Analyses of Dos-Response and Mechanistic Action of Different Anti-Cancer Drugs for Neuroendocrine Tumor Cell Lines

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Abstract

Cancer is a disease with poor response rates on available treatments. Problems with resistance and intolerance against cancer drugs are major reasons for failure of the drugs. The need to discover new cancer drugs is important. In this thesis screening of new cancer drugs and evaluation of their mechanism of action are discussed. The aim of the thesis was to find new compounds active against neuroendocrine tumors (NETs).

In paper I, we screened 1280 substances on two bronchial carcinoid cell lines and one pancreatic carcinoid cell line. Eleven of these compounds were found to have antitumor activity at low concentrations. The most active agents were brefeldin A, emetine, bortezomib and idarubicin, having IC$_{50}$ values (the concentration of the drug where > 50% of the cells die) < 1 μM. In addition, sanguinarine, Bay11-7085, mitoxantrone, doxorubicin, β-lapachone, NSC 95397 and CGP-74514A were active with IC$_{50}$ values < 10 μM.

In paper II, additional studies have been undertaken to investigate the combination effect of the most active drugs with conventional cytotoxic drugs used in clinical practice. If synergistic or additive effects are found, drugs with different mechanism of action and toxicity profiles may be combined, making it possible to reduce the toxic effects yet maintaining the antitumor activity.

In paper III, studies were undertaken to find the mechanistic action, apoptosis or necrosis, of the drugs NSC 95397, brefeldin A, bortezomib and sanguinarine in NETs. All four drugs were shown to induce caspase-3 activity and nuclear fragmentation/condensation in the neuroendocrine tumor cell lines, indicating that their antitumor activity was induction of apoptosis.

In paper IV, the mechanism of action was studied for CGP-74514A and emetine. Both drugs worked by induction of apoptosis. In addition, their cytotoxic activity was studied in a three-dimensional model, the in vitro hollow fiber model. The Hollow Fiber model permits more realistic simulation of in vivo drug effects in a controlled system providing data that more accurately reflects biological responses. Our results showed that the hollow fiber model may be suitable for studies of new drugs in the neuroendocrine tumor cell lines.

Keywords: Cancer drugs, Screening, Hollow Fiber model, Apoptosis

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


IV Larsson DE, Hassan S, Oberg K, Granberg D. The Cytotoxic Effect of Emetine and CGP-74514A studied with the Hollow Fiber Model and Array Scan Assay in Neuroendocrine Tumors In Vitro. Submitted

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Additional Papers


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Abbreviations

BON-1 A human pancreatic carcinoid cell line
CI Combination Index
DMEM Dulbecco’s Modification of Eagle’s Medium
DMSO Dimethyl sulfoxide
FCS Fetal Calf Serum
FDA Fluorescein Diacetate
FMCA Fluorometric Microculture Cytotoxicity Assay
hTERT-RPE1 Normal human retinal pigment epithelial cell line
hTERT Human telomerase reverse transcriptase
NCI-H727 A human typical bronchial carcinoid cell line
NCI-H720 A human atypical bronchial carcinoid cell line
IC50 Inhibitory Concentration 50% (resulting in 50% survival)
MMP Mitochondrial Membrane Potential
PBS Phosphate buffered saline
SEM Standard Error of the Mean
SI Survival Index
Introduction

Cancer is characterized by uncontrolled division of cells and the ability of these cells to invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis (1). Over the last decades, intense research has been done to identify the molecular and genetic changes of cancer cells, and the pathogenesis of neoplasia. This has lead to identification of oncogenes, tumor suppressor genes and other molecular mechanisms explaining how the cancer cells growe survive and proliferate (2).

Classification of Neuroendocrine Tumors

Neuroendocrine tumors (NETs) derive from the neuroendocrine cell system, which is widely distributed in the body and form a heterogeneous group of malignancies, which differ from each other in their biology, prognosis and histopathological differences (3-5). The broad heterogeneity characterizing NETs has always posed problems regarding their correct classification. Neuroendocrine tumors (NETs) were earlier characterized by the production of peptide hormones that could give different endocrine symptoms. NETs were classified as functioning or non-functioning. Neuroendocrine tumors (NETs) have classically been divided into carcinoids and endocrine pancreatic tumors. Carcinoids were further subdivided, depending on the localization of the primary tumor, into: foregut carcinoids originating from the lungs, thymus, stomach and duodenum (in addition, endocrine pancreatic tumors belong to the foregut group); midgut carcinoids localized in the appendix, jejunum, ileum and proximal colon; and hindgut carcinoids, which originate from the distal colon and rectum. The most frequent sites of NETs are the gastrointestinal tract (70%) and the bronco pulmonary system (25%) (6).

Recently, a new classification of NETs has been proposed and adopted by the World Health Organization (WHO) (7). In order to explain the natural history of NETs more adequately, this classification is based on a series of histopathological and biological characteristics: cellular grading, primary tumor size and site, cell proliferation markers, local or vascular invasivity, and the production of biologically active substances. The World Health Organization (WHO) classification scheme places neuroendocrine tumors into three main categories: Well differentiated neuroendocrine tumors, further
subdivided into tumors with benign and those with uncertain behavior characterized by a low grade of malignancy, well differentiated (low grade) neuroendocrine carcinomas with low-grade malignant behavior which are more aggressive because of the presence of metastases and poorly differentiated neuroendocrine carcinomas with a high grade of malignancy and a poor prognosis.

This classification enables neuroendocrine tumors in the gastroenteropancreatic tract to be diagnosed easily, but unfortunately it is not applicable to lung tumors (8, 9).

The classification of lung tumors is currently based on that of Travis et al. (10) who recognized the following categories: typical carcinoid, atypical carcinoid, small-cell lung cancer (SCLC) and large-cell neuroendocrine carcinoma (LCNC). Typical bronchial carcinoids are more benign than atypical carcinoids, but both types are able to metastasize to regional lymph nodes or distantly to the liver, bones or brain (11, 12). SCLC and LCNC are poorly differentiated neuroendocrine carcinomas with a high frequency of metastasis and poor prognosis. Carcinoids are tumors able to produce hormones and they mainly occur in the gastrointestinal tract, a substantial percentage however is found in the bronchopulmonary system (13). Pancreatic endocrine tumors, also known as islet cell carcinomas, are another type of neuroendocrine tumor which represents 1-2% of the pancreatic neoplasm’s (14). Pancreatic endocrine tumors may cause endocrine syndromes from excessive production of hormones, such as insulin, gastrin, glucagon or vasoactive intestinal polypeptide (VIP). Although localized carcinoids or islet cell tumors are surgically manageable, metastatic disease is present in nearly 50% of patients at the time of diagnosis and cause significant mortality (15).

