Cellular Pharmacology of the Novel Antitumoural Cyanoguanidine CHS 828

BY
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Abstract

The antitumoural cyanoguanidine CHS 828 has shown promising activity in a number of preclinical and clinical studies. However, the mechanisms underlying the cell death induced by CHS 828 has not been clarified. This thesis describes in vitro studies of the cellular pharmacology of CHS 828.

CHS 828 induced cell death with necrosis like features in the lymphoma cell line U-937 GTB. Addition of 3-aminobenzamide, an inhibitor of ADP-ribosylation, resulted in a decreased sensitivity to CHS 828 and a shift in the mode of cell death towards apoptosis.

Mouse fibroblasts lacking the enzyme PARP-1 were more sensitive to CHS 828 compared to normal fibroblasts. CHS 828 was able to induce p53 in normal fibroblasts but this effect does not seem to be necessary to induce cell death.

Characterization of two CHS 828 resistant cell lines indicated that they were selectively resistant to cyanoguanidines. Known mechanisms of anticancer drug resistance did not seem to account for the cyanoguanidine resistance. One possible resistance mediating protein, which was upregulated in the resistant cells, was epidermal fatty acid binding protein.

A novel high content screening assay was also developed. The assay was shown to be suitable both for screening of potential novel antitumoural substances as well for mechanistic studies. In the assay, CHS 828 induced caspase-3 activity and reduction in mitochondrial membrane potential, both signs of apoptosis, in U-937 GTB cells. However, nuclei in exposed cells did not show nuclear fragmentation, one of the hallmarks of apoptosis.

CHS 828 was also shown to indirectly inhibit the proteasome activity in U-937 GTB cells.

In conclusion, the results presented provide new insights into the metabolic and molecular events involved in cell death induced by CHS 828.

Keywords: CHS 828, Cyanoguanidines, Oncology, Pharmacology

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urn:nbn:se:uu:diva-4088 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-4088)
If we knew what it was we were doing, it would not be called research, would it?
Albert Einstein

To Johanna
List of Papers

Paper I
Lövborg H, Martinsson P, Gullbo J, Ekelund S, Nygren P and Larsson R.
Modulation of pyridyl cyanoguanidine (CHS 828) induced cytotoxicity by 3-amino benzamide in U-937 GTB cells.
*Biochemical Pharmacology* 2002 63(8):1491-8

Paper II
Lövborg H, Wojciechowski J, Larsson R and Wesierska-Gadek J
Action of a novel anti-cancer agent, CHS 828, on mouse fibroblasts: increased sensitivity of cells lacking PARP-1.
*Cancer Research* 2002 62: 4206-4211

Paper III
Development and characterization of two human tumour sublines expressing high grade resistance to the cyanoguanidine CHS 828.
*Anti-Cancer Drugs* 2004 15: 45-54

Paper IV
Lövborg H, Nygren P and Larsson R
Multiparametric evaluation of apoptosis - Effects of standard cytotoxic agents and the cyanoguanidine CHS 828.
*Accepted Molecular Cancer Therapeutics*
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>3-ABA</td>
<td>3-aminobenzamide</td>
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<tr>
<td>CHS 828</td>
<td>N-(6-(4-chlorophenoxy)hexyl)-N'′-cyano-N''-4-pyridylguanidine</td>
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<tr>
<td>E-FABP</td>
<td>Epidermal fatty acid binding protein</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCS</td>
<td>High content screening</td>
</tr>
<tr>
<td>IC50</td>
<td>Concentration resulting in 50 % survival</td>
</tr>
<tr>
<td>IKK β</td>
<td>Inhibitory kappa B kinase β</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryo fibroblast</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
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<tr>
<td>MRP</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>NF-KB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribose)polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pgp</td>
<td>Permeability glycoprotein</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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Introduction

Chemotherapy in cancer treatment
There are three major approaches in the treatment of malignancies; surgery, radiation and chemotherapy. Each treatment modality can be used separately or in combination. Chemotherapy is most commonly used as an adjuvant treatment in combination with surgery or radiation and as part of palliative cancer therapy.

The development of chemotherapy as a treatment for cancer evolved in the mid 1940’s, with the development of alkylating mustard gas analogues for treatment of lymphomas (1). Today there are about 45 cytotoxic drugs used in the clinical treatment of cancer in Sweden. The use of these drugs has had a great importance in treatment of certain cancers. However, there is still a large proportion of tumour types that respond poorly to these drugs and they often induce serious adverse effects. In an attempt to overcome these limitations of the cytotoxic drugs used today, a number of drugs have been developed based on molecular knowledge of tumour biology. These drugs are proposed to more specifically target tumour cells, thereby limiting the adverse effects that are common to the currently used drugs.

Anticancer drug development
Identification of substances with antitumour activity is based on a number of methods with different approaches:

- Empirical observations, e.g. the identification of nitrogen mustard being toxic to normal lymphoid tissue.
- Screening of compounds from chemical libraries or extracts from various plants.
- Modification of substances with known antitumour activity.
- Rational design of drugs that interact with messenger systems or biochemical pathways important for malignant transformation and cell growth.
Following identification of substances, a number of *in vitro* methods are used to determine the mechanism of action and the spectrum of the antitumoural activity. Classification of substances, that will give an idea on the mechanism of action, can be performed by correlating the activity of different drugs in a cell line panel (2). By measuring e.g. DNA damage, caspase activities, and biochemical markers in cells exposed to the drug, one can gain more insight into the mechanistic processes involved in the induction of cell death. Another method in the search for the cytotoxic mechanism of a drug is to study its effect on gene expression (3). Up- or down-regulation of particular genes will give information on pathways important for the induction of cell death. The same approach can be used in studies of protein expression (4), where it is also possible to study post-translational modifications of the relevant proteins. It is also possible to use cell lines where a particular gene is disrupted, so called knock-out, to elucidate the role of a particular gene or gene product in the induction of cell death (5).

