Bacterial toxins
for cancer treatment

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Keep It Simple, Stupid
ABSTRACT

Even though anti-cancer chemotherapy has been continuously improved during the last decades, problems with adverse effects and drug resistance still constitutes a considerable obstacle and sets a demand for new effective treatment options. Tissue homeostasis in multi-cellular organisms is maintained through intrinsic cell death, apoptosis, which removes unwanted or damaged cells. Disrupted apoptosis is an important factor in tumorigenesis and drug resistance, therefore induction or restoration of apoptotic pathways is also important for the treatment of cancer. Several naturally occurring bacterial toxins have the ability to induce apoptosis and could thus be candidates to complement or improve the therapeutic effect of other anticancer drugs.

The bacterial toxins, adenylate cyclase (AC) toxin from *Bordetella pertussis*, α-toxin from *Staphylococcus aureus* and verotoxin-1 (VT-1) from *Escherichia coli* were investigated for their ability to induce apoptosis in different tumor cell lines. Toxin induction of cell death was investigated by cell viability assays, end-stage apoptosis induction by DNA-fragmentation (TUNEL) assay. Toxin receptor expression and signal transduction pathways to apoptosis were investigated by flow cytometry, caspase enzyme activity assays and western blot. Immunohistochemistry was used for identification of toxin receptor expression in tumor tissue samples.

AC-toxin was cytotoxic and induced apoptosis in cultured malignant plural mesothelioma (MPM) and small-cell lung cancer (SCLC) cells. Low-toxic concentrations of AC-toxin enhanced cisplatin cytotoxicity and apoptosis in both cell lines.

MPM-cells with acquired cisplatin resistance were more sensitive to α-toxin than the less resistant parental MPM cell line. A low-toxic concentration of α-toxin re-sensitized resistant MPM cells to cisplatin cytotoxicity by apoptosis induced through the mitochondrial pathway without detectable activation of common up-stream apoptosis signalling proteins.

VT-1 was highly cytotoxic and induced apoptosis in globotriosylceramide (Gb3) -expressing glioma, breast cancer and non-small-cell lung cancer (NSCLC) cells but was not cytotoxic to non-Gb3-expressing cells. PPMP, an inhibitor of glucosylceramide synthesis which makes exposed cells unable to synthesize Gb3 rendered Gb3-expressing cells resistant to VT-1. MPM cells with acquired-cisplatin resistance expressed Gb3 in contrast to the absent of expression in the less resistant parental cell line. Gb3, could however be up-regulated by cisplatin in Gb3-negative MPM-cells. Presence of a low-toxic concentration of VT-1 potentiated cisplatin-induced cytotoxicity and apoptosis in the cisplatin-resistance MPM cell line. VT-1 was a potent inducer of apoptosis, probably via stress-induced Mitogen-activated protein kinase (MAPK)-signaling involving c-Jun N-terminal kinase (JNK) and p38, leading to disruption of the mitochondrial membrane integrity, activation of caspase-9 and -3, and ultimately DNA fragmentation and cell death. Gb3 expression was demonstrated in clinical specimens of glioblastoma and breast cancer making these tumor types interesting for further VT-1 studies.

We conclude that bacterial toxins may be used to induce apoptosis in several types of cancer cells. Low concentrations of verotoxin-1 and α-toxin may potentially be used to overcome acquired cisplatin-resistance in cancer patients.
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Adenylate Cyclase-toxin (I) ....................................................... 27
This thesis is based on the following original articles which are referred to in the text by their roman numerals.


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Många av de bakterier som ger upphov till infektioner i människor har förmågan att utsändra ämnena (toxiner) som på olika sätt påverkar den infekterade vävnaden. Flera av dessa toxiner har förmågan att påverka celler i vävnaden så att de dör utan att det påverkar omkringliggande vävnad. Denna celldöd kallas apoptos och är en viktig funktion som tar bort gamla och skadade celler i kroppen. När celler förlorar förmågan att gå i apoptos kan det leda till obalans i vävnaden och till att tumörer uppstår.

Målet med denna avhandling var att studera hur de bakteriella toxinerna α-toxin, AC-toxin och VT-1 påverkar cancerceller med avseende på celldöd och apoptos.


