of a new chemical entity is extremely costly and takes many years. One way of speeding up the drug development process is by repurposing drugs that are already in use for other indications. Repurposing was one approach in the work described in this thesis, as benomyl and carbendazim are
approved as agricultural fungicides [12,13] and cryptolepine is ethnomedically used for various non-cancer indications in tropical Africa [14]. Furthermore, the screening hit in paper IV, acriflavine, has been in use against microbes but has also been tested previously in cancer models [15,16]. These agents might serve as examples of repurposing drugs for cancer chemotherapy. The potential of repurposing is well illustrated by sildenafil, initially intended for use in hypertension and angina pectoris [17], but which unexpectedly provided a remedy for erectile dysfunction and has become a billion dollar selling drug for that purpose.

**Basic aspects of the molecules investigated**

Benomyl and carbendazim are benzimidazoles, a group of ‘privileged’ chemical compounds forming lead structures in various drugs [18], e.g. in omeprazole for gastroesophageal ulceration and astemizole for asthma. Benomyl and carbendazim are currently used in the food production industry as fungicides and antihelmintics. The proposed cytotoxic mechanisms of action of these compounds in fungi as microtubule inhibitors [19,20] make them interesting for cancer drug development. All benzimidazoles are thought to have a similar mode of action and differences in activity among these drugs have been considered a consequence of differences in bioavailability [21].

The stem and roots of *Cryptolepsis sanguinolenta*, a plant in tropical West Africa producing the alkaloid cryptolepine, have been extracted and used as a concoction by natives for decades for the treatment of various diagnoses, including type 2 diabetes, hypertension, fever, inflammation and various microbial diseases [14,22,23]. Other plant species with similar ingredients are also found in the Caribbean and some parts of southern Asia. Decades of human use against different forms of ailments, indicating acceptable safety, could contribute to the rapid development of cryptolepine into a cancer drug.

Acriflavine binds to DNA and has been used as anti-microbial agent but has also been tested in cancer models [15,16]. Figure 1 shows the molecular structures of the molecules investigated in this thesis.
Figure 1. Chemical structures of the various molecules investigated in this thesis. Benomyl and carbendazim belong to the chemical group of benzimidazoles, cryptolepine is a natural indoloquinoline alkaloid product, and acriflavine, a mixture of two related molecules, belongs to the acridyl group of chemical compounds.
Aims of the thesis

The overall aims of the thesis were to preclinically characterize repurposed molecules, based on proposed mechanisms of action and current use, to potential cancer drugs and to investigate HTS as a means to identify molecules with potential for development into cancer drugs.

The specific aims were to:

1. Explore the disposition and normal tissue DNA synthesis inhibitory activity of the benzimidazole compounds benomyl and carbendazim in vivo in a mouse model.

2. Characterize in vitro the cytotoxic activity of benomyl, carbendazim and cryptolepine in a human tumour cell line panel and in primary cultures of tumour cells from patients with various tumour types.

3. Investigate HTS as a tool for the discovery of molecules with promising activity against a specific cancer type, i.e. colorectal cancer (CRC).

4. Investigate the potential of various genomics-based approaches for delineating mechanisms of action of investigational cancer drugs.
Materials and methods

In vivo experiments (paper I and unpublished data)

Whole-body autoradiography (WBA)

The WBA technique with whole-body sectioning as previously described [24] was carried out for distribution studies on labeled drugs and for assessment of their effects on normal tissue cell proliferation. The method enables comparison between normal tissue distribution of a radiolabeled drug and a tumour xenograft (not reported in this thesis). Anatomical details (Fig 2) including xenografted tumours allow the detailed analysis of phenomena such as drug distribution. WBA can also be used to trace drug receptors [25,26].

WBA is quantitative and has become a versatile tool for assessment of uptake, distribution and excretion of labeled drugs and metabolites and could provide information on covalent drug binding to macromolecules. However, limitations in sensitivity and resolution need to be considered in the interpretation of data. Though mainly applied to in vivo settings, freeze-dried tissue can be incubated with a labeled drug in order to trace a receptor in vitro.

Figure 2. Picture of a 60 μm freeze-dried whole-body section in a left sagittal plane of a laboratory mouse showing anatomical details distinguishable in autoradiography of a radio-labeled compound. WBA is an expedient in vivo technique important in drug development. Organs/tissues: 1, nasal turbinates & mucosa; 2, brain; 3, spinal cord & vertebral column; 4, brown fat; 5, blood vessels; 6, liver; 7, tumour xenograft; 8, iliac bone marrow; 9, fur on skin; 10, tongue; 11, salivary gland; 12, thymus; 13, sternal bone marrow; 14, heart; 15, lung; 16, liver; 17, stomach & oesophagus; 18, intestines; 19, white fat; 20, skeletal muscle.
**In vivo measurement of $^3$H-TdR incorporation**

Following drug exposure, thymus, spleen, liver, kidney and testis were rapidly removed and frozen on dry ice. Tissues were freeze-dried followed by weighing until radioactivity measurement. Subsequent homogenization and DNA extraction procedures of the freeze-dried tissues were carried out as described previously [27]. After addition of 10 ml Instagel to an aliquot of 4 ml DNA extract from a tissue, the tritium content was measured in a Packard Tri-Carb 460 CD liquid scintillation spectrometer. Corrections for quenching were made using an external standard.

**In vivo positron emission tomography (PET)**

PET radionuclides ($^{11}$C-L-methionine, $^{18}$F-FDG, and $^{76}$Br-BFU) were supplied by the Uppsala University PET Centre and were used as tracers to monitor the effect of benomyl on protein synthesis, glucose (energy) metabolism and cell proliferation (DNA synthesis) in laboratory mice. Details of the experimental approach are described in the Results and discussion section.

**In vitro experiments (papers II, III and IV)**

**Patient tumour cells**

Permission to obtain patient tumour samples for preparation of tumour cells was obtained from the Ethical Committee of Uppsala University. Briefly, tissue from solid tumours was finely minced and tumour cells were then isolated by collagenase dispersion followed by Percoll (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation [28]. Ovarian cancer (OC) cells from ascitic fluid were collected by centrifugation, followed by purification of the cells by Ficoll-Paque and Percoll density-gradient centrifugation. Chronic lymphocytic leukemia (CLL) cells and peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density gradient centrifugation on a 1.077 g/ml Ficoll-Paque (Pharmacia Biotech) gradient [28]. Cell viability was routinely greater than 90% as determined using a trypan blue exclusion test. The proportion of tumour cells in the preparations, as judged by inspection of May-Grunwald-Giemsa-stained cytospin preparations was greater than 70%.
Human tumour cell lines

Table 1 outlines the tumour type of origin and state of drug-resistance of the tumour cell lines forming the cell line panel [29]. The hTERT-RPE1 cells are untransformed cells representing normal epithelial cells.

With the exception of the kidney tumour cell line, ACHN, and the cervix cancer cell line, HeLa, the cell lines are pairs of parental and resistant phenotypes. These pairs were used to characterize the resistance mechanisms of the experimental drugs. The cell line panel was previously characterized in our laboratory for gene expression by microarray analysis and these data were used for mechanistic considerations in papers II and III [30]. Three colon cancer cell lines, HCT-116, HT-29, and CC20 were additionally used for HTS against CRC in paper IV. Furthermore, the epithelial breast cancer cell line, MCF7 was used for the genomics-based mechanistic analysis of acriflavine in paper IV.