Methods that provide deeper knowledge on a drug at an early stage in the development process are attracting increasing interest. The development of automated high content screening (HCS) assays provides information both on the antitumoural effect and the molecular events in the cells exposed to potential drug candidates in a single experiment (6, 7).

To confirm and further explore the activity and to determine the role of metabolism and pharmacokinetics, *in vivo* studies are performed. A number of models have been developed, e.g. murine transplantable leukaemias (8) and xenografts of solid tumours in nude mice (9). With satisfying antitumoural activity pattern, toxicity and pharmacokinetic data one can proceed into clinical trials.

### Cell death processes

In the treatment of tumours a major goal is to inhibit growth and induce cell death in the tumours, preferably without causing damage to normal non-malignant cells. Cell death has for long been separated into two different pathways; a passive death termed necrosis and an active, programmed process termed apoptosis. However, recent work shows that these different cell death modes share important pathways, indicating that this classification is perhaps not sufficient to describe all aspects of cell death (10).

The term necrosis is used to describe a process, generally considered to be passive, in the cells where there are lethal changes in the nuclei and the cytoplasm. Swelling of the cytoplasm and the individual organelles lead to a disruption of the plasma membrane (11).
The process of apoptosis, has been known for a long time, but was first characterized and summarized by Kerr et al in 1972 (12). During embryonic development and tissue homeostasis apoptosis is an important process. The original description of apoptosis was based on the morphological changes observed during this type of cell death. These are characterized by nuclear condensation and fragmentation, membrane budding and formation of apoptotic bodies (12). Later work has described biochemical molecules and pathways that are, in most cases, related to this morphology. One of the most important groups of enzymes are the caspases (13). Apoptosis induced by antitumoural drugs can be triggered by a multitude of stimuli such as disturbance of the ATP and ion levels or effects on the DNA damage and repair balance.

Depending on the status of the cell and the type and concentration of drug used, the cell can switch mode of cell death. It is known that the level of ATP is a crucial determinant for the choice between apoptosis and necrosis since apoptotic cell death is dependent on ATP. Thus, Jurkat cells exposed to the experimental anticancer drug staurosporine generally undergo apoptotic cell death. However, by reducing ATP levels in the cells prior to staurosporine exposure, the cell death observed conforms to necrosis (14).

One important enzyme in the signalling and repair of DNA damage is poly(ADP-ribose) polymerase (PARP). This enzyme is activated by DNA strand breaks and forms chains of poly(ADP-ribose) using NAD⁺ as substrate, thus reducing the ATP levels in the cells (15). Inhibitors of PARP have been shown to switch the mode of cell death from necrosis to apoptosis in cells exposed to hydrogen peroxide (16).

The classification of cell death into these two major pathways has been the subject of intense discussion. It is likely that this division sometimes is too crude to be fully discriminative (17), and that increased knowledge in the molecular events involved in cell death will reveal novel and distinct cell death pathways.

Mechanistic classes of anticancer drugs

By exposing tumour cells to antitumoural drugs at suitable concentrations, in most cases apoptosis will be induced. However, there are a number of different primary toxic effects of the drugs. Drugs with different mechanisms of action are often combined in the clinic to increase effect and reduce the risk of toxicity and development of resistance.

Based one their effects it is possible to divide the drugs in different classes. However, it should be kept in mind that there are no distinct boundaries between each mechanistic group, and that each drug may induce different toxic effects in the same cell.
Alkylating agents is a class of antitumoural drugs that are highly reactive and can bind covalently to a number of biological molecules. Their reactivity with the cellular DNA is believed to be responsible for their antitumoural activity. Drugs in this class include mitomycin C, melphalan, and cyclophosphamide.

Among the antimetabolites there are well known drugs such as methotrexate and 5-fluorouracil, both inhibiting vital metabolic processes in the tumour cells. Methotrexate is believed to induce its antitumoural effect by inhibition of dihydrofolate reductase, thereby inhibiting DNA synthesis. 5-fluorouracil is inhibiting thymidylate synthase and also interferes with RNA metabolism.

Topoisomerase interactive agents include the camptothecin derivatives topotecan and irinotecan, and the epipodophyllotoxins etoposide and teniposide. Topoisomerases are enzymes responsible for unfolding of the DNA helix during replication, and inhibition of these enzymes induce DNA strand breaks that are lethal to the highly dividing tumour cells. Another class of drugs proposed to interact with topoisomerases are the anthracyclines, e.g. doxorubicin. However, these drugs are also known to directly interact with DNA, thereby causing breaks in the DNA strand.

During cell division the formation of microtubules and especially the mitotic spindle is important. The microtubule active agents affect this process in different ways, leading to arrest of the cells in the mitotic process and subsequent induction of apoptosis. The two major classes are the microtubule stabilising taxanes, docetaxel and paclitaxel, and the vinca alkaloids vincristine and vinblastine, acting by inhibiting the formation of the microtubule (18).

Recently a number of drugs designed to inhibit specific signal transduction pathways in the tumour cells have been developed, many of them targeting receptor tyrosine kinases (RTK) (19). A number of receptors, e.g. epidermal growth factor receptor, are dependent on RTK to mediate signalling transduction regulating cell growth and differentiation. There are also attempts to modulate the activity of transcription factors regulating gene expression, thereby affecting growth and differentiation. One of these pathways that have attracted great interest as a new target is the nuclear factor kappa B (NF-KB) signalling pathway (20). However, the overall outcome in the clinical trials of these new agents have, with a few exceptions, so far been disappointing (21).