Experiences of patients with metastatic bronchial carcinoids and pancreatic endocrine tumors have given indication of poor response rates on available treatments (16). Medical treatment of patients with metastatic endocrine pancreatic tumors includes different chemotherapy combinations and biotherapy such as alpha-interferon and somatostatin analogs. Streptozocin combined with doxorubicin or 5-fluorouracil has generated partial remissions in 40-60% of the patients giving a median survival of two years in patients with advanced disease. Cisplatin plus etoposide have also demonstrated significant antitumor effect in patients with endocrine pancreatic tumors. Alpha interferon causes significant tumor reduction in about 15% of patients with long duration, up to three years. Octreotide rarely leads to objective responses (17). The use of the various chemotherapeutic agents, such as doxorubicin, 5-fluorouracil, cisplatin, carboplatin, etoposide streptozotocin and temozolomide has led to minimal responses in the treatment of patients with lung carcinoids (18,19) and pancreatic endocrine tumors (20,21), mostly of short duration. Even if an initial response is obtained in patients with malignant endocrine pancreatic tumors as well as bronchial carcinoids treated with chemotherapy or biotherapy, resistance to treatment sooner or later occurs.
There is thus a need for better treatments in patients with malignant neuroendocrine tumors.

**Cell death processes**

One target in the research field of oncology is to identify molecules important for apoptosis regulation in tumor cells (22). In order to identify and evaluate promising new anticancer drugs, it is important to rapidly and easily identify substances with apoptosis-inducing properties. Apoptosis, programmed cell death, is the necessary mechanism complementary to proliferation that ensures homeostasis of all tissues. In recent years, the molecular machinery responsible for apoptosis has been elucidated, revealing a family of intracellular proteases, one of the most important groups of proteins involved in apoptosis, the cystein aspartate-specific proteases also called caspases, which are responsible directly or indirectly for the morphologic and biochemical changes that characterize the phenomenon of apoptosis (23,24). Caspases are activated by different toxic stimuli. Three major pathways have been elucidated so far, which all result in the activation of caspase-3. One is the mitochondrial/cytochrome C pathway, largely mediated through Bcl-2 family members, which results in activation of Apaf-1, caspase-9, and then caspase-3. The second pathway involves ligation of members of the TNF-receptor family (e.g., Fas, TRAIL receptors) and activates caspase-8 and subsequently caspase-3. Finally, granzyme B (a cytolytic T-cell product) directly cleaves and activates several caspases, resulting in apoptosis (25, 26). Regardless of the pathways, it ends with DNA fragmentation with formation of apoptotic bodies, which are phagocysed by macrophages (27).

**Basic aspect of the cancer drugs investigated**

Cancer chemotherapy is the use of chemical compounds with various mechanism of action against growth and survival of tumor cells. It is used to cure or relieve tumor-related symptoms and prolong the life. Chemotherapy can be given alone as the only treatment or in combination with surgery and radiotherapy (28). In this thesis eleven anticancer drugs have been evaluated to have effect on two bronchial carcinoid cell lines and a pancreatic carcinoid cell line in vitro. Six of these drugs have been further investigated.

Figure 1 shows the molecular structures of the drugs investigated in this thesis.
Brefeldin A

Brefeldin A was initially introduced as an antiviral antibiotic (29) which is currently known to play a regulatory role in the intracellular transport system. Its biological activities have been reported to inhibit intracellular protein transport from the endoplasmic reticulum (ER) to the cis-Golgi apparatus (30), to promote reversible disassembly of the Golgi complex (31) and to facilitate redistribution of Golgi-associated proteins to the ER (32). A possible antitumor effect of Brefeldin A has also been proposed (33).

Emetine

Emetine is a potent protein synthesis inhibitor and is clinically used in the treatment of protozoan infection. Emetine has shown promise as an anti-tumor agent without bone marrow suppression (34-36).

The cytotoxicity of emetine is due to inhibition of protein biosynthesis in eukaryotic ribosomes (37) and interaction with DNA (38) Evidence of emetine's activity against tumor cells first came to light in 1918 (39) and its use in phase I and II clinical cancer trials began over 30 years ago (40-42). There are some publications suggesting induction of apoptosis by emetine in the
human leukemic cell line (U937) (43), human lung epithelial A549-S cells (44) and rat hepatocytes (45). Earlier studies have also shown that emetine is a strong inducer of apoptosis and caspase activity, comparable to the cytotoxic effect of cisplatin. Moreover, emetine enhanced the cytotoxic effect of cisplatin and increased cisplatin-induced apoptosis in leukemia cells (42). Thus, emetine could be a suitable cytotoxic agent in cancer therapy, e.g. to overcome multidrug resistance or to take advantage of synergistic effects in order to minimize side-effects due to the high dosage of other cytotoxic agents.

**Bortezomib**

Bortezomib (Velcade®), a proteasome inhibitor, has shown activity in early clinical trials among patients with Non- Hodgkin’s lymphoma and multiple myeloma. In a phase II trial, 50% of patients with recurrent myeloma who received 1.3 mg/m2 Bortezomib responded with complete inhibition of myeloma cell growth (46).

In vitro and in vivo (murine xenograft) studies have shown that bortezomib is active against a variety of malignancies, including hematologic malignancies and solid tumors (ie, breast, prostate, lung, pancreas, colon, ovarian, and head and neck cancers). Bortezomib has demonstrated activity as a single agent and in combination with several cytotoxic agents, such as 5-fluorouracil, doxorubicin, and docetaxel, and with radiation, enhancing both chemotherapy- and radiation-therapy-induced apoptosis. Bortezomib has also shown activity in some cell lines resistant to standard therapies (47-57).

**Sanguinarine chloride**

Sanguinarine chloride, a Na+/K+-ATPase inhibitor has been shown to possess antimicrobial, antioxidant, anti-inflammatory, and antitumor properties and is widely used in toothpaste and mouthwash to prevent/treat gingivitis and other inflammatory conditions of the mouth (58-60). Sanguinarine was also identified as a potential inhibitor of survivin that selectively kills prostate cancer cells over “normal” prostate epithelial cells. Survivin is selectively over expressed in most common human cancers and is a member of the inhibitor of apoptosis protein family. Studies have shown that sanguinarine induces apoptosis in various human cancer cells, including prostate cancer (61-64).

**NSC 95397**

NSC 95397 is described as a Cdc25 phosphatase inhibitor (65). Progression through the cell division cycle and transitions between its various phases are
regulated by the activation of specific cyclin-dependent kinase (CDK) complexes by CDC25 phosphatases (66).