Table 1. Human tumour cell lines in the cell line panel used for drug characterization.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Diagnosis</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 8226</td>
<td>Multiple myeloma</td>
<td>Parental</td>
</tr>
<tr>
<td>8226/Dox</td>
<td>Multiple myeloma</td>
<td>Doxorubicin-selected resistance</td>
</tr>
<tr>
<td>8226/LR5</td>
<td>Multiple myeloma</td>
<td>Melphalan-selected resistance</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>T-Cell Leukemia (ALL)</td>
<td>Parental</td>
</tr>
<tr>
<td>CEM/VM1</td>
<td>T-Cell Leukemia (ALL)</td>
<td>Teniposide-selected resistance</td>
</tr>
<tr>
<td>U937/GTB</td>
<td>Histiocytic Lymphoma</td>
<td>Parental</td>
</tr>
<tr>
<td>U937/Vcr</td>
<td>Histiocytic Lymphoma</td>
<td>Vincristine-selected resistance</td>
</tr>
<tr>
<td>NCI H69</td>
<td>Small cell lung cancer</td>
<td>Parental</td>
</tr>
<tr>
<td>H69/AR</td>
<td>Small cell lung cancer</td>
<td>Doxorubicin-selected resistance</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix cancer</td>
<td>Parental</td>
</tr>
<tr>
<td>ACHN</td>
<td>Renal cancer</td>
<td>Parental</td>
</tr>
<tr>
<td>hTERT-RPE1</td>
<td>Immortalized normal retinal pigment epithelium</td>
<td>Parental</td>
</tr>
</tbody>
</table>

Drugs

Benomyl and carbendazim were purchased from Sigma-Aldrich, Germany and cryptolepine in its pure form was kindly supplied by Dr CW Wright (University of
Bradford, Pharmaceutical Faculty, UK). Acriflavine was part of the Spectrum library of 2000 compounds, supplied as 10 mM solutions in DMSO (Sigma-Aldrich). Acriflavine for the more extended drug characterization process was from Sigma-Aldrich (CAS 8063-24-9). The standard CRC active cytotoxic drugs 5-FU, irinotecan and oxaliplatin were supplied by the Uppsala University Hospital pharmacy.

In addition to the four test compounds, the mTOR inhibitor, rapamycin (Sigma-Aldrich) and the proteasome inhibitors disulfiram, lactacystin (Sigma-Aldrich) and bortezomib (Velcade, Millennium Pharmaceuticals, Cambridge, MA) were also tested in the cell line panel. Moreover, the cell line panel had been investigated previously for the effect of a number of mechanistically different established and experimental cytotoxic agents using the same experimental set up as described here [29,30]. Data from these investigations were stored in a database continuously updated with data from new agents and used to assess drug cross-resistance.

All compounds used were dissolved in DMSO to prepare stock solutions stored at -80ºC pending use for the preparation of microtitre drug plates for the fluorometric microculture cytotoxicity assay (FMCA) after dilution in phosphate buffered saline (PBS) [31].

Measurement of cytotoxicity

Cells were seeded into 384-well plates, prepared as described (5 000 cells/well for solid tumours and tumour cell lines; 40 000 cells/well for haematological malignancies). Three columns without drugs served as controls and one column with medium only served as blank. The plates were incubated at 37 ºC for 72 h after which they were analysed using the FMCA.

The FMCA was the main method used to assess the cytotoxic effect of the experimental compounds and is outlined in Figure 3. The assay is based on the presence of esterases in viable cells that convert colourless FDA to fluorescent fluorescein [32]. Fluorescence intensity after drug exposure thus indicates the amount of surviving cells when compared with unexposed control cells. The drug effect is expressed as survival index (SI%), i.e. the fluorescence of drug exposed cultures as a percentage of an unexposed control with blank values subtracted.
In contrast to the benzimidazoles and cryptolepine, acriflavine was selected after qualifying as an HTS 'hit' compound from the Spectrum annotated library. The softwares, SLIMS [33] and Vortex (Dotmatics Inc, UK) were employed to analyze the activity of the Spectrum library. Besides the FMCA, the hanging drop spheroid culture technique [34] was additionally employed using the HCT-116 cell line to assess the cytotoxicity of acriflavine and control drugs. Tests of topoisomerase inhibition were performed using kits from TopoGen Inc. (Port Orange, FLA) according to the instructions of the manufacturer. Details on these methods are provided in Paper IV.
Mechanistic characterization of the experimental drugs (papers II, III and IV)

The cell line panel was previously characterized in our laboratory for sensitivity to a large number of standard and experimental drugs [29] as well as for gene expression by microarray analysis [30]. Results were incorporated into a database used to characterize the mechanisms of action of the novel agents based on pattern of drug activity in the cell line panel as well as for calculation of drug activity and gene expression correlations.

Briefly, the IC50 for each drug in every cell line was converted to its log10IC50 and the average log10IC50 of all cell lines in the panel was calculated. From these results, a delta graph was plotted in which all cell lines of less than average sensitivity to the drug projected to the right, while those of greater than average sensitivity projected to the left [35]. Drugs with similar mechanisms of activity show similar delta graphs. Pearson’s correlation coefficients for drug – drug (log10 IC50), gene – gene (log2 expression) and drug – gene were calculated for analysis of cross-resistance between drugs and the association between drug activity and gene expression.

The mechanism of action of acriflavine was analyzed using the connectivity map (Cmap) approach. Briefly, the Cmap analyzes patterns of drug-induced gene-expression perturbations following a standard protocol of 6 h exposure in MCF-7 cell lines. More than 1 300 small molecules are included in the Cmap database [36]. High correlation between drugs points to their mechanistic relationship. Cmap has been shown to accurately predict the mechanism of action of experimental compounds and may indicate the potential utility of existing drugs for new purposes [36,37].

Data presentation and statistics

In paper I, data on control groups of mice were pooled together in the absence of significant differences. Statistical significances among experimental groups were calculated using the two-tailed Student’s t-test for differences between means.

In paper II, bar diagrams and some statistical evaluations were made using Microsoft Excel. Other statistical analyses and presentations in papers II and III were performed using GraphPadPrism (GraphPad Software, Inc., San Diego, CA). Data were processed using non-linear regression and a standard sigmoid dose-response model to
obtain logIC_{50} values. Experiments with benomyl, carbendazim and cryptolepine in solid and haematological tumour cell lines were carried out in triplicate and results were expressed as mean values ± SEM. Unpaired Student’s *t*-test was used to compare differences in drug activity between benomyl and carbendazim. Additional statistical methods are described in paper IV.

**Results and discussion**

**Paper I: Effects of benomyl and carbendazim in vivo**

Benomyl induced statistically significant and dose-dependent inhibition of [3H]-TdR incorporation in the tissue types investigated. However, a marked inter-organ difference in sensitivity to its inhibitory action was observed (Table 2). In thymus, spleen and testis, tissues with rapid cell turnover, a clear relationship between dose and effect was observed, with a no-observed effect level (NOEL) at 1.3 mmol/kg b.w. In contrast, tissues with predominantly quiescent cells, such as liver and kidney, showed lower [3H]-TdR incorporation as expected and less obvious dose-effect relationships. Interestingly, carbendazim given at an equimolar dose to benomyl induced a statistically significant inhibition of [3H]-TdR incorporation in testis only.

Table 2. Dose-response of oral benomyl or carbendazim on the inhibition of [3H]-TdR incorporation into various tissues of C57/bl mice 24 h after drug administration. The results are presented as mean ± SEM for 21 controls and 7 mice given benomyl or carbendazim. Values marked with asterisks differ significantly from controls. (*P < 0.05; **P < 0.01; ***P < 0.001).