Pharmacology of CHS 828

CHS 828 (N-(6-(4-chlorophenoxy)hexyl)-N´-cyano-N´´-4-pyridylguanidine, figure 1) is a novel antitumoural drug, chemically belonging to the cyanoguanidines. The antitumoural activity of cyanoguanidines was
observed in a routine *in vivo* screening, and CHS 828 was found to be the most suitable candidate (22). CHS 828 has shown promising antitumour activity in a number of *in vitro* and *in vivo* models (23). Studies on a wide range of cell lines and primary human tumour cells have shown high activity in the nanomolar range (23, 24). A panel of 10 human tumour cell lines, representing defined mechanisms of resistance, indicated low cross resistance with standard antitumoural drugs. The effect of CHS 828 does not seem to be influenced by mechanisms known to induce tumour resistance, P-glycoprotein (Pgp), multidrug resistance protein (MRP) or glutathione S-transferase (23).

![Figure 1 The chemical structure of N-(6-(4-chlorophenoxy)hexyl)-N'-cyano-N'-4-pyridylguanidine, CHS 828.](image)

The cell death induced by CHS 828 is relatively slow, with the first morphological signs of toxicity appearing after about 32 h. The protein synthesis inhibitor cycloheximide inhibits the effect of CHS 828, indicating that the cell death is an active process (25). The mode of cell death induced by CHS 828 is atypical. There seems to be an active controlled process involved, but cells do not display the typical morphological signs of apoptosis, i.e. nuclear fragmentation and formation of apoptotic bodies (25).

In the search of the mechanism of action attention has been paid to the effect of CHS 828 on the mitochondria. An increase in the extracellular acidification has been observed in a number of cell lines within the first hour of exposure. This seems to be the result of a block in the mitochondrial respiration leading to an compensatory increase in the glycolysis (26). However, this effect does not seem to be neither sufficient nor necessary to induce the cytotoxic effect, since concentrations well below those inducing increased acidification rate are cytotoxic. Thus, further work is needed to establish the mechanism of cell death induced by cyanoguanidines.
Aims of the investigation

In general, the studies in this thesis aim at describing the cellular pharmacology of CHS 828 to promote preclinical and clinical development of cyanoguanidines for cancer treatment. An overall aim was also to develop *in vitro* methods to improve identification and development of antitumoural drugs.

The specific aims were to:

- Elucidate the importance of various signal transduction pathways in the cell death induced by CHS 828.
- Develop and characterize cell lines resistant to CHS 828, as tools for studies of the mechanism of resistance and identification of drug targets.
- Develop a high content screening assay for multiparametric evaluation of apoptosis in cells exposed to CHS 828 as well as novel and standard cytotoxic drugs.
Materials and Methods

Materials and methods will be outlined briefly. For further details, see the individual papers.

Cell lines
Choice of cell lines were based on their sensitivity to standard antitumoural drugs and CHS 828 as well as on their performance in the assay systems used. The lymphoma cell line U-937 GTB (27) and the 8226/s myeloma cell line (28) were grown in RPMI-1640 culture medium supplemented with 10 % fetal calf serum, glutamine, streptomycin and penicillin at 37° C in an atmosphere containing 5 % CO₂. Immortalized mouse embryo fibroblasts (MEFs) obtained from PARP-1 +/+ (A-19) and from PARP-1 -/- (A-11 & A-12) mice were grown in DMEM supplemented as above in an atmosphere of 8 % CO₂. PARP-1 -/- cells (clone A-11) were reconstituted with human PARP-1 (29). The cervical adenocarcinoma cell line HeLa was grown in Minimum Essential Medium Eagle supplemented as above. Cells in log growth phase were used for all experiments. The cell lines were subcultivated and checked for growth characteristics and morphology twice weekly.

Reagents and Drugs
CHS 828 and cyanoguanidine analogues were provided by LEO Pharmaceutical Products (Ballerup, Denmark). Antibodies, chemicals and drugs were obtained from various commercial sources.

Cytotoxicity and proliferation
In paper I and III, the cytotoxic effect of inhibitors and drugs was studied using the fluorometric microculture cytotoxicity assay (FMCA) (30). This assay measures the hydrolysis of fluorescein diacetate to fluorescent fluorescein in cells with intact plasma membrane. Briefly, 96-well plates were prepared by adding drug solution to each well and were then kept
frozen at –70°C for up to 4 weeks. For experiments, cell suspension was added and the plates were incubated. After incubation the plates were washed, fluorescein diacetate was added and the generated fluorescence was measured after 40 min. Data are expressed as survival index (SI %), calculated by the formula \( \frac{\text{fluorescence}_{\text{test}} - \text{fluorescence}_{\text{blank}}}{\text{fluorescence}_{\text{control}} - \text{fluorescence}_{\text{blank}}} \times 100. \)

In paper II the sensitivity of cells to CHS 828 was determined by proliferation and cytotoxicity assays to measure the drug effect on the cells ability to divide and by dye exclusion test to assess the direct cytotoxic action of CHS 828. Cell proliferation and cytotoxicity was assessed using a colorimetric assay, which measures the ability of viable cells to cleave a tetrazolium salt, WTS-1, and by CellTiter-Glo Luminescent Cell Viability assay. The latter is based on quantification of the ATP level in the cell culture. The phenotype and the adherence of cells were evaluated under phase contrast microscopy. To specifically study cytotoxicity, trypan blue was added and the accumulation of dye was evaluated with a standard microscope.

