Pharmacological Studies of CHS 828 and Etoposide Induced Tumour Cell Death

BY

PETRA MARTINSSON
Dissertation for the Degree of Doctor of Philosophy (Faculty of Medicine) in Clinical Pharmacology, presented at Uppsala University in 2001

abstract


Antitumour properties of the cyanoguanidine CHS 828 and analogues were discovered in 1997. CHS 828 is presently in clinical phase I/II trials. This thesis encompasses in vitro studies of the kinetics and mode of cell death induced in the human cell line U-937 GTB, by CHS 828 and the standard antitumour drug etoposide.

Etoposide induces apoptosis in U-937 GTB within 4 h. The cells exhibited apoptotic morphology, including condensed and fragmented nuclei and formation of apoptotic bodies, activation of caspase 3 and 8, and DNA fragmentation, visualised by TdT-mediated dUTP nick end-labelling (TUNEL).

CHS 828 induced few and weak signs of apoptosis. Metabolic activity was the only parameter affected during the first 24 h of exposure. After ~30 h, proliferation (DNA synthesis) and protein synthesis ceased, and viability started to decrease towards 10% at 72 h. Morphology and ultrastructure of dying/dead cells showed predominant necrosis. The decrease in viability was postponed by protein synthesis inhibition or maintenance of ATP levels by 3-aminobenzamide. In addition, 3-aminobenzamide switched morphology towards apoptosis.

Continuous co-exposure to CHS 828 and etoposide resulted in impressive cell kill synergy in U-937 GTB cells at effect levels of 30-70%. Pre-exposure to CHS 828 for 18 h or more, on the other hand, resulted in diminished cell kill and inability to activate the apoptotic machinery upon etoposide stimulation, evaluated by morphology and caspase activity.

In summary, CHS 828 induced cell death is predominantly non-apoptotic, does not involve caspases and can be postponed by maintained protein synthesis and ATP levels.

Key words: Cancer chemotherapy, cell death, caspase, etoposide, CHS 828.

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ISSN 0282-7476
ISBN 91-554-5157-8

Printed in Sweden by Uppsala University, Tryck&Medier, Uppsala 2001
When I found the skull in the woods, the first thing I did was call the police. But then I got curious about it. I picked it up, and started wondering who this person was, and why he had deer horns.

- deep thoughts by Jack Handy
Temporal effects of the novel antitumour pyridyl cyanoguanidine (CHS 828) on human lymphoma cells.
_Eur J Cancer_ 37; 260-67, 2001

Cell death with atypical features induced by the novel antitumoral drug CHS 828, in human U-937 GTB cells.

iii. **Petra Martinsson**, Gunnar Liminga, Peter Nygren, Rolf Larsson.
Characteristics of etoposide induced apoptotic cell death in the human U-937 human lymphoma cell line.
_Anti-Cancer Drugs_ 12, 699-705, 2001

iv. **Petra Martinsson**, Sara Ekelund, Peter Nygren, Rolf Larsson.
Combination of CHS 828 and etoposide in vitro – from cytotoxic synergy to complete inhibition of apoptosis.
Manuscript

v. Henrik Lövborg, **Petra Martinsson**, Joachim Gullbo, Sara Ekelund, Peter Nygren, Rolf Larsson.
Modulation of cell death and metabolic effects by 3-aminobenzamide in U-937 GTB cells exposed to CHS 828.
Submitted
pharmacological studies of CHS 828 & etoposide induced tumour cell death

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<tbody>
<tr>
<td>3-aba</td>
<td>3-aminobenzamide</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphocytic leukaemia</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptosis protease activating factor-1</td>
</tr>
<tr>
<td>ΔΨ_m</td>
<td>mitochondria membrane potential</td>
</tr>
<tr>
<td>carboxy-H&lt;sub&gt;2&lt;/sub&gt;DCFDA</td>
<td>5-(and-6)-carboxy-2',7'- dichlorodihydro-fluorescein diacetate</td>
</tr>
<tr>
<td>caspase</td>
<td>cysteine dependent aspartate directed protease</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide m-chlorophenylhydrazone</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>combination index</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myelocytic leukaemia</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>FDA</td>
<td>fluorescein diacetate</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMCA</td>
<td>fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathion</td>
</tr>
<tr>
<td>ICE</td>
<td>interleukin-1 beta-converting enzyme</td>
</tr>
<tr>
<td>LAPS</td>
<td>light-addressable potentiometric sensor</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>N&lt;sup&gt;0&lt;/sup&gt;-Monomethyl-L-arginine, monoaacetate salt</td>
</tr>
<tr>
<td>MiCK</td>
<td>microculture kinetics</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance-associated protein</td>
</tr>
<tr>
<td>MTP</td>
<td>microtitre plate</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>OD</td>
<td>optical density / absorbance</td>
</tr>
<tr>
<td>PAR</td>
<td>poly(ADP-ribose)</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylerine</td>
</tr>
<tr>
<td>PT</td>
<td>permeability transition</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RLU</td>
<td>relative light units</td>
</tr>
<tr>
<td>SBU</td>
<td>the Swedish Council on Technology Assessment in Health Care (Statens Beredning för medicinsk Utvärdering)</td>
</tr>
<tr>
<td>SI</td>
<td>survival index</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>Topo</td>
<td>topoisomerase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>Vit C</td>
<td>L-Ascorbic acid</td>
</tr>
</tbody>
</table>
introduction & background