As Cdc25 phosphatases promote cell cycle progression and are over-expressed in numerous rapidly dividing cancer cells, one might expect a correlation between over expression and the rate of proliferation. In fact, no correlation has been seen the majority (67-72). Thus, the role of Cdc25 over expression is more complicated than that of a simple driver of cell proliferation. It is quite likely that Cdc25 over expression in tumors is required to circumvent many of the checkpoints that would otherwise hinder cell proliferation.

NSC95397 is the most potent Cdc25 inhibitor to date (IC-50s of 22–125 nM). This compound blocks the G2/M transition and shows growth inhibition against human carcinoma cell lines (66).

**CGP-74514A hydrochloride**

Cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs) work simultaneously to regulate the cell cycle. CGP74514A is a new trisubstituted purine derivative that has been reported to function as a potent and selective inhibitor of CDK1 (73). For progression of the cell cycle phases, formation of complexes between cyclins and CDKs is necessary and CKIs have an inhibitory role against this. In addition to inhibiting cell cycle progression, CDK inhibitors are effective inducers of apoptosis in neoplastic cells (74).

**Measurement of Cytotoxicity**

The cytotoxic effect of different drugs can be studied by the fluorometric microculture cytotoxicity assay (FMCA) (Figure 2). The assay is based on the presence of esterases in viable cells that convert colourless fluorescein diacetate (FDA) to fluorescent fluorescein. Fluorescence intensity after drug exposure thus indicates the amount of surviving cells when compared to unexposed control cells.
Figure 2. Schematic illustration of the main steps of the Fluorometric Microculture Cytotoxicity Assay (FMCA)

Multiparametric evaluation of apoptosis

The current focus of drug discovery in the area of oncology is to identify molecules important for apoptosis regulation in tumor cells (22). Study of mechanistic action for tumor cell death; how the cells go to apoptosis or necrosis can be done by in vitro study. Thus, in the process of identifying and evaluating promising new anticancer agents, it is important to rapidly and easily identify substances with apoptosis-inducing properties. One approach to identify and evaluate substances that induce apoptosis would be to use automated information-rich image-based analysis, Array Scan, multiparametric apoptosis assay often called high-content screening (75). This assay is based on measurement of fluorescence intensity and localization on a cell-by-cell basis.
Cytotoxicity Measurement by Hollow Fiber assay

Many of the in vitro models used in preclinical anticancer drug development are based on monolayer or suspension cultures of human tumor cell lines. These models are technically simple and useful in selecting potentially active compounds for further study. However, they do not mimic the complex microenvironment, heterogeneity and proliferative properties of solid tumors and this may contribute to incorrect predictions of in vivo efficacy (76). Anticancer drugs can be effective in solid tumors only if they can penetrate several cell layers and retain their activity in the tumor microenvironment (77). To address this, three-dimensional in vitro solid tumor models have been developed such as the in vitro Hollow Fiber models (78). This assay, which is based on culturing tumor cells in polyvinylidene fluoride hollow fibers, was developed by Hollingshead (79).

The introduction of hollow fibers represents an important advancement in the field of in vitro cytotoxicity. It is believed that in vitro testing methods are useful, time and cost effective tools for drug discovery but many of the available tests are not effective for examining both time and concentration, and do not closely mimic human kinetics. This is because they do not properly take into account pharmacodynamic actions (what a drug does to the body) and pharmacokinetic actions (what a body does to the drug). The Hollow Fiber model has changed this. The hollow fiber model has exceeded the standard in vitro cytotoxicity methods by mimicking changes in the concentration of the drug over time as they occur in humans. Hollow Fiber technology offers higher reproducible control of both concentration and time of drug exposure in complex growth, infection, treatment and sampling regimens.

This system permits more realistic simulation of in vivo drug effects in a dynamically controlled system providing data that more accurately reflects biological responses (80).
Aims of the thesis

The overall aims of the thesis were to preclinically investigate new anticancer drugs on neuroendocrine tumors, based on mechanism of action, apoptosis and the cytotoxic effect on the cell lines using single drug or combination treatment. The overwhelming aim is to find more effective treatment for patients with endocrine tumors.

The specific aims were to:

1. Examine new anti-cancer drugs with effect on bronchial carcinoids and pancreatic endocrine tumors.

2. Evaluate potential synergistic effects for the new anti-cancer drugs; NSC 95397, emetine, CGP-74514A hydrochloride, brefeldin A and sanguinarine chloride, when combined with four standard cytotoxic drugs already used in the clinic for treatment of neuroendocrine tumors.

3. Study the mechanistic effect for the anti-cancer drugs; NSC 95397, bortezomib, brefeldin A and sanguinarine chloride for the apoptosis and morphologic changes with the ArrayScan assay.

4. Characterize in vitro the cytotoxic activity of emetine and CGP-74514A with Hollow Fiber assay and the apoptosis and morphologic changes with the ArrayScan assay.
Material and Methods

Human tumor cell lines (paper I, II, III and IV)
The human pancreatic carcinoid cell line BON-1 cells, derived from a lymph node metastasis of a human pancreatic carcinoid tumor, was cultured in a (1:1) nutrient mixture of Dulbecco’s Modification of Eagle’s Medium (DMEM) and Kaighn’s modification medium (F12K) (Invitrogen, Sweden). The human typical bronchial carcinoid cell line NCI-H727 and the human atypical bronchial carcinoid cell line NCI-H720 was obtained from ATCC (LGC Promochem, Sweden) and maintained in RPMI 1640 medium (Invitrogen, Sweden). The cell lines were supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 1% glutamine and 1% penicillin/streptomycin (Sigma Aldrich) and cultured in a 5% CO2 humidified atmosphere at 37 ºC. For comparison, we also studied a normal human retinal pigment epithelial cell line, hTERT-RPE1 (a human RPE cell line that stably expresses human telomerase reverse transcriptase (hTERT)) in study I. The epithelial cell line hTERT-RPE1 was cultured in DMEM.