Measurement of PARP-activity

Cells were incubated in cell culture flasks with indicated drugs. Reaction- and lysis-buffer containing radioactive nicotinamide adenine dinucleotide, \( ^{32}\text{P-NAD}^+ \), was added to collected cell pellets. After 5 min the reaction was stopped and the pellets were washed and the incorporation of \( ^{32}\text{P-NAD}^+ \) into the precipitated proteins was measured by scintillation counting.

Poly(ADP-ribose) formation

U-937 GTB cells were incubated with drugs, centrifuged onto glass slides and fixed. Slides were washed in ethanol and allowed to air dry. The cells were incubated with poly(ADP-ribose)-binding monoclonal antibody, and secondary anti-mouse FITC-antibody. Stained cells were photographed using a Nikon fluorescence microscope with a digital camera.

Measurement of ATP

ATP was measured using the commercially available ApoGlow™ kit. Cells were incubated with drugs and the plate was loaded into a dispensing luminometer and ATP levels were analysed by adding luciferin/luciferase mixture and measuring the generated luminescence.
Measurement of extracellular acidification

Acidification rate was investigated in the Cytosensor Microphysiometer as described earlier (26). U-937 GTB cells were immobilized in the cell capsule during the experiments. The capsules were installed into the sensor chambers and perfused with culture medium. Medium without drug was used to establish a baseline value. Drugs or PBS for control were added to the modified medium and continuously perfused through the sensor chambers containing the cells. The rate of acidification of the medium during the recording time was calculated as -µV/s and was presented as percent change from the initial value at start of the experiment.

Morphology

Microscope slides of cells were prepared using a Cytospin 3 centrifuge. The cells were stained according to May-Grünewald-Giemsa. Cells were viewed with a standard microscope (paper I). Cells in Petri dishes were fixed in paraformaldehyde and stained by the DNA-binding dye Hoechst 33258. Hoechst stained specimens were used for detection of morphological changes in nuclei characteristic for apoptosis using a fluorescence microscope (paper II).

Colorimetric Caspase-3 assay

U-937 GTB cells were incubated with drugs and the cells were collected. Caspase-3 activity in whole cell lysates was measured using a caspase-3 colorimetric kit. The assay measures caspase-3-like dependent cleavage of DEVD-p-nitroanilide into the chromophore p-nitroanilide that is detected by absorbance measurement.

Macromolecular synthesis

In paper II the effect of drugs on cell progression through S-phase, measured as incorporation of ³H-thymidine, was determined. Cells were exposed for 24h to various concentrations of CHS 828 and pulse labelled with ³H-thymidine before harvesting.

In paper III the synthesis of DNA and proteins was measured to study cell line proliferation. The Cytostar-T® plate is a 96-well microtiterplate with scintillants molded into the transparent bottom. When labelled precursors, e.g. ¹⁴C-labeled thymidine and leucine, is absorbed to the intracellular
compartment of the cells adherent to the bottom of the wells, the radioisotope generates a detectable signal (31).

Immunoblotting
Proteins dissolved in a sodium docecyl sulphate (SDS) buffer were separated on SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene difluoride membranes. Blots were incubated with specific primary antibodies and the immune complexes were detected autoradiographically using appropriate peroxidase-conjugated secondary antibodies. In some cases, blots were stripped and used for several sequential incubations.

Ultrastructural evaluation
Cells for transmission electron microscopy were harvested in log growth phase, and processed for ultrastructural analysis using a conventional protocol.

Differentiation analysis and cell cycle phase distribution
In the U-937 cell lines the expression of the monocytic differentiation markers, CD11c/p150.95, CD14 and CD49f/α-6 integrin, were measured using indirect immunofluorescence and flow cytometry. Analysis of the cell-cycle phase distribution of U-937 cells was performed by staining of the nuclei with propidium iodide. The stained nuclei were subsequently analyzed by flow cytometry to calculate the percentage of cells in each cell cycle phase.

Drug uptake
The accumulation of CHS 828 in cells was measured using 3H-labeled drug. Cells in medium were incubated with labelled drug for various times and the uptake was stopped by washing in ice-cold PBS. The amount of accumulated drug in the cell pellets was analyzed by scintillation.
Analysis of Pgp- and MRP like efflux proteins

Functional analysis of Pgp- and MRP like efflux proteins, was performed as described by Liminga et al (32) with minor modifications. Calcein/AM is accumulated in normal cells with subsequent generation of fluorescent calcein. Cells that express efflux proteins have reduced accumulation of calcein/AM, and thereby diminished generation of fluorescence. Briefly, cells were washed and resuspended in PBS containing glucose. The cell suspensions were exposed to calcein/AM and the generated fluorescence was measured.

Two-dimensional protein gel electrophoresis

To study differential expression of possible resistance mediating or target proteins, two-dimensional gel electrophoresis was performed. Briefly, cells were collected and lysed in electrophoresis buffer. Proteins were separated on gels based on their charge and size. The gels were stained with silver according to Shevchenko et al (33). Gels of sensitive and resistant cell lines were compared visually (34), and spots that were considerably and reproducibly up- or down-regulated in the resistant cell lines were further characterized.

Multiparametric evaluation of apoptosis

To evaluate multiple markers of apoptotic processes a multiparametric single-cell assay was developed. HeLa cells were plated in 96-well plates with flat optical bottom, and left to attach over night before addition of drugs. For U-937 GTB cells drugs were added immediately after plating. One h before the end of drug exposure FAM-VAD-FMK, for pan-caspase analysis, or FAM-DEVD-FMK, for caspase-3 analysis, was added. These probes are fluorescent and bind covalently to active caspases (35) MitoTracker Red was added to evaluate mitochondrial membrane potential (MMP). Nuclei were stained with Hoechst 33342 and the plates were washed and fixated.