De undersökta toxinerna kunde effektivt döda cancerceller genom apoptos. Detta gällde även celler som inte längre svarade på vanlig behandling. Eftersom dessa toxiner även kan vara mycket giftiga krävs mer forskning om deras mekanismer innan de kan komma till klinisk användning utan att riskera att de även skadar andra organ i kroppen.
Abbreviations

α-toxin  Alpha-toxin from *Staphylococcus aureus*
AC-toxin  Adenylate Cyclase-toxin from *Bordetella pertussis*
BCA  Bicinchoninic acid
cAMP  Cyclic AMP
Caspase  Cystein-aspartic-acid-protease
Cisplatin  Cis-diamminedichloroplatinum(II)
FDA  Fluorescein diacetate
FMCA  Fluorometric microculture cytotoxicity assay
Gb3  Globotriaosylceramide
JNK  c-Jun N-terminal kinase
LDH  Lactate dehydrogenase
MAPK  Mitogen-activated protein kinase
MAPKK  Mitogen-activated protein kinase kinase
MAPKKK  Mitogen-activated protein kinase kinase kinase
MDR1  Multidrug-resistant-1
MEBCD  Methyl-β-cyclodextrin
MPM  Malignant pleural mesothelioma
NSCLC  Non-small-cell lung cancer
PPMP  DL-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol
PS  Phosphatidylserine
SCLC  Small-cell lung cancer
SAPK  Stress-activated protein kinase
TUNEL  TdT-mediated dUTP nick end labeling
VT-1  Verotoxin-1
Introduction

Bacterial Toxins

The concept of using bacterial toxins in cancer treatment is not new. During the early 1960's there were several clinical trials with toxin therapy of malignancies such as sarcoma and malignant melanoma [1-3]. In 1970 Moolten and Cooperband showed that antibody-conjugated *Diphtheria* toxin could selectively kill cells expressing specific antigens [4]. Today both *Diphtheria* toxin and exotoxins from *Pseudomonas aeruginosa* have undergone clinical trials in patients with leukemia and malignant astrocytoma [5-8].

One reason why a number of bacterial toxins are interesting as therapeutic agents is a highly potent ability to induce apoptosis [9-13]. However the potency of these toxins also sets a demand to find ways to circumvent the obvious risk of adverse effects; this could be done using modified toxins with increased specificity against antigens over-expressed in the tumor and/or by locally administrated treatment [14-16]. Toxins targeted against specific cells or signal transduction pathways could improve cancer therapy and possibly reduce the risk of adverse effects which today in some cases surpasses the chance of favorable treatment.

AC-toxin of *Bordetella pertussis*

Adenylate cyclase-toxin (AC-toxin) is the main virulence factor of *Bordetella pertussis*, the causative agent of whooping cough. It was identified as a toxin 1982 by Confer and Eaton who showed that AC-toxin could inhibit phagocytosis and thereby help to protect the bacteria from the inflammatory response of the host [17].
AC-toxin is a single 1,706-amino-acids long polypeptide which binds with high affinity to the CD11b receptor [18]. It is known to hold two cytotoxic properties, hemolytic ability through induction of small cat-ion-selective pores in the cell membrane and also the ability to deliver an enzymatic AC domain directly through the cell membrane into the cytosol where AC is activated by binding to endogenous calmodulin and then catalyzes unregulated conversion of ATP to cyclic AMP (cAMP) [19, 20]. It has been shown that AC-toxin can induce apoptosis but the mechanisms are still not fully understood [12, 21]. cAMP has however been shown to induce growth arrest and apoptosis in assorted cancer cell lines [22, 23], possibly through a Smac/Diablo-dependent pathway [24].

α-toxin of Staphylococcus aureus

Even though Staphylococcus aureus often is present in the normal flora of the skin and colonization is usually not associated with disease, it still ranks amongst the most frequent causes behind bacterial infection in humans. Infection may lead to several diseases, ranging in gravity from superficial skin infections to life-threatening septicaemias and is also a common finding in hospital-acquired infections [25, 26].

S. aureus secretes a variety of membrane-damaging toxins including alpha-toxin (α-toxin) also known as α-hemolysin [27]. Cytotoxicity of α-toxin is mediated through pore formation in the cell membrane [28]. The bacteria secretes water-soluble monomers of the toxin which later binds to the cell membrane where it assembles into barrel-shaped heptamers (Fig. 1) [29]. No specific receptor for α-toxin binding has been reported but caveolin has been suggested to be involved in binding and assembly [30-32]. The cytotoxic response to α-toxin is dependent of cell type but also toxin concentration. In higher concentrations large Ca^{2+}-
permissive pores rapidly results in necrosis while lower concentration may induce apoptosis [11, 33, 34].

Figure 1: Schematic illustration of α-toxin assembling on the plasma membrane. 1. Monomer binds to the membrane. 2. Formation of heptameric pre-pore. 3. Fully assembled heptameric pore.

VT-1 of Escherichia coli

Verotoxin-1 (VT-1, shiga-like toxin 1) is a ribosome-inactivating protein and member of the shiga-toxin family. VT-1 is produced by highly pathogenic strains of Escherichia coli and is nearly identical with Shiga-toxin from Shigella dysenteriae [35-37].