The cancer incidence in Sweden has been registered since 1958. At present, the number of new cancer cases (43000 in 2000) increases by approximately 2% per year, due to a growing elderly population, better diagnostic methods, but also due to a true incidence increase. Approximately half of these patients will die from their disease. Cancer causes 22% of all deaths yearly. The number of cancer cases is expected to continue to increase (Glimelius et al. 2001). Globally, the year 2000 was estimated to bring about 10 million new cancer cases and 6 million patients were expected to die from their cancer (Ferlay et al. 2001).

chemotherapy in cancer treatment

Surgery and radiotherapy cure approximately 40% of all cancer patients. The remaining 60% suffer from systemic malignant disease and may therefore require systemic treatment, notably chemotherapy and/or hormone treatment (Verweij et al. 2000). Chemotherapy can be used prior to, simultaneously with, or after treatment with another modality (Boyd 1993). In a recent report by The Swedish Council on Technology Assessment in Health Care (SBU), the use of chemotherapy in some major tumour types in Sweden was reviewed (Glimelius et al. 2001). It concludes that the literature on the effects of cancer chemotherapy is extensive, and that the use of chemotherapy is of utmost importance for the possibility of cure of certain tumour types. In other tumours, chemotherapy increases the possibility of cure when added to local and regional treatments, particularly surgery. Cancer chemotherapy may also improve both patient survival and well being, to variable extent (Verweij et al. 2000, Glimelius et al. 2001). Thus, there is no doubt an essential role for chemotherapeutic drugs in contemporary clinical oncology. In a few diagnoses, e.g. acute lymphocytic leukaemia (ALL) and testis cancer, chemotherapy has become the backbone of curative treatment. However, 90% of all chemotherapy cures, occur in only 10% of cancer types (DeVita Jr. 1997). In a larger number of cancers relevant survival benefits, as well as relevant improvements in quality of life, can be achieved. Hence, there is also no doubt a need for more and better drugs. In 2000, Sweden had 44 registered chemotherapeutic compounds for use in cancer patients (Nygren 2001). According to the SBU report, chemotherapeutic drugs can be classified into seven categories, referring to their mechanism of action (Box 1). The vast majority of these drugs work principally through modulation of DNA synthesis,
transcription, or mitotic spindle formation (Boyd 1993).
Two successful “new classes” of agents introduced recently are topo I (rather than older, topo II) inhibitors and microtubule stabilisers (Verweij et al. 2000), making them fall into already established categories. Overall, available cancer chemotherapeutic drugs can be considered monotonously similar regarding spectrum of clinical antitumour activity and toxicity, as well as mechanism of action. It has been proposed that any quantum leap in effective cancer chemotherapy will require the discovery and development of new anticancer drugs with unprecedented antitumour activities, specificities and mechanisms of action (Boyd 1993).

For recently introduced drugs, the time frame for the process of development of a new drug was 10-15 years, from discovery of anticancer activity to approval for use in patients. At best, a few drugs per year are added to the chemotherapeutic armamentarium (Nygren 2001).

The development of the area of anticancer drug discovery basically reflects an evolution from highly empirical approaches, based on serendipitous findings and testing of randomly selected compounds, to the current, more focused testing of natural products, rationally synthesised agents, and biological products against panels of well-characterised tumour cell lines or molecular targets (DeVita Jr. 1997). The history of successful cancer chemotherapy started with the warfare experience during World War II, of toxic effects of nitrogen mustard on normal lymphoid tissues, which via experimental follow-up led to the first documented treatment of a patient with lymphoma (Gilman 1963).

Empirical observations also led to the development of the vinca alkaloids in the 1960’s. Later, serendipitous findings resulted in the use of cisplatin and glucocorticoids in cancer therapy (Boyd 1993).

Although targeted synthesis of substances based on rational molecule design and biochemical reasoning is an attractive theory, this approach has rendered disappointingly few clinically useful drugs. Rare examples are some of the antimetabolites (Boyd 1993). Screens devised to

**Box 1. mechanisms of chemotherapeutic drug action (number of substances in each group)**
- alkylators and alkylator-like drugs (16)
- antimetabolites (9)
- DNA interacting agents (1)
- membrane pertubators (1)
- topoisomerase (topo) inhibitors (9)
- microtubule interacting agents (7)
- amino acid depletors (1)

From (Nygren 2001)

**Box 2. drug discovery paths**
- empirical observations and experimental follow-up
- rational or targeted synthesis
- screening
- analogue synthesis

From (Boyd 1993)
address targets at the molecular, biochemical, and cellular levels, both in vitro and in vivo, have been essential for the experimental evaluation of serendipitous findings as well as from rational or targeted synthesis. The American National Cancer Institute (NCI) have provided such screening support since 1955. However, only few of the commercially available anticancer drugs today have resulted from this extensive screening programme, with taxol as a brilliant example (Boyd 1993).