Plates with stained and fixed cells were analyzed using the ArrayScan® HCS system (Cellomics Inc.). This system is a computerized automated fluorescence microscope that automatically identifies stained cells using image analysis algorithms and reports the intensity and distribution of fluorescence in individual cells. Automatic focusing, image acquisition and analysis were performed to collect data on a user-defined number of cells. Images and data regarding intensity and texture of the fluorescence within each cell, as well as the average fluorescence of the cell population within
the well, were stored in a database. This assay allows for correlation of three or more apoptosis related parameters within individual cells and also reports cell density.

**Proteasome activity**

U-937 GTB cells were exposed to CHS 828 or proteasome inhibitors in microtiter plates. The cell-permeable fluorogenic peptide SUC-Leu-Leu-Val-Tyr-AMC (SUC-LLVY-AMC) was added to a final concentration of 40 µM. The peptide is a substrate for the chymotrypsin-like protease activity of the proteasome (36), and becomes fluorescent upon cleavage. The plates were incubated for 2 h at 37°C and fluorescence was read at 380/460 nm.

The enzymatic activity of the 20S proteasome was measured by adding CHS 828 or known inhibitors to the pure enzyme and subsequently adding SUC-LLVY-AMC. By monitoring the increase in fluorescence over time, it is possible to estimate the enzymatic activity (36).
Results and discussion

This section will present and discuss the results obtained in the studies of the pharmacological profile of CHS 828 and the feasibility to use a number of cell based assays in the mechanistic evaluation of anticancer drugs.

Modulation of CHS 828 cytotoxicity (paper I)

ADP-ribosylation is a process that is important for a number of cellular events, especially in the response to DNA damage. The major ADP-ribosylating enzyme is PARP, an enzyme activated by breaks in the DNA strand, thereby consuming NAD$^+$ and ATP by forming chains of poly(ADP-ribose) (37). In paper I it was demonstrated that adding the ADP-ribosylation inhibitor 3-aminobenzamide (3-ABA) to U-937 GTB cells exposed to CHS 828, resulted in a 100-fold increase in IC$_{50}$ values compared to CHS 828 exposure alone (figure 2). At the same time 3-ABA induced a shift in the mode of cell death from necrosis to apoptosis at higher CHS 828 concentrations.

![Figure 2](image-url)

*Figure 2* Dose-response curves for U-937 GTB cells exposed to CHS 828 alone or in combination with 3 mM 3-ABA for 72 h using the FMCA (from paper I).
It is known that toxicity induced by some DNA damaging substances, e.g. hydrogen peroxide, can be inhibited by 3-ABA. As in the case of CHS 828 this is accompanied by a shift in the cell death process from necrosis to apoptosis (16). This effect may be due to inhibition of PARP, that is highly activated by DNA strand breaks. 3-ABA can in these cases partly inhibit cell death and maintain the ATP level, thus giving sufficient energy to let the apoptotic machinery run.

This led us to speculate that CHS 828 could induce PARP activity. To test this hypothesis we analyzed both the activity of the PARP enzyme in cells exposed to CHS 828, and the formation of the product poly(ADP)-ribose. However, neither of these assays showed any signs of activation of PARP.

Since the effect of 3-ABA on CHS 828 induced cytotoxicity did not seem to be due to PARP-inhibition we wanted to further characterize metabolic events in exposed cells. Measurement of ATP levels and extracellular acidification rate showed that 3-ABA did not change the initial increase in extracellular acidification rate but delayed the metabolic collapse induced by CHS 828. It was also evident that caspase-3 activity was increased in cells exposed to CHS 828 and 3-ABA in combination compared to cells exposed to CHS 828 alone. The maintained metabolic status as well as the increased caspase-3 activity could explain both the increase in IC₅₀ as well as the shift towards an apoptotic morphology at higher concentrations. However, these effects did not seem to be related to the PARP inhibiting effect of 3-ABA. We speculate that 3-ABA exerts its effect by modulating glycolytic enzymes, via interference with other ADP-ribosylating enzymes.

One enzyme that has been shown to be regulated by ADP-ribosylation is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (38). The role of PARP, ADP-ribosylation and GAPDH in CHS 828 induced cytotoxicity needs further exploration.

Cytotoxicity of CHS 828 on PARP-1-deficient cells (paper II)

The generation of mice lacking the gene for PARP-1 (29) has given opportunities to more specifically evaluate the role of PARP in a number of processes, e.g. the mechanism of action of CHS 828. We evaluated the effect of CHS 828 in normal mouse embryo fibroblasts (MEF) as well as MEF lacking PARP-1 (PARP-1 -/-). PARP-1 -/- MEF were more sensitive to CHS 828 both in assays measuring combined cytotoxicity and proliferation (WST-1 and ATP measurement) and in the cytotoxicity assay (trypan blue exclusion). Since PARP-1 is important in the process of DNA repair (37) one might speculate that PARP-1 -/- MEF are more sensitive to CHS 828 due to reduced DNA repair. These data further strengthen the conclusion
from paper I that 3-ABA protects cells from CHS 828 cytotoxicity via a mechanism different from PARP inhibition.

To further explore the effect of CHS 828 in normal and PARP-1 -/- MEF, its effect on DNA synthesis was investigated. Twenty-four h exposure to CHS 828 significantly reduced the incorporation of ³H-thymidine into DNA in both wild-type and PARP-1 -/- MEF. However, a ten-fold higher concentration of the drug was necessary to inhibit the incorporation of ³H-thymidine in normal mouse fibroblasts compared to PARP-1 -/- MEF. This once again shows that PARP-1 -/- MEF are more sensitive to the antiproliferative and cytotoxic effects of CHS 828.