The toxin is composed of an A subunit linked to a pentameric ring of B subunits which binds selectively to globotriaosylceramide (Gb3/CD77) a membrane-bound glycolipid expressed by a limited range of cell types [38, 39]. Elevated expression of Gb3 has been reported in several human cancers [40] as well as in metastatic cells [41]. When the B subunits bind to Gb3, VT-1 is internalized via clathrin-coated pits and is then transported via the trans-Golgi network and Golgi apparatus to the endoplasmatic reticulum. There an enzymatically active fragment of the A subunit (A₁) is released and translocated out to the cytoplasm,
Introduction

where the $A_1$-fragment inhibits protein synthesis by interference between elongation factors and the ribosome (Fig. 2) [42, 43].

Shiga-toxins have been shown to induce apoptosis in several cancer cell lines such as astrocytoma, myelogenous leukaemia, colon cancer, and cervical cancer [44-47] but also in normal epithelial cells [48].

Figure 2: Schematic sketch of VT-1 internalization and trafficking. 1. Toxin B subunits binds to Gb3 on the plasma membrane. 2. VT-1 is internalized in a clathrin-coated vesicle. 3. VT-1 is transported through the trans-Golgi network and Golgi apparatus to the endoplasmatic reticulum. 4. A fragment ($A_1$) of the $A$ subunit is released and translocated to the cytoplasm. 5. $A_1$ interacts with ribosomes and inhibits protein synthesis.
**Introduction**

**Apoptosis**

Regulated death on a cellular level is crucial for the longevity of an organism. That highly regulated cell death is called apoptosis and was first defined in 1972 by Kerr *et al.* as “a general mechanism of controlled cell deletion which is complementary to mitosis in the regulation of animal cell populations” [49]. In other words, apoptosis is needed to preserve tissue homeostasis, keeping a balance between cell death and cell growth. Another crucial role of apoptosis is to identify and delete damaged cells, allowing the organism to remove cells without inflammation [49].

Apoptosis is identified by specific morphological features such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and finally formation of apoptotic bodies. Malfunction of apoptosis regulation may lead to a variety of human pathologies including cancer and neurodegenerative disorders [50] and is also involved in drug resistance [51].

![Figure 3: Apoptotic signaling is divided into the intrinsic and the extrinsic pathways which both lead to activation of caspase-3.](image-url)
Introduction

Caspases, Bcl-2 and Mitogen-activated Protein Kinases

Extensive enzymatic activity and cellular signaling precedes apoptotic cell death (Fig. 3). A family of proteases named cystein-aspartic-acid-proteases (caspases) plays a central role. The caspases are divided into two major groups, the initiator- and the effector-caspases. The initiator-caspases includes, but are not limited to caspase-2, -8, -9 and -10. When an initiator-caspase is activated it will cleave and activate members of the effector-caspase group, which includes caspase-3 and -7. The activated effector-caspases will ultimately dedicate the cell to apoptotic cell death [52]. Caspase-activation is usually said to be mandatory for apoptosis but caspase-independent apoptosis has been shown as early as 1998 [53].

There is also a huge number of signaling proteins involved in apoptosis. Two important families are the Bcl-2 and the mitogen-activated protein kinase (MAPK) families. The Bcl-2 family includes pro- and anti-apoptotic members which can be divided into three subfamilies, Bcl-2, BAX and BH3-only [54, 55]. The Bcl-2 subfamily in general inhibits apoptosis by preventing release of cytochrome c from the mitochondria and by inhibition of other pro-apoptotic Bcl-2 family members [56-58]. Bax and BH3-only subfamilies are pro-apoptotic and has both been shown to trigger cytochrome c release [59-62] The BH3-only subfamily is known to be involved in stress-induced pathways to apoptosis [63, 64].

MAPK pathways regulate several cellular processes including metabolism, survival, mitosis and apoptosis [65, 66]. Common for these proteins is that they are activated by a cascade mechanism where a MAPK kinase kinase (MAPKKK) phosphorylates a MAPK kinase (MAPKK) which in turn phosphorylates the MAPK which in turn is activated and can exert its function [65, 67].
Stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 are two groups of MAPKs which are both known to be involved in stress-induced apoptosis signaling (Fig. 4) [68-73].

Cancer and Current Treatment

Cancer is, with more than forty thousand cases and over twenty-two-thousand annual fatalities, the second most common cause of death in Sweden. Breast cancer is the most frequent type in women, but lung cancer is the most common cause of tumor-associated death. Prostate cancer is most common in men, both in incidence and as cause of death [74]. Tumors arise from a complex course of events including genetics, lifestyle and other stimuli such as ionizing radiation or exposure to certain chemicals. However the most important known risk factors is tobacco smoke and diet [75].

Cancer therapy consists of chemotherapy, radiotherapy and surgery, which are usually combined for better treatment effect. Lately, a great number of new therapeutic options which takes advantage of the increased knowledge in molecular biology have been or are in process of being released. However, in
Introduction

most cases they have not yet had full impact in the clinic and there are still major obstacles in the treatment of many malignancies.