Lastly, the development of analogues to known, active compounds, usually with similar effect and mechanism of action has contributed to the modern arsenal of cytotoxic drugs. Most of the anticancer compounds that were found active in phase II clinical trials in 1970-85 were analogues to known drugs (Marsoni et al. 1987). Etoposide is an example of analogue development that led to a new mechanistic class of anticancer compounds, the topo II inhibitors (Boyd 1993).

Future strategies will probably consist of the best of historical approaches in combination with new concepts. The increasing understanding of cellular and molecular biology, including signalling pathways, and the recently concluded Human Genome Project offers an array of potential new targets for targeted screening, drug design and synthesis. The most recent success of cancer chemotherapy development is the tyrosine kinase inhibitor STI-571 (Gleevec™, imatinib mesylate). It was the first anticancer drug to come out of molecular biology research and it specifically interacts with cellular signalling pathways in chronic myelocytic leukaemia (CML) cells (Druker et al. 2001). It has been called “the wave of the future” and “the poster boy of targeted therapy” (Mauro et al. 2001, Pestell 2001).

At least part of the improvement in chemotherapy treatment for malignant disease over the years can be attributed to the use of combinations of drugs (DeVita Jr. 1997). Theoretically, combination chemotherapy accomplishes four additional objectives over single drug treatment. (1) Maximal cell kill within the range of toxicity for each drug tolerated by the patient. (2) A broader range of coverage of resistant clones in a heterogeneous tumour. (3) Preventing or slowing the development of new resistant clones (Boyd 1993, DeVita Jr. 1997). (4) Positive interactions at the cellular level.

Cancer cells frequently possess or acquire refined strategies to resist the cytotoxic effect of anticancer drugs. These include overexpression of membrane transport molecules, such as the P-glycoprotein (Pgp) and

combination chemotherapy & drug interactions
multidrug resistance-associated protein (MRP), glutathion (GSH)-
dependent increased activity of cellular detoxification systems, altered
function of nuclear targets, such as topo II, as well as altered tubulin
binding/function (Beck et al. 1997). In addition to these mechanisms of
cancer cell resistance to chemotherapeutic treatment recent evidence
suggest intrinsic or acquired inability to activate the apoptotic cell death
machinery (Hickman 1996, Beck et al. 1997), which is of particular
interest to this thesis. Concern has been expressed over the notion that
all anticancer drugs kill by apoptosis and that some cells therefore be
fundamentally resistant to treatment induced cell death (Hannun 1997).
However, some of this concern has been alleviated by the finding that
apoptosis resistant cells may die by other mechanisms when exposed
to initially lethal insults (Blagosklonny 2000).

Therapeutic substances (medicines), can influence each other’s effects
either pharmacokinetically (absorption, distribution, metabolism,
excretion) or pharmacodynamically (interaction at the target). In vitro
studies, such as in this thesis, can cover the pharmacodynamic
interactions and broadly classify them as antagonistic, additive or
synergistic.

The isobole method for evaluating interaction is based on
the sigmoid relationship that characterises the response of
many biological targets to increasing concentrations of a
molecule. This is also valid for the response of such targets
to a combination of agents maintained in constant
proportion to each other. Isoboles at specified effect levels
represent zero interaction between the two agents, i.e.
simply the sum of the individual effects. Based on such
isoboles, a set of combinations of concentrations for two
drugs can be chosen (Fig 1). These combinations are then
tested in vitro using a total cell kill assay. The observed effects are
transformed into corresponding single drug concentrations. A
combination index (CI) is calculated in order to assess the difference in
observed and predicted concentrations (Berenbaum 1989).
The additive model predicts the effect of a combination of two drugs to
be equal to the product of the survival indices of the two drugs tested as
single agents (Valeriote et al. 1975). A ratio between the observed
survival index and that predicted by the additive model is calculated for
all combinations.
In general terms, the ultimate fate of any human cell can assume one of two shapes; passive death by necrosis or active death by apoptosis. The morphological alterations of each response are, by definition, distinct. However, accumulating evidence indicate that essential biochemical mechanisms are shared by the two responses (Lockshin et al. 2000). Rather than exclusive and separate processes, these two end points are hence the extremes of a continuum, and lately many arguments against the antithesis between apoptosis and necrosis have been put forward (reviewed in (Zamzami et al. 1997). There are also descriptions of intermediate or mixed forms of the two types of cell death (Zakeri et al. 1993, D’Herde et al. 1996, Kitanaka et al. 1999). However, as models for cell death these two extremes are useful and commonly considered and depicted as two distinct entities (Fig 2). The details of the process and end result of either form also depends on whether the cell is in its physiological context, as part of an organism, studied *in vivo*, or removed from its normal surroundings, studied *in vitro*.

The term necrosis refers to the result of an external force killing a passive cell (Majno et al. 1995). Necrosis is characterised by irreversible changes in the nucleus (karyolysis and pyknosis) and in the cytoplasm (loss of structure, fragmentation) (Majno et al. 1995). There is swelling of organelles and the whole cell and finally rupture of the plasma membrane and release of cytoplasmic constituents (McConkey...
1998). \textit{In vivo} the end of the story is a cell with a disintegrated membrane and loss of all cellular functions. Cellular debris is all that remains. \textit{In vitro} the disintegrated cell will spill its contents, attracting inflammatory cells, causing inflammation. Cellular necrosis is a pathological event for the organism, over which it has no control (Darzynkiewicz et al. 1998a). It is important for the continued discussion to bear in mind that a snapshot of a completely disintegrated cell (cell debris) does not prove that the cell died from a necrotic process (Fig 2).