Drugs and Reagents (paper I, II, III and IV)
In paper I; as a screening study, we first evaluated 1280 drugs obtained from the LOPAC1280™ library (Sigma Aldrich, St. Louis, MO). The library was screened at 10 µM in three tumor cell lines. Drugs with survival index (SI) above 60% were eliminated from further studies. Survival index was defined as the fluorescence of experimental wells in percent of control wells with blank values subtracted. SI = 100 x (treated cells - blank)/ (control cells – blank). The primary screening resulted in 18 candidate drugs with SI-value of less than 60%. These drugs were chosen for further dose-response experiments in the three tumor cell lines. The drugs selected from the initial screening, their mechanisms of action and the solvents are shown in (Table 1) (paper I). Drugs were purchased from Sigma-Aldrich. Doxorubicin was supplied by the local pharmacy (Uppsala, Sweden). All drugs were tested at five 10-fold dilutions ranging from 0.01 to 100 µM or 0.001 to 10 µM for the tumor cell lines and the epithelial cell line hTERT-RPE1.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Solvent</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apomorphine</td>
<td>Sterile water</td>
<td>Non-selective dopamine receptor agonist</td>
</tr>
<tr>
<td>Bay 11-7085</td>
<td>DMSO</td>
<td>Inhibits cytokine induced IkB· (inhibitor of NFkB) phosphorylation</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>DMSO</td>
<td>Proteasome inhibitor-ubiquitin-proteasome pathway</td>
</tr>
<tr>
<td>Brefeldin A</td>
<td>Ethanol, 95%</td>
<td>Inhibitor of protein translocation from the endoplasmic reticulum (ER) to the Golgi apparatus</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>DMSO</td>
<td>DNA topoisomerase I inhibitor</td>
</tr>
<tr>
<td>Cantharidin</td>
<td>DMSO</td>
<td>Protein phosphatase 2A inhibitor</td>
</tr>
<tr>
<td>CGP-74514A</td>
<td>DMSO</td>
<td>Cyclin-dependent kinase-1 (Cdk1) inhibitor</td>
</tr>
<tr>
<td>Colchicine</td>
<td>Sterile water</td>
<td>Inhibitor of tubulin (prevents tubulin polymerization)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>PBS</td>
<td>Inhibitor of DNA topoisomerase II</td>
</tr>
<tr>
<td>Emetine</td>
<td>Sterile water</td>
<td>Protein synthesis inhibitor at the level of translation</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>Sterile water</td>
<td>Inhibitor of DNA metabolism</td>
</tr>
<tr>
<td>β-Lapachone</td>
<td>DMSO</td>
<td>Inhibition or activation of DNA topoisomerase and inhibition of NF-kB activity</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Ethanol, 95%</td>
<td>Inhibitor of DNA metabolism, DNA synthesis inhibitor</td>
</tr>
<tr>
<td>NSC 95397</td>
<td>DMSO</td>
<td>Selective, irreversible Cdc25 dual specificity phosphatase inhibitor</td>
</tr>
<tr>
<td>Ouabain</td>
<td>Sterile water</td>
<td>Inhibitor of Na+/K+-ATPase</td>
</tr>
<tr>
<td>Parthenolide</td>
<td>DMSO</td>
<td>Inhibits serotonin release from platelets</td>
</tr>
<tr>
<td>Sanguinarine chloride</td>
<td>Methanol</td>
<td>Inhibitor of Na+/K+-ATPase</td>
</tr>
<tr>
<td>Vincristin</td>
<td>PBS</td>
<td>Inhibitor of tubulin (inhibit microtubule assembly)</td>
</tr>
</tbody>
</table>

DMSO, dimethyl-sulphoxide; PBS, phosphate-buffered saline, pH 7.4.

In paper II; five of these compounds, with different mechanisms of action, were chosen for further studies aiming at evaluating potential synergistic effects when combined with four standard cytotoxic drugs already used in the clinic for treatment of neuroendocrine tumors. The five chosen compounds were NSC-95397, a selective Cdc25 dual specificity phosphatase inhibitor; emetine, a protein synthesis inhibitor and DNA interacting agent; CGP-74514A hydrochloride, a cyclin-dependent kinase-1 inhibitor; brefeldin A, the inhibitor of the protein transport from the endoplasmic reticulum to the Golgi apparatus and sanguinarine chloride, a Na+/K+-ATPase inhibitor (59,38). The four standard drugs were doxorubicin and etoposide which are DNA topoisomerase II inhibitors, oxaliplatin which is alkylating and inhibits DNA synthesis by disrupting DNA replication and transcription, and docetaxel, a microtubule stabilizer and mitotic progression inhibitor. The studies were performed on three neuroendocrine tumor cell lines; the atypical bronchial carcinoid NCI-H720, the typical bronchial carcinoid NCI-H727
and the human pancreatic carcinoid cell line BON-1. The compounds NSC-95397, emetine, CGP-74514A hydrochloride, brefeldin A, sanguinarine chloride and the standard drugs; etoposide, oxaliplatine and docetaxel were purchased from Sigma-Aldrich, while doxorubicin was supplied by the local pharmacy (Uppsala, Sweden). All compounds and drugs were prepared according to the manufacturer’s instructions.

In paper III, measurement of apoptosis was used to investigate the apoptosis resulting from NSC 95397, brefeldin A, bortezomib and sanguinarine in the typical bronchial carcinoid NCI-H727 and the human pancreatic carcinoid cell line BON-1.

In paper IV, measurement of apoptosis was used to investigate the apoptosis for emetine and CGP-74514A and an *in vitro* hollow fiber model were used to study the effect and mode of induced cell death of emetine and CGP-74514A in the human endocrine carcinoids, typical lung carcinoid cell line and atypical lung carcinoid cell line.

**FMCA (fluorometric microculture cytotoxicity assay) (paper I, II)**

The fluorometric microculture cytotoxicity assay (FMCA) is a method to study the cytotoxic effect or sensitivity of different drugs. Tumor cells are cultured in drug-prepared 384-well plates. Three columns without drugs served as controls and one column with medium only served as blank. The plates were incubated at 37°C for 72 h after which they were analyzing using the FMCA. The assay is based on the presence of esterases in viable cells that convert colorless fluorescein diacetate (FDA) to fluorescent fluorescein by cells with intact plasma membranes. The fluorescence, which is proportional to the number of living cells, is measured. 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.

**IC\textsubscript{50}-values (paper I, II)**

The IC\textsubscript{50} values (inhibitory concentration 50%), estimated from the log concentration–effect curves in Graph Pad Prism (GraphPad software Inc., CA, USA) using non-linear regression analysis, for all tested drugs in the three cell lines were determined. The concentration range for the individual drugs, the mean IC\textsubscript{50} values for all 3 cell lines, as well as their drug ratios (test drug/standard drug) is shown in Table 2 (paper II).