Cisplatin

First recognized in the 1970s and still in widespread clinical use worldwide, cisplatin (cis-diamminedichloroplatinum(II)) is one of the most successful anti-cancer drugs to date [76]. Cisplatin is a cytotoxic drug which after entering the cell binds to nuclear DNA and forms inter- and intra-strand DNA crosslinks which can halt DNA-replication and in many cases leads to apoptosis [77, 78]. However, the exact mechanisms of cisplatin-induced apoptosis is not yet fully understood and despite that most studies focus on the interaction between cisplatin and nuclear DNA there are still a number of other possible targets, such as mitochondrial DNA, and membrane proteins [79].

Despite the usefulness of cisplatin there are nonetheless limitations in treatment, one being quite severe adverse effects including nephrotoxicity, neurotoxicity, and emetogenesis [80]. Nephrotoxicity can to a degree be managed by hydration combined with diuretics, and serotonin-receptor antagonists have been seen to reduce nausea and vomiting. Neurotoxicity is however still a significant problem which limits the dose of cisplatin that can be administrated [81, 82].
Another drawback of cisplatin is the high frequency of tumor drug resistance [81]. Cisplatin resistance may be inherent but is in many cases acquired after an initial promising treatment response [83]. Several different mechanisms are known to be involved in cisplatin resistance and often are more than one simultaneously active (Table 1).

### Table 1: Examples of known cisplatin resistance mechanisms [84, 85]

<table>
<thead>
<tr>
<th>Basis of resistance</th>
<th>Mechanism of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced intracellular accumulation</td>
<td>Increased efflux</td>
</tr>
<tr>
<td></td>
<td>Decreased uptake</td>
</tr>
<tr>
<td>Resistance to apoptosis</td>
<td>Increase of inhibitor of apoptosis protein</td>
</tr>
<tr>
<td></td>
<td>Decrease of pro-apoptotic factors</td>
</tr>
<tr>
<td>Increased DNA-repair</td>
<td>Alterations in mismatch repair</td>
</tr>
<tr>
<td></td>
<td>Alterations in nucleotide excision repair</td>
</tr>
<tr>
<td>Inactivation of cisplatin</td>
<td>Increased detoxification</td>
</tr>
<tr>
<td></td>
<td>Decreased drug binding</td>
</tr>
<tr>
<td>Increased tolerance to DNA-damage</td>
<td>Decrease of DNA post-replication mismatch repair</td>
</tr>
</tbody>
</table>
Aims

The principal aims of the thesis were to:

- Test if pro-apoptotic bacterial toxins could be used against tumor cells either as single agents or to improve current treatment options.
  - To study the pro-apoptotic properties of adenylate cyclase-toxin, alpha-toxin and verotoxin-1 in assorted cell lines.
  - To investigate involved mechanisms of cytotoxicity and apoptosis.
  - To elucidate the possible clinical relevance of adenylate cyclase-toxin, alpha-toxin and verotoxin-1-induced apoptosis in tumor cells.
Cell Lines and Inducers of Apoptosis

Table 2: Summary of cell lines used.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Origin</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT4C</td>
<td>Glioma (rat)</td>
<td>III</td>
</tr>
<tr>
<td>H1299</td>
<td>Non-small-cell lung cancer</td>
<td>II, V</td>
</tr>
<tr>
<td>H1299res</td>
<td>Cisplatin-resistant sub-line of H1299</td>
<td>V</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast adenocarcinoma</td>
<td>IV</td>
</tr>
<tr>
<td>P31*</td>
<td>Malignant pleural mesothelioma</td>
<td>I, II, V</td>
</tr>
<tr>
<td>P31res</td>
<td>Cisplatin-resistant sub-line of P31</td>
<td>II, V</td>
</tr>
<tr>
<td>SF-767</td>
<td>Glioma</td>
<td>III</td>
</tr>
<tr>
<td>T47D</td>
<td>Breast carcinoma</td>
<td>IV</td>
</tr>
<tr>
<td>U1690</td>
<td>Small-cell lung cancer</td>
<td>I</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>Glioma</td>
<td>III</td>
</tr>
<tr>
<td>U-343 MG</td>
<td>Glioma</td>
<td>III</td>
</tr>
</tbody>
</table>

*This cell line is in paper II referred to as P31wt

Table 3: Summary of toxins and other inducers of apoptosis used.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Inducers of apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>21</td>
</tr>
<tr>
<td>II</td>
<td>21</td>
</tr>
<tr>
<td>III</td>
<td>21</td>
</tr>
<tr>
<td>IV</td>
<td>21</td>
</tr>
<tr>
<td>V</td>
<td>21</td>
</tr>
</tbody>
</table>
Materials and Methods

Cytotoxicity Assays

Fluorometric Microculture Cytotoxicity Assay, FMCA (I-V)

Cell viability after exposure to test substances was investigated by a fluorescein diacetate- (FDA) (Sigma-Aldrich, St Louis, MI, USA) based assay. Cells were cultured in 96-well plates for 24 h before test substance was added for incubation up to 72 h, followed by incubation for 45 min at 37 °C with 10 mg/L FDA. Fluorescence was measured using wavelengths of 485 for excitation and 538 nm for emission.