The ability of CHS 828 to induce p53 response in MEF was investigated. Exposure to CHS 828 for 24 h induced a strong induction of p53 in normal but not in PARP-1 -/- MEF (figure 3). This pattern of p53 induction has previously been seen in MEF exposed to doxorubicin. In that case the lack of p53 induction in PARP-1 -/- MEF was due to an overexpression of Pgp, limiting the intracellular level of doxorubicin (39). However, CHS 828 does not seem to be a substrate for Pgp (23). Our present results support this fact, since addition of modulators of Pgp did not change the pattern of p53 induction in PARP-1 -/- and wild type MEF.

![Figure 3](image)

Figure 3 Immunoblot analysis of p53 in PARP-1 +/- (A-19) and PARP-1 -/- (A-11 and A-12) MEF after 24 h exposure to CHS 828.

To further characterize the effect of CHS 828 on MEF the expression and localization of some proteins that are vital for regulation of the cell cycle were investigated. It was demonstrated that the protein MCM7 is partly excluded from the nuclei of both normal and PARP-1 -/- MEF after 24 h of CHS 828 exposure. The nuclear localization of MCM7 and other members of the MCM family during G1 are essential to initiate DNA replication and its nuclear exclusion is necessary to prevent re-replication of the DNA (40). The other protein studied was p27kip1, that inhibits the progression from G1
to S phase (41). The level of p27kip1 was reduced in both normal and PARP-1
−/− MEF after 24 h of CHS 828 exposure. The role of these cell cycle
regulators in the induction of cell death induced by CHS 828 needs further
evaluation.

Development and characterization of CHS 828 resistant
cell lines (paper III)

In the study of the cellular pharmacology of novel antitumoural drugs it is
useful to have access to pairs of cell lines with differential sensitivity to the
studied drug. To study mechanisms important for cellular resistance and to
seek possible targets and pathways important for CHS 828 induced
cytotoxicity, two pairs of cell lines were developed. By exposing U-937
GTB and 8226/s to increasing doses of CHS 828 two resistant sublines were
selected, denoted U-937/CHS and 8226/CHS respectively. The sublines
demonstrated a more than 400-fold increase in IC₅₀ compared to the parental
cell lines. Both sublines expressed a more than 1000-fold increase in IC₅₀
towards six other closely related cyanoguanidines. However, the
cyanoguanidine resistant cell lines did not demonstrate any major resistance
towards standard cytostatic drugs of different mechanistic classes. This
indicates that the changes rendering the cell lines resistant to CHS 828 are
specific to cyanoguanidines.

In an effort to identify the cause of resistance, a number of studies were
performed. The general appearance of the resistant cells was not
significantly altered compared to their parental cell lines. Neither cell
population doubling time nor macromolecular synthesis (DNA and protein
synthesis) were altered between resistant and sensitive cell lines. To exclude
that the difference in sensitivity was due to differentiation of the cells, U-
937/CHS was analysed for expression of differentiation markers expression
(42) and the distribution of the cells within the different phases of the cell
cycle. Neither of these cellular characteristics were altered.

The intracellular concentration of the drug is important for the induction
of cell death. One common mechanism of resistance towards a number of
antitumoural drugs is expression of efflux proteins limiting the intracellular
concentration of the drug (18). The drug accumulation in the resistant
sublines was not reduced, rather the opposite in the case of the 8226 cell line
pair. Even though CHS 828 does not seem to be a substrate for Pgp (23) a
functional assay for Pgp measuring the accumulation of calcein/AM inside
the cells also confirmed that expression of Pgp was not causing the
resistance.

Since general cellular characteristics and classical resistance mechanisms
could not explain the acquired resistance, studies were made on the protein

16
expression profiles in the cell lines. Two-dimensional gel electrophoresis has previously been used to identify resistance mediating proteins (43, 44). By visually comparing the spot intensity between resistant and sensitive cell lines proteins possibly important for resistance or even target molecules could be identified. The analysis performed revealed a number of differentially expressed proteins. However, only a few of them, all in the myeloma cell line pair, were present in enough quantity to allow identification and are presented in table 1.

Table 1: Differentially expressed proteins in 8226/s and 8226/CHS identified by 2D-gel electrophoresis (from paper III)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mw</th>
<th>Differential expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin λ light chain</td>
<td>25</td>
<td>↓</td>
</tr>
<tr>
<td>Parvalbumin α</td>
<td>12</td>
<td>↓</td>
</tr>
<tr>
<td>Heat shock protein 27</td>
<td>31</td>
<td>↓</td>
</tr>
<tr>
<td>Epidermal fatty acid binding protein</td>
<td>14</td>
<td>↑</td>
</tr>
</tbody>
</table>

Mw = Molecular weight (kDa)
↓ = Down-regulated in 8226/CHS vs 8226/s
↑ = Up-regulated in 8226/CHS vs 8226/s

Immunoglobulin λ light chain is a protein present in large amounts in 8226/s but only in small amounts in the resistant cell line. The possible connection to resistance is not established, but it is known that 8226/s, due to the origin of the cell line, has a very active synthesis of this protein (28). It is also unclear whether down-regulation of parvalbumin α and heat shock protein 27 is related to observed resistance. Heat shock proteins are generally induced in cells exposed to stress as a means of protection (45).