The mean of the estimated IC\textsubscript{50} of the drug in the 3 cell lines for each drug was used to select its concentration range for the combination studies in study II.
Table 2. The tested drugs, concentration range, mean IC$_{50}$-values for all three neuroendocrine tumor cell lines and drug ratios.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration range tested (µM)</th>
<th>IC$_{50}$-value (µM)</th>
<th>Drug ratios (tested drug/standard drug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC-95397</td>
<td>0.34–86</td>
<td>5.4</td>
<td>1:0.5</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.19–48</td>
<td>3.0</td>
<td>1:30</td>
</tr>
<tr>
<td>Etoposide</td>
<td>6.6–1,696</td>
<td>106</td>
<td>1:19</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>2.5–630</td>
<td>39</td>
<td>1:7</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.62–160</td>
<td>10</td>
<td>1:2</td>
</tr>
<tr>
<td>Emetine</td>
<td>0.0062–1.6</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.19–48</td>
<td>3.0</td>
<td>1:30</td>
</tr>
<tr>
<td>Etoposide</td>
<td>6.6–1,696</td>
<td>106</td>
<td>1:1,06</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>2.5–630</td>
<td>39</td>
<td>1:394</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.62–160</td>
<td>10</td>
<td>1:100</td>
</tr>
<tr>
<td>CGP-74514A</td>
<td>0.12–32</td>
<td>2.0</td>
<td>1:2</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.19–48</td>
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</tr>
<tr>
<td>Oxaliplatin</td>
<td>2.5–630</td>
<td>39</td>
<td>1:20</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.62–160</td>
<td>10</td>
<td>1:5</td>
</tr>
<tr>
<td>BrefeldinA</td>
<td>0.0062–1.6</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.19–48</td>
<td>3.0</td>
<td>1:30</td>
</tr>
<tr>
<td>Etoposide</td>
<td>6.6–1,696</td>
<td>106</td>
<td>1:1,06</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>2.5–630</td>
<td>39</td>
<td>1:394</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.62–160</td>
<td>10</td>
<td>1:100</td>
</tr>
<tr>
<td>Sanguinarine</td>
<td>0.062–16</td>
<td>1.0</td>
<td>1:3</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.19–48</td>
<td>3.0</td>
<td>1:3</td>
</tr>
<tr>
<td>Etoposide</td>
<td>6.6–1,696</td>
<td>106</td>
<td>1:106</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>2.5–630</td>
<td>39</td>
<td>1:39</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.62–160</td>
<td>10</td>
<td>1:10</td>
</tr>
</tbody>
</table>

Combination studies (paper II)

The combination studies were designed as suggested in the CalcuSyn software manual (81). We investigated nine different concentrations of each drugs by diluting the highest concentration two-fold to span a wide effect range, on both side of IC$_{50}$. With this design, the drugs were combined at equipotent concentrations with a fix ratio. IC$_{50}$ of one drug were combined with IC$_{50}$ of the other drug and so on. Fixed concentration ratios of the drugs were used with two-fold serial dilutions in nine steps for combinations and for single drug containing wells.

To characterize the combination effects between the 5 compounds and the 4 standard cytotoxic agents, data were analyzed according to the median-effect method of Chou and Talalay, using the software CalcuSyn Version 2 (Biosoft, Cambridge, UK) (82). Each dose–response curve (individual agents as well as combinations) was fit to a linear model using the median effect
equation. The extent of drug interaction between the drugs was expressed using the combination index (CI) for mutually exclusive drugs:

\[ \text{CI} = \frac{d_1}{D_1} + \frac{d_2}{D_2} \]

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 75% effect was chosen for presentation. Synergy was defined as CI significantly lower than 1 and antagonism as CI significantly higher than 1. When the confidence interval included 1 the interaction was defined as additive.

Array Scan (multiparametric apoptosis assay) (paper III, IV)

Cell death characteristics, can be studied by a multiparametric single-cell assay. We have studied the mechanistic effect for the cell death; apoptosis for the most interesting drugs for the to neuroendocrine tumor cell lines BON-1 and NCI-H727. This was done with the multiparametric apoptosis assay, Array Scan HCS. Cells are seeded and exposed to drugs in 96-well plates and probes are added to stain apoptotic markers: FAM-DEVD-FMK to stain activated caspase-3, MitoTracker Red to evaluate mitochondrial membrane potential (MMP) and Hoechst 33342 to stain nucleus. Analysis are performed by the ArrayScan high content screening system (Cellomics Inc.) which is a computerized automated fluorescence imaging microscope that automatically identifies stained cells and reports the intensity and distribution of fluorescence in individual cells. Automatic focusing, image acquisition and analysis are performed to collect data on a user-defined number of cells. Images and data regarding intensity and texture of the fluorescence within the individual cells, as well as the average fluorescence of the cell population within a well are stored in a database for easy retrieval and analysis.

In this work, we present evaluation of caspase-3 activity, fragmentation/condensation and nuclear morphology in cells exposed to the cytotoxic drugs, NSC 95397, brefeldin A, sanguinarine chloride, emetine and CGP-74514A hydrochloride.
Hollow Fiber Assay (paper IV)

We have studied if the effect of the drugs on the endocrine carcinoid cell lines can be transferred to the solid tumors by the Hollow Fiber Assay.

The assay is based on implanting tumor cells cultured in polyvinylidene fluoride hollow fibers in vitro or in vivo for the living cell density assessment.

In paper IV we have applied an in vitro Hollow Fiber model for the assessment of growth and drug sensitivity for the bronchial carcinoid cell lines.

The effect of the assay can be analyzed by fluorometric microculture cytotoxicity assay (FMCA).

Statistical analysis (paper I, II and IV)

The IC\textsubscript{50}-values for drugs in the cell lines were determined from log concentration effect (survival index %) curves in GraphPad Prism using non-linear regression analysis. Statistical analysis was performed using the GraphPad Prism software. Comparison of activity between two groups, here the activity in the cell lines, was made with an unpaired Student’s t test and ANOVA with Bonferroni post-hoc test were used to compare three or more treatments groups. Significance level was set to p < 0.05. Comparison of activity between two groups in paper II was made with two-sided unpaired Student’s t test. One-sample t tests were used to determine if the CI differed from 1. SI values at different drug concentrations for the various tumor cells tested were compared using an unpaired Student's t-test in paper IV.
Results and Discussion

The Screening study (paper I)
The aim of the Paper I was to examine new anti-cancer drugs with effect on bronchial carcinoids and pancreatic endocrine tumors. The fluorometric microculture cytotoxicity assay (FMCA) were used for screening of 1280 drugs from the library (LOPAC1280™) on the three neuroendocrine cell lines and the normal human retinal pigment epithelial cell line, hTERT-RPE. The screening study resulted in 18 candidate drugs with SI-value of less than 60%. To assess the activity of the hit compounds, dose-response experiments were performed. The activity of the compounds was determined by their IC50-values (the concentration of the drug where > 50% of the cells dies). Compounds with IC50-values < 10 µM were selected as active. Eleven of 18 compounds were sensitive with IC50-values < 10 µM, shown in Table 3 (study I). Brefeldin A, emetine, bortezomib and idarubicin were the most active agents in vitro, with IC50 values < 1 µM in all four cell lines, while sanguinarine chloride showed IC50 values between 0.5 and 2.µM. In addition, Bay 11-7085, mitoxantrone, doxorubicin, β-lapachone, CGP-74514A and NSC 95397 showed IC50 values < 10 µM in the three tumor cell lines.