It has been proposed that the term necrosis be used exclusively for the changes occurring in the cells after they have died, regardless of the pre-lethal process (Levin 1998). The prefixes \textit{oncotic} should then be used for the process usually referred to as necrosis and \textit{apoptotic} when apoptosis has preceded cellular disintegration (Majno et al. 1995). However, in this thesis the term necrosis refers to the whole process as depicted in Fig 2.

Apoptotic cell death is by no means a new concept (Flemming 1885, Nissen 1886, Kerr et al. 1972, Hockenbery 1995, Majno et al. 1995), but one that has received much attention the past decade, especially in the cancer context (Dive et al. 1992, Fisher 1994, Huschtscha et al. 1995, McDonell et al. 1995, Dixon et al. 1997, Kaufmann et al. 2000). It is defined in much more detail than necrosis, partly because of its specificity in pathways, and partly because of the extensive interest it has received.

Cells maintain a delicate balance of factors that could potentially kill them (intracellular levels of Ca\textsuperscript{2+}, nitric oxide (NO) etc) and factors that aim to keep them alive (ionic pumps, repair enzymes etc). Should this balance shift too far towards cell death most cells will launch a genetically conserved cell death program, activating specific signalling pathways and die by apoptosis. This physiological mode of cell death, crucial during normal development and tissue homeostasis, allows the organism to eliminate excess or dying cells without an inflammatory response. It is the sacrifice of an individual for the benefit of the community.

Originally, apoptosis was described, based on its striking morphological appearance, with condensed, marginated chromatin and fragmented nuclei, budding and release of apoptotic bodies (Kerr et al. 1972). However, it is important to remember that \textit{in vitro} also the apoptotic cell will eventually disintegrate and assume necrotic appearance since there
are no phagocytic cells present to dispose of it. In this case, the result of apoptosis and necrosis are the same.

The net growth of an untreated cancer is directly related to both its proliferate capacity and its rate of apoptosis (Reed 2000). With chemotherapeutic treatment of cancer, we wish to stimulate the latter. Recent research has shown that chemotherapy induced cell death, with few exceptions, is executed as apoptosis (Hickman 1992, Sachs et al. 1993, Kerr et al. 1994, Lutzker et al. 1996, Hannun 1997, Debatin 2000, Kaufmann et al. 2000). Some cell types are also considered more susceptible to apoptotic cell death than others are (Hickman 1992, Blagosklonny 2000). Hence, much effort has been put into finding or designing chemotherapeutic substances that specifically launch the apoptotic machinery of cancer cells. Also, two important questions have been raised in association with this research; 1) are cancer cells which are primarily resistant to chemotherapy unable to activate their apoptotic machinery? 2) Can this inability be reversed and thereby make cancer cells susceptible to chemotherapy?

Apoptosis is the result of an active process by the individual cell, leading to stereotypic morphological features (Kerr et al. 1972) (Box 3). The apoptotic program is initiated either via cell surface death receptors (CD95/Fas receptor or tumour necrosis factor receptor-I) directly coupled to the cell death machinery or damage to DNA or other critical biochemical targets within the cell (Box 4). It is propagated by key proteases, known as caspases, named so because of their specificity (cysteine dependent aspartate directed proteases) (Harvey et al. 1998, Nunez et al. 1998, Earnshaw et al. 1999). Pre-existing pro-caspases are specifically cleaved to form an active protease. Caspase 8 (Salvesen 1999) and caspase 9 (Kuida 2000) are the most apical caspases of separate, yet not entirely exclusive signalling pathways (Sun et al. 1999). Eventually they both activate caspase 3 and/or caspase 6 and/or caspase 7 (Porter et al. 1999), leading to the specific hallmarks of apoptosis (Ghibelli et al. 1995, Darzynkiewicz et al. 1998b) (Fig 3 and box 3). Other factors in the programmed cell death course may or may not be dependent on
caspase activity, such as mitochondria transmembrane depolarisation (Li et al. 2000), Bid cleavage (Han et al. 1999), cytochrome c release from the mitochondria intermembrane space (Bossy-Wetzel et al. 1998, Sun et al. 1999) and phosphatidylserine (PS) exposure on the outer leaflet of the cell membrane (Kagan et al. 2000). The caspase 8 pathway is initiated by CD95-/Fas-ligand binding to and causing trimerisation of the CD95/Fas receptor (Friesen et al. 1996, Friesen et al. 1999). These interact physically with the caspases via regions in their cytoplasmic domain (“death domains”). The caspase 9 pathway is activated indirectly, either by upregulation of the cell death receptor pathways, or by effects on other vital cellular targets. It involves mitochondria, with release of inner membrane bound cytochrome c, apoptosis protease activating factor-1 (Apaf-1) and disruption of the mitochondria membrane potential ($\Delta \psi_{m}$) (Reed 1997, Sun et al. 1999, Kroemer et al. 2000, Kuida 2000).