LDH (II, IV)

Cellular leakage of lactate dehydrogenase (LDH) was measured to detect necrosis induction. Cells were cultured in 96-well plates for 24 h before test substances were added and cells were incubated for 6 or 24 h. LDH-content of medium was then analyzed using Roche’s Cytotoxicity detection kit, LDH (Roche Applied Science, Mannheim, Germany) following manufactures instructions.

Apoptosis – Detection and Quantification

Morphological Assessment (III-V)

Morphological changes in cells exposed to test substances were studied by phase-contrast microscopy and photographed at 40x magnification (Olympus CK2 microscope).
Materials and Methods

Phosphatidylserine Externalization (I)

Phosphatidylserine (PS) externalization was used as a marker for early apoptosis and was detected by annexin V binding. Cells were cultured in culture dishes for 24 h before test substances were added and 24 h of incubation followed. PS externalization was then detected with fluorochrome-conjugated annexin V (R&D Systems, Minneapolis, MN, USA) using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

DNA-fragmentation Analysis (II-V)

Apoptosis-specific DNA-fragmentation was used as marker of final-stage apoptosis. Cells were cultured in culture dishes for 24 h before test substances were added and 72 h of incubation followed. DNA fragmentation was quantified by TdT-mediated dUTP nick end labeling (TUNEL) using Roche’s In situ cell death detection kit, TMR red (Roche Applied Science) according to manufactures instructions and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems).

Detection of Pro- and Anti-apoptotic Proteins (II-V)

Western blotting was used to analyze expression levels of pro- and anti-apoptotic proteins. Cells were cultured in culture dishes for 24 h before test substances were added and 24 h of incubation followed. Cell lysate was obtained with lysis buffer (R&D systems) supplemented with a complete protease inhibitor EDTA-free cocktail tablet (Roche Applied Science). Bicinchoninic acid (BCA) Protein
Materials and Methods

Assay kit (Pierce Biotechnology Inc, Rockford, IL, USA) was used for determination of total protein concentration. Gel electrophoresis and immunoblotting was then performed according to protocol.

### Caspase Activity Assays (I-V)

Caspase -3, -8, and -9 enzyme activities were measured with fluorometric assays (R&D Systems). Cells were cultured in culture dishes for 24 h before test substances were added and 24 h of incubation followed. Cells were then lysed and caspase enzyme activity was analyzed according to manufactures instructions with aid of a LS55 Luminescence spectrometer (PerkinElmer) using 400 nm excitation and 505 nm emission wavelengths.

### Analysis of Mitochondrial Membrane Potential (III)

Disruption of mitochondrial membrane potential was analyzed as a marker for mitochondria-depended apoptosis. Cells were cultured in culture dishes for 24 h before test substances were added and 24 h of incubation followed. Mitochondria were then stained using JC-1 (Stratagene, La Jolla, CA, USA) according to manufactures instructions and analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems).
Detection and Manipulation of Toxin Receptors

CD11b (I)

CD11b expression on the cell membrane was investigated using a FITC-labelled monoclonal antibody (Immunotech, Marseille, France), using FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) for detection.

Caveolin-1 (II)

For quantification of caveolin-1 expression cells were first fixated in 2% para-formaldehyde and then permeabilized in 90% methanol at -20 °C. The cells were then incubated with anti-caveolin-1 antibody (Cell Signaling Technology, Danvers, MA, USA) followed by alexa fluor-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). Analysis was done using FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems).

Disruption of Caveolin-1 Functionality (II)

Cholesterol content in the lipid rafts was reduced using methyl-β-cyclodextrin (MEBCD) to disrupt caveolin-1 functionality. Cells were pre-treated with 1 mmol/L MEBCD for 24 h before any other experimental procedure.
Materials and Methods

Gb3 Detection (III-V)

Gb3 expression on the cell membranes was investigated using a monoclonal antibody (Immunotech) and a conjugated secondary antibody (Invitrogen). Analysis was completed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) for detection.

Inhibition of Gb3 Synthesis (III-V)

Gb3 expression was inhibited using PPMP (DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) a chemical inhibitor of glucosylceramide synthesis. Cells were cultured in presence of 2 μmol/L PPMP for 72 h prior to any other experimental procedure.