<table>
<thead>
<tr>
<th>Organ</th>
<th>Benomyl (mmol/kg body weight)</th>
<th>Carbendazim (mmol/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corn oil</td>
<td>1.3</td>
</tr>
<tr>
<td>Thymus</td>
<td>2133 ± 186</td>
<td>1569 ± 277</td>
</tr>
<tr>
<td>Spleen</td>
<td>5207 ± 683</td>
<td>4817 ± 1200</td>
</tr>
<tr>
<td>Liver</td>
<td>81 ± 5</td>
<td>55 ± 13*</td>
</tr>
<tr>
<td>Kidney</td>
<td>71 ± 4</td>
<td>45 ± 2**</td>
</tr>
<tr>
<td>Testis</td>
<td>894 ± 64</td>
<td>821 ± 64</td>
</tr>
</tbody>
</table>
WBA 10 min after p.o. administration of $^{14}$C-labeled benomyl or carbendazim to C57/bl mice showed similar distribution patterns, though drug accumulation by more tissues was apparent for benomyl compared to carbendazim (Fig 4). Lymph nodes and spleen were intensely labeled with $^{14}$C-benomyl. For both compounds, radioactivity in the liver, the gastrointestinal tract (GIT), kidney and retina was prominent. Tissues with high cell turnover, such as thymus and bone marrow, showed very low levels of drug uptake within 10 min of drug administration. The central nervous system (CNS) also lacked radiolabeling in the WBA during this period.

Unpublished data on the \textit{in vivo} distribution of the benzimidazoles

In order to provide information about the reasons for the absence of benzimidazole radioactivity in the adult CNS, and to evaluate the possibility of secretion as an additional mode of excretion of these agents in mammals [38], pregnant mice near term were used as models (Fig 5). This was based on the premises that the blood brain barrier (BBB) is immature in the foetus and that radioactive benzimidazoles and metabolites transferred from the dam to the foetus through the umbilical vein become compartmentalized in the foetal intestines if secretion occurred in the foetus in parallel with absorption via the umbilical artery [39]. The foetal CNS was found devoid of radioactivity suggesting that...
the BBB could not account for the absence of radioactive benzimidazoles in the adult CNS. The finding of intense radioactivity labeling in the foetal intestine, and also in eyes (not shown) but barely visible in other tissues including the amniotic fluid [40,41], might indicate that benzimidazoles, and possibly their metabolites, are secreted into the intestine [42]. Furthermore, absence of radioactivity in the gall bladder was noted, whereas higher levels of radioactivity in the gastrointestinal lumen persisted, with much lower compared with higher levels (not shown) observable in the oesophageal and secreting portions of stomach respectively, with increasing time.

Figure 5. WBA of (A) 14C-carbendazim and (B) 14C-benomyl in pregnant mice at d17 of gestation, showing tissue distribution 1 h (A) and 24 h (B), after p.o. administration. Note that at 1 h, carbendazim shows a distribution pattern similar to benomyl after 10 min (Fig 4). Radioactivity in all tissues decreased considerably 24 h after p.o. administration for both drugs.

Unpublished data on the effects of benomyl in vivo as assessed by PET

Results from experiments on the effect of benomyl in normal tissues using three different PET tracers are shown in Figure 6. 18F-FDG was used to monitor glucose and thus energy metabolism, 11C-L-methionine to monitor protein synthesis and 76Br-BFU to monitor cell proliferation (DNA synthesis). Taken together, the results are consistent with benomyl induction of hypoxia in vivo, based on an increase in the uptake of 18F-FDG (Fig 6A). Benomyl also induced a decrease in the uptake of 11C-L-methionine and 76Br-BFU (Figs 6B and C) [43]. This is compatible with findings in paper II, with the effects of benomyl showing relatively high correlation with those of rotenone, a hypoxia inducing agent and with the expression of genes associated with oxidative stress.
Figure 6. Effects of benomyl on the accumulation of various PET tracers. (A) $^{18}$F-FDG (2 MBq), was injected i.p. 1 h before animal sacrifice followed by whole-body sectioning and PET autoradiography as described [44]. Histograms representing various tissues of mice given a single dose p.o. benomyl (2.55 mmol/kg b.w.) and measurements 1, 3, 6, 12, 24 h after drug dosing. (B) Procedure as in A but with $^{11}$C-L-methionine (5 MBq), a protein synthesis tracer. Data are expressed as a percentage of unexposed control. (C) Procedure as in A and B but with $^{76}$Br-BFU (2 MBq), a PET cell proliferation (DNA synthesis) tracer. There was a sharp decrease in the uptake of $^{76}$Br-BFU in tissues containing rapidly proliferating cells 1 h after benomyl dosing. $^{76}$Br-BFU uptake returned to normal levels within 48 h. X-axis = SUV (% of control).

Abbreviations: Myocard, myocardium; Br fat, brown fat; Saliv gl, salivary gland; Nasal muc, nasal mucosa.

**Paper II: Characterization in vitro of the cytotoxic activity of benomyl and carbendazim**

The activity profiles, expressed as delta-graphs of benomyl and carbendazim in the tumour cell line panel were similar (Fig 7), though benomyl was more potent than carbendazim. The mean IC$_{50}$ values in the cell lines were 15 ± 5.7 and 50 ± 9.0 μM (mean ± SEM) for benomyl and carbendazim, respectively.

![Figure 7](image)

Figure 7. Delta-graphs for (A) benomyl and (B) carbendazim in the human tumour cell line panel. Note the similarity in activity profile of the two benzimidazole derivatives with similar chemical structures.

Benomyl was also more potent than carbendazim in patient tumour cells (Fig 8). In these cells, the mean SI% values for benomyl and carbendazim were 42 ± 4.8 % and 72 ± 4.0%, respectively, for solid tumours, and 31 ± 5.0% and 70 ± 4.6 % for haematological tumours. Carbendazim thus showed less difference in activity between patient haematological and solid tumours, a pattern also observed in the cell line panel.
However, for carbendazim, cancer of the breast, CRC and NSCLC showed lower mean SI values than the hematological samples.

Figure 8. Cytotoxic activity, expressed as survival index, of 100 μM benomyl or carbendazim in patient tumour cells.
From the cell line panel drug activity database, drug - drug correlations between benomyl or carbendazim and mechanistically different standard and experimental cytotoxic agents were calculated. High to moderate correlation coefficients were observed for drugs representing different mechanistic groups, indicating multiple mechanisms of action of the benzimidazoles.

In particular drugs inducing mitochondrial dysfunction and thus oxidative stress, such as rotenone and antihelminthic agents, showed high to moderate drug - drug activity correlations with benomyl and carbendazim. The activity of thapsigargin, a putative Ca$^{2+}$-ATPase inhibitor in the event of oxidative stress, and also known to induce apoptosis through endoplasmic reticulum stress [45-48], correlated closely with that of both benomyl and carbendazim (not shown). Even though the benzimidazoles are referred to as putative microtubule agents, the activity correlations with drugs belonging to other mechanistic groups were at least as high as those with the microtubule inhibitors.

Drugs with defined mechanisms of resistance mostly showed resistance factors in the ten to several hundred fold range. In comparison, the benzimidazoles were approximately only 2-3 fold less active in pairs of cell lines expressing P-gp, glutathione and tubulin associated drug resistance, indicating that acquired drug resistance might be less of a problem for these benzimidazoles.

A number of genes were found to be associated with the activity of benomyl and carbendazim based on cut-off correlation coefficients of $\geq 0.7$ or $\leq -0.7$. In general, there was good agreement between the two drugs for gene correlations, corroborating their close relationship with respect to activity as well as structure.

Based on the microarray based measurements of gene expression in the cell lines of the cell line panel, drug activity - gene expression correlations were calculated for groups of genes reported in the literature to be involved in the action of cytotoxic drugs of the major mechanistic classes. This was used in an effort to mechanistically classify benomyl and carbendazim, as shown in Table 3 for genes related to oxidative stress. The mean absolute correlations are considered to reflect how closely a drug is related to the mechanistic pathway. Rotenone, a classical oxidative stress inducing agent showed the highest correlations, followed by the benzimidazoles, indicating that oxidative stress might be part of their mechanism of action.
Drug activity of both benomyl and carbendazim was found to correlate closely with the expression of genes associated with oxidative stress, DNA interaction and epigenetic activity. These findings point to multiple mechanisms behind the cytotoxic action of these benzimidazoles.