In addition to the model cell death modes apoptosis and necrosis, and the proposed intermediate modes common in developmental cell death, the course of induced cell death (by cancer chemotherapy or radiation) can switch between the two extremes. The decision between apoptosis and necrosis upon lethal drug stimulation is dependent on the severity of the insult, but also on intra- and extracellular factors and environment. Some essential factors may act as switches between the two types of cell death. One is cellular ATP level (Leist et al. 1997, Lelli et al. 1998, McConkey 1998, Lee et al. 1999, Leist et al. 1999). An ATP level of 30-75% during execution of cell death, will promote apoptosis, while ATP depletion >70% will lead to necrosis. The execution of apoptosis requires ATP for activation of caspase 9 and for active transport of apoptotic factors into the nucleus (Eguchi et al. 1999) and possibly also for activation of caspase 8 under certain circumstances (Ferrari et al. 1998). Another switch factor is differential alterations in intracellular $Ca^{2+}$, where transient increases vs. low sustained increases vs. uncontrolled massive influx produce proliferation, apoptosis and necrosis, respectively (McConkey 1998). The level of oxidative stress can also act as a switch between apoptosis, associated with low to moderate concentrations of reactive oxygen species (ROS) and necrosis, associated with higher levels (McConkey 1998).

**Box 4. biochemical hallmarks of apoptosis**

- DNA fragmentation
- PS externalisation
- caspase 3 activity
- deceased $\Delta \psi_{m}$
- cytochrome C release

"the switch"
NO is a small hydrophobic molecule, with many important signalling properties. Many of its physiological effects are mediated by the cyclic guanosine monophosphate (cGMP) production resulting from NO reacting with the haem group of guanylyl cyclase. Unregulated NO production can cause cell death through a number of reactions (Fig 4). Oxidative stress is the major cause of damage associated with elevated NO, largely from the formation of ONOO-, which is far more reactive and damaging than its precursors. Mitochondria are affected by NO in three principal ways: reversible inhibition of respiration, irreversible inactivation of mitochondrial enzymes, and the induction of mitochondrial permeability transition (PT). DNA synthesis is blocked by NO and DNA damaged by ONOO-, eventually leading to poly(ADP-ribose) polymerase (PARP) activation and ATP depletion. Dysregulation of cytosolic calcium by NO is a result of both mitochondrial damage and ATP depletion (Murphy 1999).

Etoposide is a topo II inhibitor, used primarily in the treatment of leukaemia, but also in a number of solid tumours, e.g. lung cancer and testicular cancer (FASS 2001). Its mechanism of action starts with stabilisation of covalent topo II-DNA complexes, induction of DNA strand breaks in these complexes, subsequently leading to activation of stress-associated signalling pathways, cell cycle arrest and activation of caspases (Kaufmann 1998). Previous reports have described the various parts of the apoptotic cell death pathway induced by etoposide in U-937 GTB cells. The dependency on proteases with interleukin-1 beta-converting enzyme (ICE)-like activity (the caspases) was found early (Mashima et al. 1995, Dubrez et al. 1996) and the morphology and DNA degradation has been described elaborately (Dini et al. 1996, Garcia-Bermejo et al. 1998, Shrivastava et al. 2000). Etoposide has been shown to induce Bcl-2 protein (cell death suppressor) cleavage by caspase 3 (Fujita et al. 1998), to produce a pro-apoptotic fragment. Sharp induction of metabolic activity characterises etoposide (Ekelund et al. 1998) and over-expression of Bcl-XL protects U-937 GTB from undergoing etoposide-induced apoptotic cell death (Schmitt et al. 1998).
Etoposide has also been shown to induce PS externalisation and release of cytochrome c, together with activation of caspase 3 and PARP cleavage (Sun et al. 1999). There is also evidence of autocrine stimulation of the CD95/Fas pathway, with induction of CD95-ligand and upregulation of CD95 in various cell types treated with therapeutic concentrations of etoposide (Friesen et al. 1999). However, also Fas-resistant cell lines retain the ability to undergo apoptosis in a normal fashion after treatment with etoposide (Kaufmann 1998). It has been established previously that when U-937 cells are exposed to etoposide, caspases (Ghibelli et al. 1995, Mashima et al. 1995, Dubrez et al. 1996), and ultimately endonucleases (Ghibelli et al. 1995, Garcia-Bermejo et al. 1998, Shrivastava et al. 2000) are responsible for the observed morphological features of apoptosis.

Pyridyl cyanoguanidines are known as potassium channel openers and the structural prototype pinacidil (N-1,2,2-trimethylpropyl-N'-cyano-N''-4-pyridylguanidine, Fig 5) is a very potent hypotensive compound due to this effect. Leo Pharmaceutical Products performed modifications of this molecule resulting in compounds that had lost the channel opening property, but had gained antitumoural effects (Schou et al. 1997). The cyanoguanidine moiety was found crucial for the antiproliferative effect and the chain length proved important for the degree of activity. An in vitro model system of three cell lines was used for investigation of antiproliferative activity: MCF-7 (breast cancer), NCI-H460 (large cell lung cancer) and NYH (small cell lung cancer). In vivo activity was determined in rats and nude mice bearing Yoshida ascites tumours. Based on excellent preclinical activity in these models, CHS 828 (N-(6-chlorophenoxyhexyl)-N'-cyano-N''-4-pyridylguanidine) was selected as a lead candidate for further investigation (Schou et al. 1997).