To conclude, in Paper I, our results indicated that in vitro screening using annotated compound libraries may be used for identification of compounds with antitumor activity in neuroendocrine tumor models. Our study demonstrated that Bay 11-7085, bortezomib, brefeldin A, CGP-74514A, doxorubicin, emetine, idarubicin, β-lapachone, mitoxantrone, NSC 95397, and sanguinarine showed antitumor effect in the human bronchial carcinoid and pancreatic carcinoid cell lines in vitro.
Table 3. In vitro sensitivity of the drugs with IC50 values <10µM in the tumor cell lines

<table>
<thead>
<tr>
<th>Drug</th>
<th>NCI-H720 IC50</th>
<th>NCI-H727 IC50</th>
<th>BON-1 IC50</th>
<th>hTERT-RPE1-IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brefeldin A</td>
<td>0.071</td>
<td>0.092</td>
<td>0.52</td>
<td>0.29</td>
</tr>
<tr>
<td>Emetine</td>
<td>0.094</td>
<td>0.15</td>
<td>0.11</td>
<td>0.40</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>0.55</td>
<td>0.63</td>
<td>0.38</td>
<td>0.73</td>
</tr>
<tr>
<td>Idarubicin</td>
<td>0.71</td>
<td>0.87</td>
<td>0.99</td>
<td>0.26</td>
</tr>
<tr>
<td>Sanguinarine</td>
<td>0.57</td>
<td>1.0</td>
<td>1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Bay 11-7085</td>
<td>1.6</td>
<td>3.4</td>
<td>2.2</td>
<td>0.97</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>1.6</td>
<td>3.5</td>
<td>2.1</td>
<td>0.69</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.4</td>
<td>5.4</td>
<td>3.4</td>
<td>2.7</td>
</tr>
<tr>
<td>β-Lapachone</td>
<td>2.1</td>
<td>2.7</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>CGP74514A</td>
<td>2.2</td>
<td>3.2</td>
<td>1.9</td>
<td>13</td>
</tr>
<tr>
<td>NSC 95397</td>
<td>1.4</td>
<td>8.3</td>
<td>8.9</td>
<td>3.5</td>
</tr>
</tbody>
</table>

H720, atypical bronchial carcinoid cell line; H727, typical bronchial carcinoid cell line; BON-1, pancreatic carcinoid cell line; hTERT-RPE1, normal human retinal pigment epithelial cell line.

The combination analyses (paper II)

The aim of Paper II was to evaluate potential synergistic effects for the new anti-cancer drugs; NSC 95397, brefeldin A and sanguinarine chloride, when combined with four standard cytotoxic drugs already used in the clinic for treatment of neuroendocrine tumors. Synergistic effect is the interaction between two or more substances that produces an effect greater than the sum of their individual effects. It is opposite of antagonism. Additive effect is the term used when two or more drugs are taken at the same time and the action of one plus the action of the other results in an action as if just one drug had been given. Antagonism is a phenomenon where two or more substances in combination have an overall effect which is less than the sum of their individual effects.

Table 4 summarizes the combination effect for NSC-95397, emetine, CGP-74514, brefeldin A and sanguinarine combined with the four standard drugs in the three neuroendocrine tumor cell lines.

The number of synergistic interactions of the five drugs with the four standard drugs in the typical NCI-H727, the atypical NCI-H720 and the pancreatic neuroendocrine cell line BON-1 was 11/20, 7/20 and 5/20 respectively.
Table 4. Combination effect of NSC-95397, emetine, CGP-74514A, brefeldin A, and sanguinarine with the standard chemotherapeutic drugs in neuroendocrine tumor cell lines: pancreatic carcinoid tumor (BON-1), typical broncial carcinoid (NCI-H727) and atypical broncial carcinoid (NCI-H720) in vitro

<table>
<thead>
<tr>
<th>Combination</th>
<th>BON-1 Effect</th>
<th>NCI-H727 Effect</th>
<th>NCI-H720 Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC-95397 + Doxorubicin</td>
<td>Synergistic</td>
<td>Synergistic</td>
<td>Synergistic</td>
</tr>
<tr>
<td>NSC-95397 + Etoposide</td>
<td>Synergistic</td>
<td>Synergistic</td>
<td>Synergistic</td>
</tr>
<tr>
<td>NSC-95397 + Oxaliplatin</td>
<td>Additive</td>
<td>Synergistic</td>
<td>Synergistic</td>
</tr>
<tr>
<td>NSC-95397 + Docetaxel</td>
<td>Additive</td>
<td>Synergistic</td>
<td>Additive</td>
</tr>
<tr>
<td>Emetine + Doxorubicin</td>
<td>Additive</td>
<td>Additive</td>
<td>Additive</td>
</tr>
<tr>
<td>Emetine + Etoposide</td>
<td>Synergistic</td>
<td>Synergistic</td>
<td>Synergistic</td>
</tr>
<tr>
<td>Emetine + Oxaliplatin</td>
<td>Synergistic</td>
<td>Additive</td>
<td>Synergistic</td>
</tr>
<tr>
<td>Emetine + Docetaxel</td>
<td>Synergistic</td>
<td>Synergistic</td>
<td>Synergistic</td>
</tr>
<tr>
<td>CGP-74514A + Doxorubicin</td>
<td>Additive</td>
<td>Synergistic</td>
<td>Additive</td>
</tr>
<tr>
<td>CGP-74514A + Etoposide</td>
<td>Additive</td>
<td>Additive</td>
<td>Additive</td>
</tr>
<tr>
<td>CGP-74514A + Oxaliplatin</td>
<td>Additive</td>
<td>Additive</td>
<td>Additive</td>
</tr>
<tr>
<td>CGP-74514A + Docetaxel</td>
<td>Additive</td>
<td>Antagonistic</td>
<td>Additive</td>
</tr>
<tr>
<td>Brefeldin A + Doxorubicin</td>
<td>Additive</td>
<td>Synergistic</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>Brefeldin A + Etoposide</td>
<td>Additive</td>
<td>Synergistic</td>
<td>Synergistic</td>
</tr>
<tr>
<td>Brefeldin A + Oxaliplatin</td>
<td>Additive</td>
<td>Additive</td>
<td>Additive</td>
</tr>
<tr>
<td>Brefeldin A + Docetaxel</td>
<td>Antagonistic</td>
<td>Synergistic</td>
<td>Additive</td>
</tr>
<tr>
<td>Sanguinarine + Doxorubicin</td>
<td>Additive</td>
<td>Synergistic</td>
<td>Additive</td>
</tr>
<tr>
<td>Sanguinarine + Etoposide</td>
<td>Additive</td>
<td>Additive</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>Sanguinarine + Oxaliplatin</td>
<td>Antagonistic</td>
<td>Additive</td>
<td>Antagonistic</td>
</tr>
<tr>
<td>Sanguinarine + Docetaxel</td>
<td>Additive</td>
<td>Additive</td>
<td>Antagonistic</td>
</tr>
</tbody>
</table>

NSC-95397

The combination of NSC-95397 and the four standard cytotoxic drugs showed synergy in the two lung carcinoid cell lines NCI-H727 and NCI-H720. The interaction with NSC-95397 and doxorubicin or NSC 95397 and etoposide in the pancreatic carcinoid cell line BON-1 also showed synergy. The combination with docetaxel showed additive effect in the atypical carcinoid cell line NCI-H720. It was additive with oxaliplatin and docetaxel carcinoid cell line BON-1. The number of synergistic interactions of NSC-95397 with all four standard drugs was 9/12.