**Paper III: Characterization in vitro of the cytotoxic activity of cryptolepine**

The cytotoxic potency of the natural alkaloid, cryptolepine, was higher than that of the benzimidazoles. The mean IC$_{50}$ for cryptolepine in the cell line panel was 0.9 ± 0.2 μM. Most of the tested cell lines of haematological origin were more sensitive to cryptolepine than those of solid tumour origin and the normal cell line hTERT-RPE1 (Fig 9).
Cryptolepine was also hematologically active in the patient samples: the mean IC$_{50}$ for patient samples of haematological malignancies was $1.0 \pm 0.2$ μM compared with $2.8 \pm 1.2$ μM for solid tumours. However, many individual samples from solid tumours, especially those from breast, CRC and non-small cell lung cancer (NSCLC), were more sensitive than samples from hematological tumours (Fig 10).
Cryptolepine showed drug resistance factors within the 1-2 range, indicating that drug resistance associated with activity directed to topo-II-, GSH-, Pgp-, MRP- or tubulin may have little impact on the activity of cryptolepine.

The activity of cryptolepine in the cell line panel correlated strongly to the DNA intercalating and topoisomerase inhibitor, acriflavine, followed by zinc pyrithione, a zinc ionophore that promotes the activity of p53, induces oxidative stress, starves tumour cells of iron and activates proapoptotic proteins. Other agents showing high correlation coefficients to cryptolepine included the topoisomerase inhibiting anthracyclin antibiotics, daunorubicin and doxorubicin.

Rotenone and arsenic trioxide, believed to act via disruption of the respiratory chain and induction of chemical hypoxia, ROS formation and oxidative stress, showed high drug - drug activity correlations with cryptolepine. Cryptolepine has been implicated in bioreductive cytotoxic activity, leading to the induction of apoptosis [49], and correlating strongly with the effect of acridinyl, a potent hypoxia-selective agent. Amine oxidases are known to produce ROS from their substrates, spermine and spermidine [50], and these agents also showed high drug - drug activity correlations with cryptolepine. Proteasome inhibition seems to be part of the cytotoxic activity of cryptolepine as disulfiram and lactacystin showed high to moderate drug - drug activity correlations with cryptolepine. Cryptolepine has been shown to inhibit telomerase [51], which is in agreement with the high correlation with curcumin, known to inhibit telomerase, protein kinase C, HIF-1α and NFκB [52-57]. The protein kinase inhibitor, rottlerin, had a drug - drug activity correlation of 0.75 with cryptolepine (not shown). High correlations were also observed for the microtubule inhibiting agents, vincristine and benomyl.

The drug activity – gene expression correlations supported the notion of a multi-mechanistic activity of cryptolepine including the expression of genes such as HIF-1α, VEGF, GLUT1 Glutathione S-transferase p.i., Glutathione peroxidase 1, Glutaredoxin 3 and MAP kinase isoforms for hypoxia induction and ROS formation, Calcyclin binding protein for Ca²⁺ mobilization, NADH dehydrogenase and Thioredoxin-like 4A for bioreductive reaction, Metallothionein gene isoforms and Tumour protein p53 inducible protein 3, reflecting the cytotoxic role of labile Zn²⁺ and the mobilization of p53.
Mechanisms underlying the cytotoxic activity of the quinone doxorubicin might be similar to cryptolepine, another quinone. Doxorubicin induces cardiomyopathy. To investigate the hypothesis that cryptolepine might also be cardiotoxic, correlations between drug activity and metallothionein gene isoform expression (the products of which confer myocardial cytoprotection [58,59]) were calculated (Table 4). Clear differences between cryptolepine and doxorubicin were observed whereas rotenone was very similar to cryptolepine, despite the seemingly different cytotoxic pathways [23,49,60-63]. The association of zinc pyrithione to cryptolepine but not doxorubicin [58] also indicates that cryptolepine might have an advantage over doxorubicin with respect to cardiac safety. In cancers treated with anthracycline based chemotherapy, such as multiple myeloma and breast cancer, cryptolepine may prove to be a superior alternative therapy.

<table>
<thead>
<tr>
<th>ACCID</th>
<th>Gene name</th>
<th>Symbol</th>
<th>Cryptolepine</th>
<th>Acriflavine</th>
<th>Doxorubicin</th>
<th>Cisplatin</th>
<th>5-FU</th>
<th>Rotenone</th>
</tr>
</thead>
<tbody>
<tr>
<td>297392</td>
<td>Metallothionein 1X</td>
<td>MT1X</td>
<td>0.7509</td>
<td>0.6438</td>
<td>0.3538</td>
<td>0.3296</td>
<td>0.4859</td>
<td>0.7731</td>
</tr>
<tr>
<td>214162</td>
<td>Metallothionein 1H</td>
<td>MT1H</td>
<td>0.7394</td>
<td>0.6672</td>
<td>0.391</td>
<td>0.3187</td>
<td>0.5014</td>
<td>0.7330</td>
</tr>
<tr>
<td>1472735</td>
<td>Metallothionein 1E</td>
<td>MT1E</td>
<td>0.7278</td>
<td>0.6217</td>
<td>0.3533</td>
<td>0.3504</td>
<td>0.5568</td>
<td>0.7730</td>
</tr>
<tr>
<td>232772</td>
<td>Metallothionein 1B</td>
<td>MT1B</td>
<td>0.6697</td>
<td>0.6412</td>
<td>0.3356</td>
<td>0.2570</td>
<td>0.4548</td>
<td>0.7798</td>
</tr>
</tbody>
</table>

Unpublished data on the properties of cryptolepine in vivo and in vitro

Cryptolepine distributed normally throughout tissues after injection of 0.3 MBq (5 μl)/g b.w., corresponding to 5 mg/kg b.w. of ³H-cryptolepine in 20 g mice followed by WBA. No drug-associated adverse events were observed.

³H-cryptolepine was observed in tissues with rapid cell renewal, such as bone marrow and in the epithelium of the intestinal tract as early as 5 min after i.v. injection (Figs 11A and B). The gall bladder was also intensely labeled. Other tissues including the liver and kidney (not shown) were also labeled after 5 min. Quantitation of the WBAs
was performed to study the differential absorption and excretion rate from various tissues over time (Fig 11C). Almost all tissues studied showed rapid absorption of \( ^3 \)H-cryptolepine from blood, reaching a peak around 1 h, after which excretion appeared to start. \( ^3 \)H-cryptolepine was localized in all tissues investigated, except the central nervous system. This was also found in the foetuses of pregnant mice (not shown), suggesting that the BBB, immature in the foetuses, cannot account for the absence of uptake. However a non-radiolabelled metabolite of cryptolepine might penetrate the BBB as has been observed for the structurally very similar drug ellipticine [64,65, Fig 14].
Figure 11. WBA (A) showing the thorax and abdominal regions of the mouse with specific uptake of $^3$H-cryptolepine in the sternal and vertebral bone marrows and intense uptake of $^3$H-cryptolepine in para-aortal gangliae and the gall bladder 5 min after i.v. injection. The abdominal region (B) showed general uptake of $^3$H-cryptolepine in liver and specific uptake in intestinal epithelium 5 min after i.v. injection. (C) shows densitometric quantitation from autoradiograms of the changes in $^3$H-cryptolepine accumulation over time (5 min – 8 days after $^3$H-cryptolepine injection) in various tissues of the mouse. Abbreviations: m, min; h, hour; d, day.