In a study using a panel of ten cell lines, representing differential resistance patterns, the effects of CHS 828 showed low correlation with the activity patterns of known anticancer agents, and no sensitivity to known mechanisms of multidrug resistance was observed (Vig Hjarnaa et al. 1999). Cytotoxicity was in the same range as that of standard cytotoxic compounds in NYH and MCF-7 cells, while normal lung fibroblasts and endothelial cells were significantly less sensitive. In the
cell line panel, a characteristic plateau-shaped dose-response relationship was observed. Comparing the activity pattern to that of 100 standard and investigational cytotoxic drugs, low to moderate correlations were observed.

The same study showed that CHS 828 administered to nude mice inhibited the growth of xenotransplanted human MCF-7 tumours and caused regression of NYH tumours. The study concluded that CHS 828 is an effective new antitumour agent, with a potentially new mechanism of action, and able to discriminate between malignant and normal cells (Vig Hjarnaa et al. 1999).

Using a newly modified in vivo rat model, anti-tumour effect, haematological toxicity and pharmacokinetics of CHS 828 were investigated. This hollow fibre method enables implantation of human tumour cells (from cell lines or patients) in immunocompetent Sprague-Dawley rats (Jonsson et al. 2000, Jonsson et al. 2001). Toxicity was low, and administration once daily for five days was shown superior to a single high dose. The anti-tumour effect was good for primary tumour cells from patients with chronic lymphocytic leukaemia (CLL) and ovarian carcinoma, while no effect was seen in the highly drug resistant breast cancer cell lines (Jonsson et al. 2000, Jonsson et al. 2001).

In vitro studies showed that CHS 828 exposure cause an immediate increase in metabolic activity, measured as extracellular acidification rate (Ekelund et al. 2000), a partly different response compared to many standard cytotoxic compounds (Ekelund et al. 1998).

Cell death parameters in breast cancer cell lines (mainly MCF-7) exposed to CHS 828 showed that although CHS 828 induce DNA fragmentation and PS externalisation in these cells, the morphology was not that of classical apoptosis (Hansen et al. 2000). It should be noted here that MCF-7 lack caspase 3.

One clinical Phase I study of CHS 828 in patients with solid tumours has been concluded to date. This Swedish study included 16 patients and CHS 828 was administered daily for five days, in cycles of 28 days. An accelerated study design was employed and a Phase II dose of 20 mg x 5 days was set. Dose limiting toxicities were diarrhoea, oesophagitis, constipation, thrombocytopenia and thrombosis. No tumour responses were observed, but seven patients showed stable disease (Ahlgren et al. 2001). Another Phase I study, performed by EORTC, has to date reached doses of 350 mg on a single day dosing schedule, with only one occurrence of dose limiting toxicity, haematuria
(Ravaud et al. 2001). A third Phase I study is ongoing, as well as a Phase II study in patients with CLL.
pharmacological studies of CHS 828 & etoposide induced tumour cell death
The general aim of this investigation was to characterise the mode of \textit{in vitro} cell death induced by the novel antitumoural substances CHS 828 and etoposide.

The specific aims were

- to characterise the mode and kinetics of CHS 828 and etoposide induced cytotoxicity using the U-937 GTB cell line as a model
- to further explore the biochemical pathways for CHS 828 and etoposide induced cell death.
- to study the effects of the combination of CHS 828 and etoposide.
- to evaluate the microculture kinetics (MiCK) assay as a tool for monitoring apoptotic cell death.
- to study the role of ATP for CHS 828 induced cytotoxicity.
pharmacological studies of CHS 828 & etoposide induced tumour cell death

**materials & methods**

This section will be kept to a minimum of that needed to grasp the general concept of the methods to be able to follow the results and discussion section. For more detailed descriptions, please see the individual articles (i-v).

**cells**

The main biological model throughout this work was a human lymphoma cell line, U-937 GTB, established from a male patient with histiocytic lymphoma in the 1970’s (Sundström et al. 1976). These cells are highly sensitive to CHS 828 and also readily inducible to undergo active cell death with classical apoptotic features, in response to various cytotoxic and physical stimuli (Lennon et al. 1991, Bicknell et al. 1995, Dini et al. 1996, Dubrez et al. 1996, Lai et al. 1998, Ashush et al. 2000, Liminga et al. 2000, Okuma et al. 2000, Shrivastava et al. 2000, Yang et al. 2000). Where indicated, a U-937 GTB subline highly resistant to CHS 828 (U-937/CHS), the myeloma cell line RPMI 8226/S, the leukaemia cell line CEM/S, and the lung cancer cell line NCI H69 were used. All cell cultures were maintained in RPMI 1640 culture medium, supplemented with foetal calf serum, glutamine and antibiotics. Cultures were kept under standard incubating conditions and were monitored and passaged twice weekly. All experiments were performed on cells in log phase, attained by adding fresh medium to the cultures the day before each experiment. The U-937/CHS cells were exposed to 1 µM CHS 828 every 5 weeks to maintain resistance.