All NSC-95397 combinations with doxorubicin and etoposide were synergy which may indicate that these combinations may be the possible candidates for further pre-clinical and clinical studies. It has previously been reported that NSC-95397 showed synergy effect with paclitaxel in breast cancer cell line (22) and in the combination of other Cdc25 inhibitors with paclitaxel have additive effect on colon cancer cells (38). Docetaxel is a drug not commonly used in patients with carcinoids and there have been no published...
reports, preclinical or clinical, on docetaxel-containing combinations in the
treatment of patients with neuroendocrine tumors. Our results indicate that
docetaxel as well as oxaliplatin are suitable candidates for combination with
NSC-95397 with a synergy and additive effect. It is important to remember
that additive interactions, not only synergistic, could be of clinical benefit if
the drugs have non-overlapping toxicity profiles. For NSC-95397 no reports
are available regarding its toxic side effects. Combining drugs with synergis-
tic or additive interaction effects and non-overlapping side effects may ena-
ble dose reduction of toxic chemotherapeutic agents, yet maintaining enough
antitumor effect.

**Emetine**

The interactions for emetine were mainly synergy with etoposide, oxaliplatin
and docetaxel while they were additive with doxorubicin in all cell lines.
Such combinations are thus interesting objects for further studies. The num-
ber of synergistic interactions of emetine with all four standard drugs was
8/12. In Figure 3, emetine and etoposide combinations showing synergism
are visualized as concentration-effect curves, bar graph of chosen concentra-
tion, isobolograms and CI plots, in BON-1 (Figure 3 A-D, left panel) and
H727 (Figure 3 E-H, right panel) cell lines.
Figure 3. Combination of emetine and etoposide in the human pancreatic carcinoid cell line, BON-1 (A–D, left panel) and in the human typical bronchial carcinoid cell line NCI-H727 (E–H, right panel).
CGP-74514A hydrochloride

Almost all interactions for CGP-74514A hydrochloride and the standard cytotoxic drugs were additive. This agent may thus also be a possible candidate for further studies.

The synergistic interactions of CGP-74514A hydrochloride, with the four standard drugs were few, 1/12, and the number of additive interactions were 10/12.

Brefeldin A

The most interactions for Brefeldin A were also additive and the synergistic interactions with the four standard drugs were few, 4/12 while the numbers of additive interactions were, 6/12.

Sanguinarine chloride

The synergistic interactions of sanguinarine chloride with the four standard drugs were also few, 1/12, and the numbers of additive interactions were, 8/12 for the three cell lines. The interactions with etoposide were additive, while the interactions with oxaliplatin and docetaxel showed antagonistic effect for the atypical carcinoid cell line NCI-H720. Sanguinarine and oxaliplatin combinations showing antagonism are visualized as concentration–effect curves, bar graph of chosen concentration, isobolograms and CI plots in Figure 4, in BON-1 (Figure 4 A-D , left panel) and H720 (Figure 4 E-H , right panel) cell lines.

To summarize, in paper II, the interaction-effects between the five investigated drugs and the four standard cytotoxic agents were mostly synergistic or additive. NSC-95397, the Cdc25 dual specificity phosphatase inhibitor and emetine, the protein synthesis inhibitor and DNA interacting agent had the most attractive interactions (synergy) in combination with the four standard cytotoxic drugs in the three cell lines tested. The synergy interactions of NSC-95397 and emetine in the pancreatic and atypical bronchial carcinoid cell lines is worth noting as the pancreatic carcinoid cell line previously has been shown to be the least sensitive cell line to the five drugs (59) and clinical studies have demonstrated a worse prognosis for patients with atypical compared to patients with typical bronchial carcinoids (83).

The three remaining compounds CGP-74514A, brefeldin A and sanguinarine displayed less synergy interactions, with combination index < 1 in most of the interactions with the standard drugs. They thus seem less attractive to combine with the standard chemotherapeutic agents we tested.
Figure 4. Combination of sanguinarine and oxaliplatin in the human pancreatic carcinoid cell line, BON-1 (A–D, left panel) and in the human atypicabronchial carcinoid cell line NCI-H720 (E–H, right panel).
Array Scan (multiparametric apoptosis assay)
(paper III, IV)

Our results show that NSC 95397 bortezomib, brefeldin A, sanguinarine, emetine and CGP-74514A, induced high level of caspase-3 activity, apoptotic changes in nuclear morphology with nuclear fragmentation/condensation and a dose-dependent increase in fragmentation/condensation after 24h, 48h and 72 h in the neuroendocrine cell lines. Cells exposed for drugs for 48 h showed marked increase in the typical signs of apoptosis: chromatin condensation and nuclear fragmentation with less necrosis. After 24 h, a modest decrease in MMP was observed for the drugs at the lowest tested concentrations compared to the untreated controls. In summary, in Paper III and IV our data indicated induction of apoptosis with the tested drugs by intrinsic or extrinsic pathways in the neuroendocrine tumor cell lines. The experiments have demonstrated that the tested drugs; NSC 95397 bortezomib, brefeldin A, sanguinarine, emetine and CGP-74514A had a considerable apoptotic effect in the studied human neuroendocrine tumor cells \textit{in vitro}. At longer exposure times, these drugs activated the extrinsic apoptotic pathway and at shorter drug exposure times (24 h), the intrinsic apoptotic pathway resulting in nuclear condensation and fragmentation.