Due to the longer persistence of $^3$H-cryptolepine in adrenal medulla compared to cortex, neuroendocrine tumour cell lines were cultured as spheroids for further preliminary work on the anti-cancer activity of cryptolepine. Three-dimensional spheroid-cultured tumour cell lines are probably more representative of cancer than mono-layer cultures, and are thus an appropriate complimentary model for studying experimental drugs under consideration for use in solid tumours. Preliminary findings on the effect of cryptolepine on $^3$H-TdR uptake in spheroid cultures of the neuroendocrine tumour cell lines, BON and LAN, are shown in Figure 12. These data indicate that cryptolepine is active in spheroids (see legend to Figure 12).
Figure 12. $^3$H-TdR accumulation in spheroids of (I) BON and (II) LAN cell lines cultured in an incubator for 7 days at 37°C with 5% CO$_2$. Culture medium was changed every other day. Incubation of the spheroids with the indicated concentrations of cryptolepine was carried out for 24 h. After rinsing with Tris buffer (1 min x 3), radioactivity in the spheroids was measured in a β-scintillation counter. Results were calculated as relative uptake (activity in spheroid / spheroid volume / activity in 1 μl incubation medium with unexposed control set to 100%). Panels III and IV show growth inhibitory effect of various concentrations of cryptolepine 15 days after incubation of BON (III) and LAN (IV) spheroids in drug-free medium. Initially, spheroids were incubated for 24 h in cryptolepine-containing medium at the concentrations 1.0, 2.5 and 5.0 μM. Drug-treated spheroids were next rinsed with Tris buffer and provided with fresh medium. After a second rinsing 24 h later, spheroid volumes were measured repeatedly every other day until the 15th day after the first rinsing. Day 0 represents the 24th hour after the drug treatment and first rinsing of the spheroids, and day 1 the second rinsing 24 h later. Using a Zeiss inverted microscope, each spheroid size was measured using an ocular scale manipulated by a micrometer. Spheroid volumes were set to 100% on day 0, i.e. 24 h after cryptolepine exposure. BON is a human pancreatic carcinoid cell line, and LAN a human neuroblastoma cell line. Abbreviation: Drug conc, drug concentration; vol, volume.

**Paper IV: HTS as a method to identify diagnosis specific cancer drugs**

The 2 000 molecule Spectrum library was screened in colon cancer cell lines and counter-screened in two normal cell lines to elucidate whether this strategy, using stringent criteria for hit selection, could identify a CRC active candidate drug. Hit identification was to be followed by further characterization of drug activity in patient tumour samples and mechanism of action was studied using the Cmap approach. Based on >70% inhibition of viability in all three colon cell lines included in the screen, < 30% inhibition in the normal cells and after consideration of drugability, the acridine, acriflavine was identified as the screening hit.

In the validation step, using patient tumour cells of CRC, CLL and OC origin, and by comparing established CRC active drugs, acriflavine showed unprecedented activity against CRC.
Acriflavine was also shown to be advantageous as measured by low drug resistance factors for established resistance mechanisms as well as retained activity in spheroids of the colon cancer cell line, HCT 116.

Acriflavine showed high drug - drug activity correlation (0.92) with cryptolepine, proposed to be a strong topoisomerase II inhibitor (paper III). Consistent with this finding, acriflavine was identified as a topoisomerase inhibitor in the Cmap analysis based on its high gene expression signature correlation with hycantone, mitoxantrone, daunorubicin
and ellipticine (Table 5). This was then confirmed by direct measurements of topoisomerase I and II inhibition in a cell-free system.

The molecules showing strong negative correlation with acriflavine (Table 5) theoretically indicate agents which might ablate or confound the cytotoxic activity of acriflavine. Diclofenamide, clopamide and chenodeoxycholic acid might act in a similar fashion to lycorine by interfering with the plasma membrane ion flux required for acriflavine-induced apoptosis [66-71].

Table 5. Small molecules most highly ranked for positive and negative correlations in Cmap analyses of gene signature perturbations in the MCF-7 cell line. See paper IV for details.

<table>
<thead>
<tr>
<th>CMAP name</th>
<th>Concentration</th>
<th>Score</th>
<th>Mechanism of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hycanthone</td>
<td>11μM</td>
<td>1</td>
<td>DNA alkylating/ MDR reversal/ Plasma membrane receptor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>internalization inhibition</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>8μM</td>
<td>0.913</td>
<td>Topoisomerase inhibition</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>1μM</td>
<td>0.896</td>
<td>Topoisomerase inhibition</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>16μM</td>
<td>0.874</td>
<td>Topoisomerase inhibition</td>
</tr>
<tr>
<td>Diclofenamide</td>
<td>13 μM</td>
<td>-0.814</td>
<td>Carbonic anhydrase inhibition (Ca²⁺-activated-K⁺ channel activation)</td>
</tr>
<tr>
<td>Clopamide</td>
<td>12 μM</td>
<td>-0.815</td>
<td>(NaCO₃⁻/Cl⁻) anion exchanger inhibition</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>10 μM</td>
<td>-0.845</td>
<td>Negative effects on potassium absorption</td>
</tr>
<tr>
<td>Lycorine</td>
<td>12 μM</td>
<td>-0.882</td>
<td>K⁺ channel interference</td>
</tr>
</tbody>
</table>

Ellipticine has a molecular structure highly similar to cryptolepine but both differ from acriflavine (Fig 14). The Cmap database lacks data on cryptolepine but the strong correlation between acriflavine and ellipticine indicates that a common mechanism of action does not necessarily require structural similarity [72,73].
Based on the drug–drug activity and drug activity–gene expression correlations observed for cryptolepine, several additional mechanisms of action for acriflavine are possible, such as NF-kappaB, microtubule, protein synthesis, and HSP90 inhibition as well as DNA intercalation, epigenetic activity, induction of chemical hypoxia, HIF-1α destabilization and the up-regulation of Bax and Bim.

Despite mechanistic similarities between acriflavine and cryptolepine, these molecules clearly differed in the genes associated with their activity in the cell line panel. Some of these genes are listed in Table 6. The high correlations with the endocytosis and chemotactic associated genes for acriflavine is interesting in view of the knowledge that acriflavine promotes cell surface changes, thereby inducing agglutination in tumour cells, and also that its binding capacity is controlled by the acrA gene of *E. coli* [74,75].
Table 6. Examples of genes with differential drug activity - gene expression correlations between acriflavine and cryptolepine.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>ACCID</th>
<th>Gene activity</th>
<th>ACRI</th>
<th>CRYPTO</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMI</td>
<td>755228</td>
<td>Endocytosis- and actin/cytoskeleton-associated</td>
<td>0.8632</td>
<td>0.4817</td>
</tr>
<tr>
<td>NODAL</td>
<td>1571493</td>
<td>Connective tissue activity, TGF-beta coding</td>
<td>0.8527</td>
<td>0.5471</td>
</tr>
<tr>
<td>RHOD</td>
<td>591907</td>
<td>Protein kinase associated, endocytosis- and actin/cytoskeleton-associated</td>
<td>0.8162</td>
<td>0.6232</td>
</tr>
<tr>
<td>DHRSX</td>
<td>855029</td>
<td>Oxidoreductase activity</td>
<td>–0.8442</td>
<td>–0.2197</td>
</tr>
<tr>
<td>CCL13</td>
<td>80146</td>
<td>Chemotactic activity; immuno-regulation and inflammation</td>
<td>–0.7979</td>
<td>–0.5327</td>
</tr>
</tbody>
</table>

Table 7 shows six different functional categories of genes whose expression profile was modulated by acriflavine, after exposure to MCF-7 cells for 6 h. The relatively high up-regulation of these genes and the variability in function of each group, indicate acriflavine-induced multiple cytotoxic mechanisms besides topoisomerase inhibition. Acriflavine, similarly to daunorubicin, has been found to modulate the activity of the endosomal/lysosomal system [76]. Support for the role of endocytosis in the cytotoxic activity of acriflavine is further provided by a high 5.03-fold change in the expression of the transferrin gene [77].
Table 7. Six different functional categories of genes from the Cmap database whose expression was clearly upregulated by acriflavine after exposure to MCF7 cells for 6 h. Note the highest change for the SLC7A9 gene associated with a PKC - arginine - nitric oxide- ER stress pathway of apoptosis induction [78-83].