**drugs & reagents**

The main drug, CHS 828 (N-(6-(4-chlorophenoxy)hexyl)-N’-cyano-N”-4-pyridylguanidine) was a kind gift from Leo Pharmaceuticals, Denmark. A stock solution of 10 mM in dimethyl sulfoxide (DMSO) was kept at -20°C and diluted fresh for each experiment, in decreasing DMSO concentrations. Etoposide (Vepesid®, Bristol-Myers Squibb, Bromma, Sweden) as 20 mg/ml injection concentrate (ethanol solution) was obtained from the hospital pharmacy. Prepared dilution was used for up to 72 h. For experiments on the effect of caspase inhibitors, the pan-caspase inhibitor Z-Asp-DCB (Z-Asp(CH2-[2,6-dichlorobenzoyl)oxy]-methane) and the selective caspase 3 inhibitor DEVD -fmk (ApoAlert™ caspase 3 inhibitor; Clontech Laboratories Inc, Palo Alto, CA) were used. The nitric oxide synthase (NOS) inhibitor N⁶-Monomethyl-L-arginine, monoacetate salt (L–NMMA; Calbiochem; La Jolla, CA), the PARP inhibitor 3-aminobenzamide (3-aba) and the ROS inducer menadione (Sigma-Aldrich Co, St. Louis, MO), together with the NOS
inducer phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich Co) and the antioxidant L-Ascorbic acid (Vit C; Sigma-Aldrich Co) were used in the ATP-associated studies.

The non-clonogenic fluorometric microculture cytotoxicity assay (FMCA) is based on measurement of fluorescence generated by the hydrolysis of fluorescein diacetate (FDA) to fluorescein by esterases in cells with preserved membrane integrity. In the original setting, it quantifies viability in undisturbed single cell suspension cultures exposed to cytotoxic drugs (Larsson et al. 1992). Drug solution is added at 1/10 of final cell suspension volume in a 96-well microtitre plate (MTP). After 72 h (or as specified) FDA is added and fluorescence measured after 40 min incubation. Results are expressed as Survival Index (SI%) = (mean fluorescence of exposed triplicates - mean blank fluorescence) / (mean fluorescence of controls - mean blank fluorescence) * 100.

In cells with intact membrane integrity 5-(and-6)-carboxy-2',7'-dichlorodihydro-fluorescein diacetate (carboxy-H$_2$DCFDA) is cleaved by the same esterases as FDA and fluorescence is produced when the cleavage product encounters a reactive oxygen intermediate. The assay is similar to the FMCA, with a 60 min incubation with carboxy-H$_2$DCFDA instead of 40 min of FDA.

The microculture kinetics (MiCK) assay measures changes in optical density (OD) in a cell culture, due to early apoptotic changes in cell morphology, mainly cellular and nuclear condensation. The correlation of results obtained by the MiCK assay to established apoptosis markers has been demonstrated (Kravtsov et al. 1998, Kravtsov et al. 1999).

Equation 1
\[
\frac{(\text{fluorescence}_{\text{test}} - \text{fluorescence}_{\text{blank}})}{(\text{fluorescence}_{\text{control}} - \text{fluorescence}_{\text{blank}})} \times 100 = \% \text{ fluorescence}
\]
Cells, in medium without phenol red, and desired drugs were added to a flat bottomed MTP, layered with sterile mineral oil to prevent evaporation and contamination, and placed in a SpectraMax™ Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). OD at 600 nm was measured automatically every 14 min for 48-72 h. During this monitoring the plates were kept at 37 °C, protected from light. The essential information lies in the shape of the curve produced by investigated cultures in relation to the curve produced by unexposed control cultures (Fig 6).

**TdT-mediated dUTP nick end labelling (TUNEL)**

Terminal deoxynucleotidyl tranferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) provides enzymatic *in situ* labelling of DNA strand breaks. TdT catalyses polymerisation of fluorescein labelled nucleotides to free 3’-OH DNA ends in a template independent manner. Analysis was performed on cytospin slides of U-937 GTB cells, exposed to drugs as desired. TUNEL was performed stringent to the commercial protocol (*In situ* cell death detection kit, fluorescein; Boehringer Mannheim GmbH, Mannheim, Germany). Subsequently the slides were rinsed in PBS, air-dried and mounted with antifade mounting medium (Vectashield®, Vector Laboratories, Inc., Burlingame, CA) and coverslips. Analysis of TUNEL staining was performed in a confocal microscope.

** mitochondria membrane potential (Δψ<sub>m</sub>)**

Change in Δψ<sub>m</sub> was assessed using JC-1, a lipophilic cation that is accumulated and retained in mitochondria, thus switching from green to red fluorescence. The electron transport chain uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as positive control. Red and green fluorescence was measured in a 96-well MTP reader. Results were calculated after fluorescence values had been corrected for cell density as (red fluorescence of test triplicates - red fluorescence of blanks) / (green fluorescence of test triplicates - green fluorescence of blanks) for each experiment. These means were averaged together and divided by mean control fractions and expressed as percentages.