The only identified up-regulated protein was epidermal fatty acid binding protein (E-FABP) that was present in large amounts in 8226/CHS but absent in 8226/s. E-FABP belongs to a family of proteins important for intracellular regulation and transport of fatty acids (46). Due to some structural similarities between CHS 828 and fatty acids it is possible that CHS 828 is bound to the overexpressed E-FABP. One might speculate that this results in a sequestration or removal of the drug from the target site within the cells. The observation that 8226/CHS had an increased uptake of CHS 828 might be explained by binding of the drug to E-FABP. E-FABP has been described as a potential mediator of resistance to the antitumoural drug mitoxantrone (43). However, the possible binding of CHS 828 to E-FABP and the mediation of resistance remains to be shown.
Multiparametric apoptosis profiling of CHS 828 and standard cytotoxic drugs (paper IV)

CHS 828 has previously been described to induce active cell death associated with a modest increase in caspase-3 activity, DNA fragmentation, measured by TUNEL staining, and reduction in MMP (25, 47). To further evaluate the mode of cell death a multiparametric apoptosis assay was developed. The assay is based on an automated image capture and analysis system, the ArrayScan® HCS system, that measures the intensity and pattern of fluorescence in cells labelled with various chromophores (figure 4). The system allows for rapid cell-based screening of samples in microtiter plates (48).

![Figure 4 Example of images acquired with the ArrayScan® HCS system. Left: Unexposed HeLa cells. Right: HeLa cells exposed to 0.4 µM staurosporine for 4 h. Cells are stained with DNA binding dye Hoechst 33342 (blue), MMP probe MitoTracker (Red) and FAM-VAD-FMK as marker of active caspases (green). Note the changes in nuclear morphology and MMP in cells expressing active caspases.](image)

The assay reports three fundamental aspects of apoptosis; nuclear morphological changes, caspase activation and changes in MMP. Fluorescent probes binding to DNA, the active sites of caspases and to mitochondrias reports these features. The intensities and pattern of each fluorochrome is reported for each cell analyzed as well as for the whole population. This makes it possible to correlate, e.g. the activity of caspases with nuclear fragmentation in single cells. The assay was shown to be suitable for studies of apoptotic parameters in cells exposed to standard cytotoxic drugs, as well as for screening purposes.

In this assay CHS 828 induced caspase-3 activity, a modest reduction in MMP and changes in nuclear morphology already at 48 h but more pronounced at 72 h in U-937 GTB cells. The induction of caspase-3 activity occurred at a very low concentration. Cells exposed to 1-10 µM CHS 828 had a significant reduced cell number. Individual nuclei were condensed and did not show any major signs of fragmentation. This supports the results from paper I and II where no signs of nuclear fragmentation were observed. The caspase-3 activity observed in this study was higher than previously
The present results thus confirms that the mode of cell death induced by CHS 828 share some of the features of typical apoptosis.

Proteasome activity measurements (unpublished data)
CHS 828 has recently been reported to inhibit the NF-KB signalling pathway, that is an important regulator of apoptosis (50). This observation initiated studies of the effect of CHS 828 on the proteasome, an important regulator in the NF-KB signalling. Comparison of activity patterns and shape of concentration-response curves in a panel of 10 cell lines between known proteasome inhibitors and CHS 828 did not suggest mechanistic similarity (not shown). However, CHS 828 induced a dose-dependent inhibition of the chymotrypsin-like activity of the proteasome after 24 h of exposure (figure 5). This indicates that CHS 828 modulate pathways important for the proteasome activity.

![Dose-response curve for proteasome activity in U-937 GTB cells exposed to CHS 828 for 24 h (mean of 3 experiments +/- SEM). After incubation the fluorogenic probe SUC-LLVY-AMC was added and the generated fluorescence measured (unpublished data).](image-url)
Overall discussion and concluding remarks

In this thesis novel findings are presented that provide new information on the mechanism of action of CHS 828. However, the papers in the thesis, as well as other publications regarding CHS 828, does not provide a clear-cut description of the events leading to cell death.

Previous in vitro studies of CHS 828 has focused on describing the time course and mode of cell death as well as metabolic profiling of cells exposed to the drug. The onset of the toxic effect is slow with no signs of impaired cell health during the first 24 h of exposure. During the first hour of exposure there seems to be an inhibition of the mitochondrial respiration and a compensatory increase in the glycolysis (26). However, this effect is not sufficient to explain the toxic effect of CHS 828. Sometime between 24-32 hours there seems to be a shut down of the DNA and protein synthesis levels (25) accompanied by the reduction in the ATP levels as presented in paper I. It is thus likely that CHS 828 interferes with the respiratory and glycolytic pathways of the cells, but this is not likely to be the only event triggering toxicity.

From experiments on MEF lacking PARP-1, one could speculate that there is a DNA damage induced by CHS 828 that cells without PARP-1 is not able to repair and recover from. However, there are no studies yet focusing on the DNA-damaging properties of CHS 828. The experiments also showed, for the first time for this drug, an increase in the level of p53. At 24 h there was an increase in the level of p53 in normal MEF and this has been confirmed in the human fibroblasts and the tumour cell line MCF-7 (51). The importance of p53 induction has not been further evaluated, but activation of p53 is known to be one way to induce apoptosis (52).

One proposed mechanism of action for CHS 828 has been interference with the NF-KB system (50). NF-KB is a transcription factor regulating the transcription of both pro- and anti-apoptotic genes (53). The NF-KB subunits are normally sequestered in the cytoplasm by attachment to IKBα and β (53). Under stressful conditions or by stimulation with cytokines, IKBα/β is released and the NF-KB subunits translocate to the nucleus and induces gene transcription. It has been shown that inhibition of the translocation from the cytoplasm to the nucleus can lower the threshold for apoptosis induced by antitumoural drugs or even directly induce apoptosis (54). Preincubation with CHS 828 for 24 h before stimulation with lipopolysacharide inhibits the NF-KB translocation. There is also an inhibition of NF-KB-dependent gene
transcription. The basis for these inhibitory effects of CHS 828 was suggested to be inhibition of IKB kinase β (IKK β), responsible for phosphorylating, and thereby targeting, IKBα/β for degradation. The in vitro activity of IKB kinase β was actually inhibited by CHS 828 in the nanomolar range. There was also a correlation between the antitumoural effect of CHS 828 analogues and the inhibition of IKK β (50). It is likely that inhibition of the NF-KB subunits contributes to the cell death. An indication of this is the synergistic interactions of CHS 828 with standard antitumoural drugs such as etoposide, melphalan and doxorubicin (55, 56). Other NF-KB inhibitors have been shown to potentiate the effect of standard antitumoural drugs (57, 58).