<table>
<thead>
<tr>
<th>Proposed function</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Probe Set ID</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td>UDP glucuronosyltransferase 2 family, polypeptide B4</td>
<td>UGT2B4</td>
<td>206505_at</td>
<td>4.53</td>
</tr>
<tr>
<td></td>
<td>Cytochrome P450, family 1, subfamily A, polypeptide 1</td>
<td>CYP1A1</td>
<td>205749_at</td>
<td>3.84</td>
</tr>
<tr>
<td>Oxidative stress and endocytosis</td>
<td>Transferrin</td>
<td>TF</td>
<td>203400_s_at</td>
<td>5.03</td>
</tr>
<tr>
<td></td>
<td>CDC42 effector protein (Rho GTPase binding) 3</td>
<td>CDC42EP3</td>
<td>209288_s_at</td>
<td>4.74</td>
</tr>
<tr>
<td></td>
<td>Calcium channel, voltage-dependent, T type, alpha 1G subunit</td>
<td>CACNA1G</td>
<td>207869_s_at</td>
<td>4.62</td>
</tr>
<tr>
<td></td>
<td>Placental growth factor, vascular endothelial growth factor-related protein</td>
<td>PGF</td>
<td>209652_s_at</td>
<td>3.61</td>
</tr>
<tr>
<td></td>
<td>BCL2-related protein A1</td>
<td>BCL2A1</td>
<td>205681_at</td>
<td>3.54</td>
</tr>
<tr>
<td></td>
<td>Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2</td>
<td>ACE2</td>
<td>219962_at</td>
<td>3.27</td>
</tr>
<tr>
<td></td>
<td>Carbonic anhydrase II</td>
<td>CA2</td>
<td>209301_at</td>
<td>2.71</td>
</tr>
<tr>
<td>Cell adhesion (colon cancer) in association with dynamin</td>
<td>Protocadherin 7</td>
<td>PCDH7</td>
<td>210273_at</td>
<td>3.06</td>
</tr>
<tr>
<td>Protein-protein interaction. (tumour staging)</td>
<td>SAM domain, SH3 domain and nuclear localization signals 1</td>
<td>SAMSN1</td>
<td>220330_s_at</td>
<td>4.47</td>
</tr>
<tr>
<td></td>
<td>Nescient helix loop helix 2</td>
<td>NHLH2</td>
<td>215228_at</td>
<td>4.27</td>
</tr>
<tr>
<td>WNT/β-catenin pathway</td>
<td>Connective tissue growth factor</td>
<td>CTGF</td>
<td>209101_at</td>
<td>3.16</td>
</tr>
<tr>
<td>Cationic amino acid transport</td>
<td>Solute carrier family 7 (y+ system), member 9</td>
<td>SLC7A9</td>
<td>220135_s_at</td>
<td>5.70</td>
</tr>
</tbody>
</table>

Based on known biochemical effects of acriflavine, drug activity - gene expression correlations as well as acriflavine gene expression perturbation profiles, speculations on the mechanisms of acriflavine with respect to its specific activity in CRC compared with OC and CLL will be provided.

The inhibition of the ubiquitous phase I metabolizing enzyme, epoxide hydroxylase, by acriflavine [84,85] paralleled by the up-regulated UGT2B4 and CYP1A1 genes (Table 7) is likely to contribute to the metabolite-mediated cytotoxic potency of this agent in some tumour cells, including CRC [86-88]. Sorafenib, a tyrosine kinase
inhibitor [89], also known to inhibit soluble epoxide hydroxylase [86], obtained a high drug-activity correlation of 0.72 to acriflavine.

The expression of the CCKB/gastrin receptor differs according to cell type [90], is clearly expressed in some cells of the digestive tract [91-93] and over-expressed in colon cancer, where it might be transported by endocytosis [94,95]. Antagonist-stimulated internalization of the G-protein-coupled cholecystokinin receptor has been reported [96,97]. Disruption of endocytosis-mediated transport by acriflavine might explain its specific potent activity in CRC compared with OC and CLL.

The probable targeting of the WNT/β-catenin pathway by acriflavine to compromise the viability of tumour cells was indicated by a WNT-associated gene with a relatively moderate score from the cMap database (Table 7) and by genes with high drug activity - gene expression correlations from the cDNA database (Table 8). Among these genes is Dickkopf, of the homolog 3 isotype, a key component of WNT-signaling which relays WNT-signals from receptors to downstream effectors, and also mediates receptor endocytosis [98]. WNT proteins form a pathway that plays major roles in development and differentiation and this pathway is believed to contribute to the malignant phenotype of cancers such as CRC [99-101].

Table 8. Acriflavine and cryptolepine activity - gene expression correlations specific to WNT-associated genes in the tumour cell line panel.

<table>
<thead>
<tr>
<th>ACCID</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>R for acriflavine</th>
<th>R for cryptolepine</th>
</tr>
</thead>
<tbody>
<tr>
<td>811848</td>
<td>Transforming growth factor beta 1 induced transcript 1</td>
<td>TGFB1I1</td>
<td>0.8448</td>
<td>0.8758</td>
</tr>
<tr>
<td>2490795</td>
<td>WNT1 inducible signalling pathway protein 2</td>
<td>WISP2</td>
<td>0.7732</td>
<td>0.4025</td>
</tr>
<tr>
<td>2436850</td>
<td>Wingless-type MMTV integration site family, member 7A</td>
<td>WNT7A</td>
<td>-0.042</td>
<td>0.6666</td>
</tr>
<tr>
<td>760299</td>
<td>Dickkopf homolog 3 (Xenopus laevis)</td>
<td>DKK3</td>
<td>0.7717</td>
<td>0.8497</td>
</tr>
<tr>
<td>770884</td>
<td>Tax1 (human T-cell leukemia virus type 1) binding protein 3</td>
<td>TAX1BP3</td>
<td>0.6934</td>
<td>0.8559</td>
</tr>
<tr>
<td>841282</td>
<td>Secreted frizzled-related protein 4</td>
<td>SFRP4</td>
<td>0.6681</td>
<td>0.6498</td>
</tr>
</tbody>
</table>

R = Pearson’s correlation coefficient

Recent work [102] has implicated that the DNA interacting [103] and anti-helminthic agent [104] hyacanthone inhibits the internalization of the plasma membrane receptor, Frizzled, associated with Wnt proteins and translocation of cytosolic β-catenin to the nucleus [100]. The Secreted frizzled-related protein 4 gene obtained a moderately
high drug activity - gene expression correlation of 0.67 to acriflavine (Table 8). These observations might support the hypothesis that acriflavine which obtained the highest gene expression signature score to hycanthone (Table 5), inhibits the internalization of tumour growth promoting factors including CCKB/gastrin [94,95].

The proposed inhibition of protein kinase C by acriflavine [79,84] might induce a sequence of events (Fig 15) that disrupt tumour viability [78-83,105-111] along a pathway in which the SLC7A9 gene is up-regulated (Table 7). The SLC7A9 gene is specifically expressed in the kidney and the gastrointestinal tract [83].

Figure 15 Simplified diagram of the proposed consequence of PKC inhibition by acriflavine, leading to up-regulation of the SLC 7A9 gene and the probable formation of RNS to induce apoptosis in CRC. Adjacent figures are reference numbers. Abbreviations; ACR, acriflavine; PKC, protein kinase C; ER, endoplasmic reticulum; RNS, reactive nitrogen species; NO, nitric oxide. Numbers refer to references.
General discussion

The unduly long time and enormous costs required to establish a new chemical entity as a drug for treatment of cancer has stimulated the repurposing approach as a way to increase the efficiency of the development of new cancer drugs.

Agents already approved for other indications or used ethnomedically, have provided knowledge and experience on their biological actions in humans. This knowledge could be used to shorten the clinical development process of such drugs for new indications. Identification of such drugs as candidates for cancer drug development and previous experience gained from them thus provide the basis for an accelerated drug development process.

Repurposing of drugs for use in cancer would benefit from efficient preclinical methods able to characterize candidate drugs with respect to their spectrum of cytotoxic activity and mechanisms of action and drug distribution. The methods described in this thesis and applied to the four molecules studied by no means provide all information needed for judgments on whether it would be worthwhile to pursue further drug development. However, the package of methods used can provide relevant information on which to base decisions.