Gb3 Detection in Tissue (III-IV)

Cryostat sections from tumor tissue were cut and postfixed in acetone. Immunohistochemistry was performed with a monoclonal anti-Gb3 antibody (Immunotech) at 1:40 dilution using the Envision system (Dako, Denmark).

Statistics

Statistical significance was tested with one-way ANOVA using ORIGIN 6.0 (paper I, III) or 7.0 (paper II, IV-V) software (OriginLab Co, North Hampton, MA, USA). The level of significance for rejecting the null hypothesis of zero treatment effect was taken to be P < 0.05.
Results and Discussion

Adenylate Cyclase-toxin (I)

AC-toxin Increases the Cytotoxic Effect of Cisplatin

FMCA analysis showed that treatment of P31 and U1690 cells with 0.1-10 mg/L AC-toxin or cisplatin for 6, 24 or 72 h induced time- and dose-dependent reduction of cell viability in both cell lines. AC-toxin (1 mg/L) induced a limited loss of cell viability in both cell lines and was therefore chosen to be tested in combination with cisplatin. AC-toxin (1 mg/L) increased the cytotoxic effect of cisplatin when cells were exposed for at least 24 h.

AC-toxin-enhanced Cisplatin Cytotoxicity Involves Increased Apoptosis

Flow cytometry analysis of annexin V-binding indicated that cisplatin (1-10 mg/L) was able to induce apoptosis in both P31 and U1690 cells. Combined exposure to cisplatin (1-10 mg/L) and AC-toxin (1 mg/L) resulted in more annexin V-binding than cisplatin exposure alone, even though annexin V-binding was not increased by AC-toxin alone. This effect was most pronounced in P31 cells where AC-toxin increased the amount of apoptotic cells induced by 5 mg/L cisplatin from 15 % to 38 %. In U1690 cells the largest effect was seen when AC-toxin was combined with 10 mg/L cisplatin (from 31 to 43 % apoptotic cells).

Caspase-activity assays confirmed activation of the apoptotic machinery by showing cisplatin (<10 mg/L, 24 h)-induced activation of caspase-3 and to a lesser degree also of caspase-8 and -9. AC-toxin (1 mg/L) could further increase activity of all three caspases in U1690 cells where it by itself activated caspase-3.
Results and Discussion

There was however no effect of AC-toxin on caspase-activity in the P31 cells. It could not be concluded from this data if apoptosis was induced in a caspase-9 dependent manner or if caspase-9 was activated following caspase-3 activation. Earlier studies showed that AC-toxin induces mitochondrial disruption which indicates primary involvement of caspase-9 [86].

Alpha-toxin (II)

α-toxin Induces Apoptosis

FMCA and TUNEL-staining showed that low concentrations of α-toxin (< 1 mg/L) strongly reduced cell viability and induced significant levels of apoptosis in both MPM (P31 and P31res) and NSCLC (H1299) cells. Furthermore, 10 mg/L α-toxin resulted in major release of LDH, suggesting that cell death through necrosis is dominant at higher concentrations of α-toxin. Interestingly, the cisplatin-resistant P31res cell line was significantly more sensitive to α-toxin-induced apoptosis than the parental P31 and the H1299 cell lines.

α-toxin Enhances Cisplatin-induced Apoptosis

P31 cells responded well to cisplatin treatment. 5 mg/L markedly reduced cell viability and induced apoptosis. The cisplatin effect was however considerably weaker in P31res cells concerning cell viability and apoptosis. The portion of apoptotic H1299 cells was low although cisplatin treatment reduced cell viability.

A low-toxic concentration of 0.1 mg/L α-toxin synergistically potentiated the effect of cisplatin in P31res cells concerning cell viability and induction of apoptosis. This was also seen in the H1299 cells but much less distinct.
Signal Transduction in α-toxin-enhanced Cisplatin-induced Apoptosis

Caveolin-1 was expressed by all three cell lines but expression which was highest in P31 cells could not be correlated to α-toxin sensitivity. Cisplatin treatment (5 mg/L, 24 h) up-regulated caveolin-1 expression in H1299 but not in P31 or P31res cells. Furthermore, MEBCD disruption of caveolin-1 functionality had some impact on α-toxin sensitivity of P31res cells which is in line with earlier observations of a role for caveolin-1 in α-toxin cytotoxicity [30]. However, this could not explain the enhanced effect noted when α-toxin was combined with cisplatin.

Apoptosis signal transduction was also investigated by caspase-3, -8 and -9 activity assays. P31 cells had cisplatin (5 mg/L, 24 h) -induced activation of all three caspases compared to only a slight increase of caspase-3 in P31res cells and no activation in H1299 cells. Importantly, while the low concentration of α-toxin (0.1 μg/L, 24 h) did not increase the activity of neither caspase-3, -8 nor -9 by itself, it significantly increased caspase-3 activity in all three cell lines and caspase-9 in P31res cells when combined with cisplatin. The α-toxin-mediated changes of caspase-activity imply caspase-involvement in the enhanced treatment effect. α-toxin-producing *S. aureus* has been shown to induce caspase-independent apoptosis [87] implying that other factors also may contribute.