Hollow Fiber Assay (paper IV)

In paper IV with the \textit{in vitro} Hollow Fiber, the human atypical bronchial carcinoid cell line NCI-H720 showed a higher absorbance signal per cell than typical bronchial carcinoid cell line NCI-H727 when these tumor cells were cultured inside the hollow fiber for 2 weeks. There was gradual increase in the signals generated by the viable cells throughout the observation period under the growth condition evaluated. A concentration-dependent decrease in SI treated with emetine or CGP-74514A was observed for both cell lines. There was a tendency for the 3-day, high-proliferating cultures to be more sensitive than the 14-day low-proliferating cultures for emetine (p <0.05 at 0.2 µM) and for CGP-74514A (p <0.05 at 4.0 µM). Since emetine and CGP-74514A showed high in vitro activity against the human atypical bronchial carcinoid cell line NCI-H720, these drugs are possible candidates for further studies, yet the activity was less towards the human typical bronchial carcinoid cell line NCI-H727. Atypical bronchial carcinoids are more prone to metastasize and recur than typical ones. It is thus more urgent to find new active drugs against atypical carcinoids. Another conclusion, from Paper IV is the confirmation of the possibility to use human tumor cells from NCI-H727 and NCI-H720 cell lines in the Hollow Fiber assay. This may hopefully facilitate and reduce the costs for developing new active drugs for patients with bronchial carcinoids.
Drug discovery is a time-consuming and a labour-intensive process that evolves through several sequential phases including target identification and validation, drug design, identification and characterization, clinical candidate selection and pre-clinical and clinical testing (84). In the development of novel therapies for cancer treatment it is important to have good insight of the molecular mechanism of the drug and events involved in the induction of cell death. The results presented in this thesis have given opportunity to go further on and do animal studies on these drugs. We are therefore planning further studies, both in animals and using the Hollow Fiber model, to validate the cytotoxicity of the eleven drugs that have shown cytotoxicity in vitro. We also aim at performing combination studies with the most interesting drugs and standard chemotherapeutic agents in animals and with the Hollow fiber model both in vitro and in vivo. We hope that this will lead to better therapeutic options for patients with neuroendocrine tumors, both by the synergistic and additive effects per se, by overcoming drug resistance and by decreasing the side effects of toxic chemotherapeutic drugs, which may possibly be given in lower doses.

Solid tumors require the formation of new blood vessels when growing to a size greater than 2 mm in diameter. NETs are highly vascularized and are also known to express endothelial growth factor receptor (VEGF) (85). The formation of new blood vessels, called angiogenesis, can be inhibited by several drugs as human endostatin which inhibits the proliferation and migration of vascular endothelial cells and thereby inhibits angiogenesis and tumor growth (86).

Bevacizumab (Avastin™, Roche) is a monoclonal antibody against VEGF with antiangiogenic properties. This drug was compared with pegylated interferon alfa-2b in 44 patients with carcinoids already taking octreotide. Among the bevacizumab treated patient, four patients (18%) achieved partial response (PR) and 17 patients (77%) had stable disease (SD). In the PEGinterferon arm, none had radiological response and 15 patients (68%) had SD. It is however important to realize that PEGinterferon, which also has antiangiogenic properties, was given in a lower dose than normally used.

Combinations between antiangiogenic and cytotoxic drugs have also been tried. In one study with thalidomide, which has antiangiogenic activity by interfering with the VEGF pathway, and temozolomide, antiangiogenic activity response were observed in patients with endocrine pancreatic carcino-
mas, in carcinoid patients (87). To further optimize the antitumor effect of the drugs studied in this thesis we would validate the combination between these drugs and standard antiangiogenic agents, both using the Hollow Fiber model and in animal experiments. This may hopefully result in new interesting treatment combinations for patients with neuroendocrine tumors, with increased activity and fewer side effects.

Vi har studerat nya cytostatika på neuroendokrina tumörcellinjer genom singel- eller kombinationsbehandling, baserat på verkningsmekanism, apoptos och cytotoxisk effekt på bronkialkarciinoilder; atypisk lungcarcinoid NCI-H720 och typisk lungcarcinoid NCI-H727 och endokrina pankreaskarciinoilder celluljen BON-1. Studierna är främst utförda med fluorometrisk microcultures cytotoxicity analys (FMCA), där fluorescens, som är proportionell till...
antalet levande celler, mäts. Mekanismen för celldöd, apoptos gjordes med multiparameter apoptos analysen Array Scan HCS.

Karakteriseringen in vitro av den cytotoxiska aktiviteten hos substanserna studerades med Hollow Fiber assay.

Många av in vitro-modeller som används i preklinisk cancer läkemedels-utveckling bygger på tumörcellinjer. Dessa modeller är tekniskt enkla och användbara för vidare studier, men de efterliknar inte den komplexa mikromiljö, heterogenitet och proliferativa egenskaper hos solida tumörer, och detta kan bidra till felaktiga förutsägelser vid in vivo effekter. Läkemedel för behandling av cancer kan vara effektiv i solida tumörer endast om de kan tränga in i flera cellager och behålla sin verksamhet i tumörens mikromiljö.


Delarbete 1

I delarbete har vi screenat 1280 droger som erhållits från LOPAC1280TM bibliotek (Sigma Aldrich, St Louis, MO). Läkemedel med överlevnad index (SI) under 60 % har tagits fram för vidare studier. Studierna utfördes på tre neuroendokrina tumörcellinjer, som jämförelse studerade vi också en normal human retinal pigmentepitel cellinje, hTERT-RPE1 med fluorometrisk microculture cytototoxicity analys (FMCA). Den första undersökningen resulterade i 18 läkemedelskandidater med SI-värde lägre än 60 %. Dessa läkemedel valdes ut för ytterligare en dos-respons experiment i tre neuroendokrina tumör cellinjer och en normal epitcelcellinje. Alla droger testades i intervallet 0,001 till 100 µM. Den cytotoxiska effekten av föreningarna bestämdes av deras IC50-värden (koncentrationen av läkemedlet där > 50 % av cellerna dör). Elva av 18 föreningar var känsliga med IC50-värden <10 µM. Sammanfattningsvis har vår studie visat att de elva substanserna, Bay 11-7085, bortezomib, brefeldin A, CGP-74514A, doxorubicin, emetine, idarubicin, β-lapachone, mitoxantron, NSC 95397, och sanguinarine har antitumör-effekt i lungcarcinoid och pankreas carcinoid cellinjer in vitro.

Delarbete 2

I det första delarbetet fann vi 11 föreningar efter en screening på 1280 cytostatika där 50 % av tumör cellerna dog efter behandling av cytostatika med koncentration <10 µM. I delarbete 2 valdes fem av dessa föreningar ut, med olika verkningsmekanismer, för vidare studier med fluorometrisk microculture cytotoxicity analys (FMCA), som syftar till att utvärdera potentiella synergistiska effekter när de kombineras med fyra etablerade cytotoxiska

Delarbete 3

Delarbete 4
liga tumörceller från NCI-H727 och NCI-H720 celllinjer och överföra cellerna till solida tumörer med hjälp av Hollow Fiber analys.
Acknowledgements

This work was carried out at the department of Medical Sciences, Endocrine Oncology and Division of Clinical Pharmacology at Academic Hospital, Uppsala University. Financial support was provided by grants from the Lion’s Cancer Research Foundation.

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