The most recently introduced among these methods in our laboratory is gene expression analyses as a basis for determining the mechanisms of action of experimental drugs. Short-term drug induced gene perturbation in a defined model system followed by computer based analysis of pattern similarities according to Cmap characterization has now repeatedly been shown to provide reliable and important mechanistic information on various types of drugs [36, paper IV].

Cmap, however, is limited by the types of molecules included in the database, which means that characterization of molecules with mechanisms of action not yet represented will not be successful. The correlation of drug activity with gene expression in a cell line panel is a more general but also a less accurate approach as illustrated in paper II. Although it seemed to provide some relevant data on the benzimidazoles, the analytical algorithm is clearly in need of further development based on biological and statistical considerations to sharpen its ability to provide clear mechanistic data.
The benzimidazole structure is a ‘privileged chemical structure’ within the context of its wide use as a backbone in several types of drugs, such as omeprazole, astemizole and albendazole, for human and veterinary use. Benomyl and carbendazim are currently used globally as agricultural fungicides with an anti-microtubule mechanism of action, which motivated their selection for pre-clinical testing as putative cancer agents. Both are well tolerated by mice, and restoration of cell proliferation, protein synthesis and energy metabolism in normal cells after benomyl exposure in vivo are favourable, supporting their further development for cancer therapy.

Benomyl and carbendazim were found to have several other mechanisms of tumour cytotoxicity besides microtubule interference and were not severely affected by the major mechanisms described for drug resistance. Benomyl and carbendazim generally showed similar patterns of cytotoxic activity, although some differences, perhaps attributable to the butylcarbamoyl side chain of benomyl that yields n-butylisocyanate, were also noted. Benomyl might be an alternative for the nitrosoureas used for treatment of glioblastoma, as these agents also yield n-butylisocyanate which seems to confer cytotoxic potency not only by ROS-induced apoptosis signaling [112,113] but also by what might be the cytotoxic activity of thiocarbamate intermediaries [114-116]. According to FDG-PET- in vivo, activity of benomyl was also traced to the brain.

Based on primary cultures of cancer cells from patients, benomyl was more potent than carbendazim; the latter already investigated in phase 1 clinical trials in solid tumours [117]. However, whereas benomyl appeared classically more potent in patient hematological tumours compared to solid tumours, carbendazim was almost equally active in both groups of diagnoses, which is an advantageous feature arguing in favour of carbendazim for cancer drug development.

Two phase I clinical trials of carbendazim in solid tumours have been reported. Using a weekly schedule of up to 19.2 g/m²/week of p.o. carbendazim, 26 patients with various solid tumours received a total of 44 cycles, with a median of 1.7 cycles per patient. The encouraging results included absence of dose-limiting toxicity, hematologic toxicity less than grade 2 and minor response/stable disease in some tumour types [117]. In a second phase I trial, a daily schedule was used but the drug could not be escalated to maximum tolerated dose due to the intolerable taste of the vehicle [118]. A new
formulation of carbendazim that permits dose escalation to MTD has been reported as ongoing.

In conclusion, preclinical findings in vitro as well as in vivo, together with the limited clinical experience obtained so far, points to a potential for these benzimidazoles for further investigations as cancer drugs.

Cryptolepine belongs to the indoloquinoline class of compounds and is a natural alkaloid that has now been synthesized. The wide use of cryptolepine in ethnomedicine dates back several decades in the tropics. This suggests multi-mechanistic properties as well as good tolerance in humans. Cryptolepine was distributed rapidly to various tissues in the mouse, specifically in tissues with rapid cell turnover, and has since been found to inhibit topoisomerase.

Characterization of the cytotoxic activity of cryptolepine in the human cell line panel and patient tumour samples showed cytotoxic potency higher than the benzimidazoles, with typical higher activity in hematological malignancies compared to solid tumours. However the difference was quite small and many samples of breast, CRC and OC were as sensitive as or even more sensitive than the hematological samples, indicating an additional potential for cryptolepine in solid tumours. This was also supported by the observation of cryptolepine activity in spheroid-cultured human tumour cell lines and its relative insensitivity to classical mechanisms of drug resistance.

Cell line based HTS of a molecular library with the focus on finding a cytotoxic agent with activity against CRC using a stringent algorithm for hit definition identified acriflavine as the candidate drug. Thus, in a spheroid cultured colon cancer cell line and in tumour cells from patients with CRC, OC and CLL, acriflavine was more cytotoxic than the clinically used standard drugs 5-FU, oxaliplatin and irinotecan. Acriflavine is a mixture of two very closely related acridine molecules widely used experimentally to trace DNA and has been applied to specific microbial indications. It has also been considered for use as a cancer drug [15,16].

The finding by ‘targeted’ HTS in paper IV of a drug with unprecedented activity in vitro in CRC is by no means proof of the efficacy of HTS for the identification of new cancer drugs in a specific diagnosis. However, the approach seems promising and would be interesting in attempts to find drugs against other tumour types. Furthermore, whether
acriflavine is selectively active in CRC or has more general activity against solid tumours remains to be elucidated, initially in a ‘phase II trial’ in vitro using a spectrum of patient samples of different tumour types with different sensitivity to chemotherapy.

Cmap analysis revealed acriflavine to inhibit topoisomerase I and II. However, correlation analyses based on drug activity and gene expression in the cell line panel indicated a more a multi-mechanistic cytotoxic property of acriflavine, including inhibition of protein kinase C, HIF-1α, NF-kappa B, iron ion transport, microtubules, heat shock protein and DNA methyltransferase. Most of these findings are in accordance with previous reports on mechanisms of action of acriflavine [15,16,79,119-122] This also provides support for the validity of the methods used in this thesis for mechanistic characterization of cytotoxic drugs. In addition, genes associated with drug metabolism, oxidative stress, endocytosis, WNT/β-catenin, metastasis and cationic amino acid transport were found to be significantly up-regulated after short-term exposure in MCF-7, all of which contribute information that may reveal the mechanisms of action of acriflavine.

Taken together, previous experience on acriflavine in humans and in animal models together with its interesting activity pattern and mechanistic features, argues in favour of its further development as a cancer drug with CRC as a potential target diagnosis. Furthermore, excellent abdominal local tolerance to acriflavine [120] together with some pharmacokinetic data [123], argues for the development of acriflavine for use in intraperitoneal chemotherapy together with cytoreductive surgery in peritoneal carcinomatosis of CRC origin.
Summary and conclusions

• Repurposing of old drugs, i.e. the use of established drugs for new indications based on theoretical considerations and/or empirical findings, could be a fruitful approach for the identification of new cancer drugs. The use of a preclinical package of methods, such as that discussed in this thesis, for the basic characterization of pharmacokinetic and pharmacodynamic properties relevant to a particular cancer indication would be necessary for selection of candidate drugs for further development.

• Based on proposed mechanisms of action, previous experience from their use and a repurposing approach to find new cancer drugs, the benzimidazoles, benomyl and carbendazim, and the alkaloid, cryptolepine, were investigated for their cytotoxic effects and pharmacokinetic properties using a set of preclinical in vivo and in vitro methods. Drug distribution, effect on normal tissues, activity against various types of tumour cells and indications of multiple mechanisms of action argue in favour of the consideration of these drugs for further development as cancer drugs.

• HTS of libraries of molecules in cell line models representing a specific tumour type with stringent criteria for selection of screening ‘hits’, might be fruitful for the identification of promising lead molecules for development into new cancer drugs. In this way, the acridine, acriflavine was identified as a drug with advantageous properties and promising activity against CRC tumour cells.