Verotoxin-1 (III-V)

VT-1 Induces Apoptosis in Cells Expressing Gb3

VT-1 has been shown to be a potent inducer of apoptosis in cells expressing the Gb3 receptor. VT-1 proved highly cytotoxic to all tested cells expressing Gb3, except the MCF-7 cell line which even though it had a weak expression was
Results and Discussion

resistant to VT-1 (Table 4). All tested cells became resistant or at least more tolerant (T47D) after inhibition of Gb3 by PPMP-exposure. DNA-fragmentation was induced in all sensitive cell lines even though the amount of DNA-fragmentation in the T47D cells was much lower then would be expected from the result in the cell viability (FMCA) assay. This pattern had earlier been seen in the H1299 cell line (data not included in thesis) and interestingly, both these cell lines are p53-mutants [88, 89] indicating a possible role of a p53-dependent pathway to apoptosis.

Table 4: Summary of VT-1 effect on tested cell lines.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Gb3-expression</th>
<th>VT-1 Sensitivity</th>
<th>LD50 after 72 h</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT4C</td>
<td>Negative</td>
<td>Negative</td>
<td>N/A</td>
<td>III</td>
</tr>
<tr>
<td>H1299</td>
<td>Week</td>
<td>Sensitive</td>
<td>&lt;0.01 μg/L</td>
<td>V</td>
</tr>
<tr>
<td>H1299res</td>
<td>Positive</td>
<td>Sensitive</td>
<td>&lt;0.001 μg/L</td>
<td>V</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Week</td>
<td>Negative</td>
<td>N/A</td>
<td>IV</td>
</tr>
<tr>
<td>P31</td>
<td>Negative</td>
<td>Negative</td>
<td>N/A</td>
<td>V</td>
</tr>
<tr>
<td>P31res</td>
<td>Positive</td>
<td>Sensitive</td>
<td>&gt;5 μg/L</td>
<td>V</td>
</tr>
<tr>
<td>SF-767</td>
<td>Strong</td>
<td>Sensitive</td>
<td>&lt;0.05 μg/L</td>
<td>III</td>
</tr>
<tr>
<td>T47D</td>
<td>Strong</td>
<td>Sensitive</td>
<td>&lt;0.01 μg/L</td>
<td>IV</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>Negative</td>
<td>Negative</td>
<td>N/A</td>
<td>III</td>
</tr>
<tr>
<td>U-343 MG</td>
<td>Strong</td>
<td>Negative</td>
<td>&lt;0.01 μg/L</td>
<td>III</td>
</tr>
</tbody>
</table>

Cisplatin Up-regulates Gb3-expression

Gb3-expression was up-regulated in the Gb3-negative cell line P31 after exposure to 5 mg/L cisplatin for 72 h. Cisplatin treatment (5 mg/L, 72 h) also resulted in elevated expression of Gb3 in P31res, H1299 and H1299res but in these cases it is uncertain if elevated Gb3-expression is due to up-regulation or selection. Gb3 has earlier been shown to be associated with multidrug resistance through the multidrug-resistant-1 (MDR1)-protein [90], identifying the relations between
Results and Discussion

cisplatin exposure and up-regulation of Gb3 as an interesting object for further studies.

**Presence of VT-1 Sensitizes Cells to Cisplatin Treatment**

The presence of a low-toxic concentration of VT-1 (0.1 μg/L, 72 h) dramatically increased cytotoxic effect when P31res cells was incubated with cisplatin (5 mg/L, 72 h). This was also reflected in a synergistic increased DNA-fragmentation when VT-1 and cisplatin was combined.

**Signal Transduction of VT-1-induced Apoptosis**

VT-1-induced apoptosis seemed to induce a similar pathway to apoptosis in the tested cells (P31res, SF-767, T47D, and U-343 MG). VT-1-exposure activated caspase-3 in all Gb3 positive cells tested. Caspase-9 was also activated in all cell lines except P31res, where only a low concentration (0.1 μg/L) of VT-1 was tested. Caspase-9 involvement was further augmented by the loss of mitochondrial membrane integrity shown by JC-1 staining of SF-767 and U-343 MG cells. In the T47D cells there was also a significant increase of caspase-8 activity, not seen in any other cell line. PPMP-exposure effectively abolished VT-1-induced caspase-activity in all tested cell lines (SF-767, T47D, and U-343 MG). The presence of pan-caspase inhibitor Z-VAD-fmk (40 μmol/L) completely inhibited VT-1-induced apoptosis in SF-767 and U-343 MG cells suggesting that VT-1-induced apoptosis is caspase-dependent.