However, CHS 828 might use other ways to inhibit NF-KB translocation in the cell. An emerging group of promising antitumoural drugs are the proteasome inhibitors (59, 60). The proteasome is responsible for degradation of a number of proteins, one is IKBα/β. By preventing IKBα/β-degradation, proteasome inhibitors prevent translocation of NF-KB and thereby induces apoptosis (59). The unpublished data presented in this thesis describe an inhibition of the proteasome chymotrypsin-like activity in cells exposed to CHS 828 for 24 h. At this time-point there are few sign of toxicity (25) and the ATP levels are only slightly reduced (paper I). The inhibition of the proteasome did not seem to be a direct action, but rather a result of interference with targets regulating the proteasome. The activity pattern of CHS 828 in a cell line panel did not correlate with known proteasome inhibitors, indicating an indirect regulation of the proteasome, perhaps at an upstream target. This is also supported by an unpublished study of the relationship between the expression of 3864 genes in a cell line panel, and the potency of CHS 828. This study indicated that the level of a number of genes coding for proteasome and ubiquitination proteins were connected to the effect of the drug.

Based on the observed inhibition of the proteasome by CHS 828 exposure, one can explain a number of the findings presented in the thesis. In paper I it was shown that an inhibitor of ADP-ribosylation, 3-ABA, protected the cells against CHS 828 cytotoxicity. Studies in neurons exposed to proteasome inhibitors show that ADP-ribosylation increases and that 3-ABA attenuates their toxicity (61). Therefore it is possible that the observed effects of 3-ABA in CHS 828 exposed cells could be mediated through proteasome regulation.

It is well known that the proteasome is responsible for degradation and turnover of a large number of proteins. One of them is p53, that accumulates in the cell after inhibition of the proteasome (59). As shown in paper II and elsewhere (51) p53 accumulates in MEF as well as the human tumour cell line MCF-7 after 24 h of CHS 828 exposure. However, the cell cycle regulator p27kip1 was reduced in MEF exposed to CHS 828, while proteasome inhibitors normally induce accumulation (59).
Another interesting notion is that the proteasome inhibitor bortezomib, the first to reach the clinic, induces a mitochondrial hyperpolarization with induction of cell death starting at 24 h (62) resembling the events described for CHS 828 (25, 49). It is also interesting to note that patients treated with bortezomib experience similar adverse events, with thrombocytopenia but modest reductions in white blood cell counts (63-65).

In figure 6 some of the features of CHS 828 presented in this thesis and elsewhere, are shown. This should not be considered a complete summary of the cellular pharmacology of CHS 828 but rather a way to graphically present some of the key topics discussed here.

The striking similarities between CHS 828 and proteasome inhibitors, its inhibitory effect of IKK $\beta$ and the importance of NF-\(\kappa\)B in cell death induced by proteasome inhibitors, indicate that NF-\(\kappa\)B-signalling may play an important role in the induction of cell death by CHS 828.

Referring to the aims, the results may be summarized as:

- Effects of CHS 828 on metabolic pathways and interference with ADP-ribosylation are likely to be involved in cell death induced by CHS 828. Inhibition of the proteasome activity could be an alternative pathway for induction of cell death.

- Established CHS 828 resistant cell lines are valuable tools for mechanistic studies and studies of factors important for the cyanoguanidine resistance. Analysis of the resistant sublines indicated that CHS 828 is not affected by classical resistance mechanisms. A possible mediator of resistance was identified as the epidermal fatty acid binding protein.

- A multiparametric high content screening assay was successfully develop for studies of apoptotic processes at the single cell level. The assay proved useful for both in-depth cell biological studies as well as for screening purposes. Analysis of cells exposed to CHS 828 indicated a pronounced activation of caspase-3, reduced mitochondrial membrane potential as well as condensation of nuclei but without subsequent nuclear fragmentation. This indicates that cell death induced by CHS 828 share some of the hallmarks of apoptosis.
Figure 6  Schematic overview of some of the cellular effects of CHS 828 described in this thesis
Future outlooks

The results presented in this thesis should be considered as an attempt to improve the general knowledge of the cyanoguanidine CHS 828. In the development of novel therapies for cancer treatment it is important to have a clear picture of the molecular targets and events involved in the induction of cell death.

The work presented in this thesis, together with previous studies, provide information on possible targets and resistance mediating factors. To optimize and develop cyanoguanidines as a new class of antitumoural drugs more work is needed in especially the following areas:

- Evaluation and characterization of mitochondrial and glycolytic pathways affected by cyanoguanidines
- Evaluation of the p53 signalling, especially the role of mutated and unmutated p53 in the induction of apoptosis induced by CHS 828
- Study the importance of the NF-KB and proteasome pathways as a target for cyanoguanidines
- Characterize cyanoguanidine resistant cell lines by analyzing mRNA expression profiles. Identified genetic changes in target molecules or resistance mediating genes have both mechanistic and clinical importance

By learning more about the basic mechanism of cyanoguanidines it would be possible to further optimize the antitumoural effect while keeping the toxic side effects at a minimum. This could result in a new interesting class of antitumoural drugs, hopefully providing some hope for improved cancer treatment.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine*. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)