• Analyses of patterns of drug activity and drug activity – gene expression correlations in a defined cell line panel, as well as computer based analysis in the Cmap of patterns of changes of gene expression in a cell line model after short-term exposure to an experimental drug, seem to be fruitful methods for mechanistic characterization of new cancer drugs.
Acknowledgements

Facilities were provided for this thesis from various institutions at Uppsala University: the Pharmaceutical Faculty, Division of Toxicology and the PET Centre, with the greatest share coming from the Medical Faculty, Department of Medical Sciences, Division of Clinical Pharmacology, the Cancer Pharmacology and Bioinformatics Unit.

Having completed this thesis successfully, my deepest gratitude is extended to my major supervisor, Professor of Clinical Oncology, Peter Nygren, who has excellently and devotedly guided me with the essentials and benefits of good medical research. Peter was never tired of my life’s restrictions and shortcomings as a post-graduate student; he handled me with encouragement and trust with a brilliant sense of responsibility and goodwill. Some evening and week-end discussions on academics and philosophy of life with humour are all a grateful and appreciated memory. My deputy supervisor, Professor of Clinical Cancer Pharmacology, Rolf Larsson, has been a pillar of encouragement and hope, and I am ever very grateful to him for recognizing and approving my initial basic ideas on the potential of most of the ‘drug possible’ compounds I worked with and thus conferring with Peter to help to build upon my academic vision. It has truly been a huge privilege and a delight indeed to achieve this academic goal under Peter and Rolf!

My former teacher at medical school and head of the Department of Medical Sciences, Associate Professor Ulla Lindquist, and senior consultant in internal medicine specializing in haematology, Associate Professor Kristina Carlson: your wisdom and humanity guided me to Rolf and Peter and ‘Thank you very much’ doesn’t sound enough, but I hope you know how sincere I am with my enormous gratitude to both of you and to your brilliant colleagues and members of staff.

Excellent technical support is always golden to the success of medical research at the bench, and for that I am very grateful to the expertise of the Medical Laboratory technicians, Lena Lenhammar whose understanding, willingness and patience for me in several aspects of FMCA and Cmap, and to Christina Leck who is always keen to maintain strictly good laboratory practice for the tumour cell line panel and chemicals, meant a lot to me. Their responsible engagements have ensured reliable results.
I am also equally grateful to the Medical Laboratory technicians, **Anna-Karin Lannerjärd** and **Annika Jonasson** who were magnificent in preparing primary cells from patient malignant samples, and for readily helping me to collect patient data for paper IV.

Associate Professor of toxicology, **Björn Hellman** has been an inspiration in my interest for research in the health sciences and I shall ever be grateful for what that has meant for me. The friendliness and eagerness of Professor **Mats Bergström**, to impart the application of the PET technology to post-graduate students combined with the enthusiasm of his wife **Elizabeth Bergström** who provided excellent technical assistance in the PET Pre-clinical laboratory has been highly admirable and fruitful to me.

All my co-authors are sincerely acknowledged for their various contributions to the success of this thesis; Professors **Stig Linder**, **Bengt Glimelius**, **Wilhelm Graf**, **Lars Pålman** and senior surgeon **Haile Mahteme**, are warmly thanked for their enthusiasm and contributions in paper IV of the thesis, not forgetting **Jenny Felth**, and **Walid Fayad**. Post Doc **Saadia Hassan** is acknowledged for her efforts in paper IV and our occasional extra-academic conversations about a spiritual meaning of life.

My research associates, Assoc. Professors **Elin Lindhagen**, **Joachim Gullbo** and **Dr Anna Eriksson**, Post Docs **Malin Wickström**, **Linda Rickardson**, **Claes Andersson**, and Post graduates **Caroline Haglund** and **Kristin Blom** are heartedly thanked for various ‘more than willing’ contributions and warm friendliness they provided. Post Doc **Mårten Fryknäs** and Assoc. Professor **Anders Isaksson** are gratefully acknowledged for our several discussions on gene expression profiling.

The staff of the bioinformatics unit for providing a warm working atmosphere and help whenever needed: Professor **Mats Gustafsson**, Assoc. Professor. **Anders Isaksson**, the hard working and highly efficient Medical Laboratory technicians, **Maria Rydåker** and **Hanna Haukkala**, the diligent Bioinformaticians **Hanna Göransson-Kultima** and **Markus Rasmussen**.

The Pharmacogenetic unit headed by Professor **Maria-Lisa Dahl** and her staff members: Assoc. Professor **Mia Wadelius**, Drs and clinicians **Gabriella Scordo**, **Ilma Bertulyte**, **Anna Lundberg**, Dr **Arzu Gunez**, Post graduates **Anna-Karin Hamberg**,
Mao Mao, and their hard working Medical Laboratory technicians, Lena Fredriksson, Gunilla Frenne, Hugo Kohinke and David Munro were indeed a happy, nice group to be with at our broad-based institute.

Senior Medical Officer Dr Thomas Nilsson, his colleagues and staff members at the Department of Clinical Nephrology are not forgotten for their efficiency by which I got back to work and hoped for the horizon.

The Osteoporosis group, headed by Professor Håkan Melhus and his staff among them Eva Hallberg and Sophie Schwann, and my former clever class mate at medical school full of interesting humour, Senior Medical Officer Dr Per Hallberg; their nice companionship at the department are appreciated.

All other colleagues including the efficient administrative staff members, Anna Foyer, Elizabeth Harrysson, Birgitta Sembrant, Eva Prado, Kristin Byron, and Enrique Vegas; they are all warmly thanked for all contributions they could provide and which are highly appreciated for my success at the department.

Raili Engdahl and Lena Norgren at Uppsala Biomedical Centre (BMC) and Alf Johansson (Astra Zeneca) formerly at BMC, are warmly acknowledged for their empathy and never ending friendship and encouragements towards my interest for academia, as my thoughts also go to other former colleagues at the Division of Toxicology, BMC, my entry zone to Sweden, and from where I formed my good impression about the country and her people; from where my academic vision was made possible. I thank you all most sincerely.

Former colleagues at the Uppsala University PET Centre, who never doubted my respect and admiration for them and returned it in a warm and faithful manner, are warmly thanked for sharing my academic interest with me.

The friendliness of the members of staff of our twin institute, Department of Clinical Chemistry created a nice academic atmosphere highly appreciated.

To Doreen, Nathalie and Lawrence, my dear “big” children who have never failed me with their faithful love and who have always cherished my well-being and provided me with a meaning to live as long as possible to share the joy of life with them. Your whole-hearted contributions to the success of this thesis is recognized as truly unequivocal; and to my brothers and sisters, for their unconditional love and pride in me.
for being the ‘family’s ambassador’ in far away Sweden. Your positive hopes in me have always been my strength in my good fights for humanity. Let’s hope that this little work for which you all, my children, and many others, have been a support to, will not be in vain for mankind at large.

The financial supports provided by the **Swedish Cancer Society** and the **Lions Cancer Research Fund** are gratefully acknowledged.
References


86. Liu JY, Park SH, Morisseau C, Hwang SH, Hammock BD, Weiss RH. Sorafenib has soluble epoxide hydrolase inhibitory activity, which contributes to its effect profile in vivo. Mol Cancer Ther 2009;8:2193-203.
113. Kirkland RA, Saavedra GM, Franklin JL. Rapid activation of antioxidant defenses by nerve growth factor suppresses reactive oxygen species during neuronal

114. Daniel KG, Chen D, Yan B, Dou QP. Copper-binding compounds as proteasome inhibitors and apoptosis inducers in human cancer. Front Biosci 2007;12:135-44.


123. Song S, Kwon OS, Chung YB. Pharmacokinetics and metabolism of acriflavine in rats following intravenous or intramuscular administration of AG60, a mixture of acriflavine and guanosine, a potential antitumour agent. Xenobiotica 2005;35:755-73.
Dedication

*Professor of Toxicology, Uppsala University, Sven Folke Ullberg, (Sweden)

*Radiochemist Josephus Peter Herbert Brown, Centre for Radioisotope Application (Ghana)

and

*My dear late parents.
Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 593

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-130330