Western blot analysis showed VT-1-induced phosphorylation of MAPK member SAPK/JNK in all tested cells (P31res, SF-767, T47D, and U-343 MG). Furthermore, MAPK kinase-3 and -6 was also phosphorylated in P31res and T47D cells (not
Results and Discussion

tested in SF-767 or U-343 MG). This indicates a role for MAPK p38 as well as SAPK/JNK in VT-1-induced apoptosis. A possible common pathway for VT-1-induced apoptosis in P31res, SF-767, T47D, and U-343 MG cells involves JNK, P38, caspase-9 and -3 (Fig. 6).

Figure 6: A possible pathway for VT-1-induced apoptosis involving JNK, P38, caspase-9 and -3.

Gb3-expression in tumor samples

Gb3-expression was investigated by immunohistochemistry on cryostat sections of glioma and breast cancer tumors. Blood vessel endothelium expressed Gb3 in a majority of glioma and all breast cancer tumor samples. Tumor cells expressing Gb3 was found in 30% of the glioma tumors and 68% of the breast cancers. In contrast, no Gb3 expression could be found in normal astrocytes.

Beside a possible apoptotic effect of VT-1 on tumor cells, presence of Gb3 in tumor blood vessels could create a possible role for VT-1 also as an anti-angiogenesis agent in tumor treatment [91].
General Discussion and Conclusions

Low-toxic concentrations of AC-toxin and α-toxin were able to increase treatment effect of cisplatin in MPM (AC-toxin and α-toxin), SCLC (AC-toxin) and NSCLC (α-toxin) cells in vitro, presumably by increased apoptosis. Especially interesting was the synergistic treatment effect of low-toxic concentrations of α-toxin and cisplatin. A specific mechanism for how AC-toxin and α-toxin potentiates cisplatin treatment could not be found, and will require further investigation. Previous studies have shown that the pore-forming drug amphotericin B may potentiate cisplatin through increased uptake [92, 93] an aspect which also would be of interest to study in AC-toxin and α-toxin-enhanced cisplatin-induced apoptosis.

VT-1 is the most interesting of the three toxins due to a higher specificity of toxicity. VT-1 was also the strongest inducer of apoptosis and a low concentration of the toxin could in most cases eradicate sensitive tumor cell populations. Further studies are however needed to find safe and effective methods to deliver the toxin to the target cells. VT-1 seems to induce a similar signal transduction mechanism, involving MAPK protein JNK and p38 phosphorylation together with caspase-3 and -9 activation, irrespective of which tumor type the cells originates from.

Our finding that Gb3 is elevated in cisplatin-resistant MPM cells and that cisplatin can up-regulate Gb3-expression indicates that Gb3-targeted therapy could be an approach to target malignant cells which are resistant to cisplatin. Also in cisplatin-resistant MPM cells a low-toxic VT-1 concentration combined with cisplatin had synergistic effect on apoptosis induction. A note-worthy observation relating to this is that in line with earlier studies [94, 95] we found involvement of JNK and p38 in the response to cisplatin exposure and when cisplatin failed to activate JNK or p38 in cisplatin-resistant MPM cells, a low-toxic concentration of
General Discussion and Conclusions

VT-1 was sufficient to activate both JNK and p38, possibly sensitizing the cells to cisplatin cytotoxicity.

The screening for Gb3-expression in tumor tissue showed cases of Gb3-positive gliomas as well as a high prevalence of Gb3 in breast cancer. In combination with the cell line findings of elevated Gb3-expression in cisplatin-resistant cells this encourage to further studies of possible Gb3-targeted therapy.

Conclusions

- Adenylate cyclase-toxin induces apoptosis in MPM and SCLC cells in vitro and could possibly increase the effect of cisplatin through increased apoptosis.

- Alpha-toxin induces apoptosis in MPM and NSCLC cells in vitro. A low-toxic concentration of alpha-toxin sensitizes cisplatin-resistant MPM cells to cisplatin treatment through activation of caspase-3 and -9 which lead to increased apoptosis.

- Verotoxin-1 induces apoptosis in tumor cells expressing the Gb3-receptor. Gb3 was present in a broad range of cancer cell lines, including glioma, breast cancer and NSCLC cells. Cisplatin-resistant MPM cells have an increased expression of Gb3 and were sensitized to verotoxin-1-induced apoptosis. Low-toxic concentrations of verotoxin-1 may be used to potentiate cisplatin-induced apoptosis in cisplatin-resistant MPM cells. Verotoxin-1-induced apoptosis signaling seems to include MAPK activation and a caspase-9 dependent pathway to apoptosis.

- Gb3 is expressed in both glioma and breast cancer tissue and up-regulation Gb3 in cisplatin-resistance makes for a possible clinical application which however would require further studies.
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References


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