**caspase activity**

Caspase activity was assayed by colorimetric detection of p-nitroanilidine (pNA) after cleavage of the peptide substrates DEVD-pNA (Asp-Glu-Val-Asp), IETD-pNA (Ile-Glu-Thr-Asp) or LEHD-pNA (Leu-Glu-His-Asp) specific substrates for caspases 3, 8 and 9 respectively. All reagents were part of commercial “Caspase Colorimetric Assays” (R&D
Systems Inc., Minneapolis, MN) for the three separate caspases. After drug exposure, cells were collected by centrifugation. Supernatants were removed by decanting and the pellets were frozen and kept in –70 °C until analysis. The assay was performed according to the commercial protocol, on one aliquot each for the separate caspases. Absorbance was measured at 405 nm in an ELISA reader (SpectraMax™Plus) after 90 min of incubation in the dark, followed by 20 s shaking. Activity was calculated as (test absorbance)/(control absorbance) with average absorbance of blank wells subtracted and expressed as percentages.

In these in vivo experiments, polyvinylidene fluoride hollow fibres were filled with U-937 GTB cells and heat-sealed. The fibres were incubated in vitro for 48 h before implantation subcutaneously on male Sprague-Dawley rats. On the day after implantation (day 0) and four subsequent days, the rats received 75 mg/kg of CHS 828 by oral gavage. On day 5, fibres were retrieved and cell density determined by staining with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for 4 h. MTT is converted to formazan by metabolically active cells, the amount of formazan being proportional to the number of living cells. After rinsing the fibres and drying them, the formed formazan was extracted with DMSO and OD read at 570 nm. Rats were monitored for 2 weeks with respect to weight and haematological parameters. The study included 2 control rats and 2 CHS 828 treated rats, each one carrying 3 fibres with U-937 GTB and one empty fibre. Results are expressed as net growth (%), calculated for each fibre as: (OD_{retrieval day} - \text{mean OD}_{implantation day})/\text{mean OD}_{implantation day}. Hence, a net growth of –100% represents total cell kill, while a value greater than 0% represents net growth in the fibre.

All preparations for morphological evaluation were made from cultures growing in 96-well MTP or culture flasks, as indicated. Cytospin slides were air dried and stained with May-Grünwald/Giemsa. These preparations were examined and/or photographed in a Nicon Eclipse E400 microscope, equipped with a digital colour video camera. Cells were classified as viable (intact membrane structures), apoptotic (condensed cytoplasm and condensed, fragmented nucleus) or necrotic (compromised membrane integrity, decomposed nucleus and membrane structures).
After continuous exposure to 0.1 µM CHS 828 and rinsing in PBS at desired time points, cells were processed for ultra structural analysis. The cells were fixed in 2% (w/v) glutaraldehyde in a 0.1 M sodium cacodylate buffer supplemented with 0.1 M sucrose, followed by 1.5 h post-fixation in 1% (w/v) osmium tetroxide in cacodylate buffer. After dehydration, the specimens were embedded in epoxy resin. Ultra thin sections (50 nm) were prepared with an ultratome equipped with a diamond knife, from three separate locations in each specimen. The sections were placed on copper grids covered with a film of polyvinyl formal plastic, and photographed in a Philips 201 transmission electron microscope (Lukinius et al. 1995). These preparations were kindly prepared at the Dept of Genetics and Pathology, Uppsala University.

Protein and DNA synthesis were measured with a Cytostar-T® plate (Amersham International plc, Buckinghamshire, UK), a pre-made scintillating MTP (Harris et al. 1996, Graves et al. 1997). Cells were suspended in fresh media containing [14C]-leucine for protein synthesis experiments or [14C]-thymidine for DNA synthesis experiments. Radioactivity was measured with a Wallac 1450 MicroBeta® trilux liquid scintillation counter (Wallac OY, Turku, Finland).

The isobole method was used for evaluation of drug interaction between CHS 828 and etoposide since both drugs produced sigmoid concentration-response curves. Concentration-response curves were created for CHS 828 and etoposide, respectively and isoboles for the effect levels 30, 40, 50, 60 and 70% were constructed. Based on the five isoboles, five CHS 828/etoposide combinations were chosen and tested by the FMCA. To account for the intra-assay variability of the FMCA, an interaction with a CI exceeding 1,2 was classified as sub-additive, under 0,8 as synergistic and for CI between 0,8 and 1,2 the interactions were denoted additivity (Lepri et al. 1991, Jonsson et al. 1998).

In addition, the same set of data was analysed by the additive model. A ratio between the observed survival index and that predicted by the additive mode of >1,2 was classified as a sub-additive interaction, <0,8 a synergistic interaction and ratios between 0,8 and 1,2 additive, in accordance with the isobole method CI (Lepri et al. 1991).
PARP activity was assayed as the polymerisation of radio labelled nicotinamide adenine dinucleotide (32P-NAD) by cell lysates after whole cell exposure to 1 µM CHS 828. The polymerised product was pelleted by centrifugation at 1200g and quantified by scintillation counting.

Formation of the PARP product poly(ADP-ribose) (PAR) was investigated in situ in cells exposed to 1 µM CHS 828. Slides were incubated with a primary PAR binding 10H monoclonal antibody, washed and exposed to the secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody. The result was photographed in a fluorescence microscope.

Cellular ATP content was assayed using the commercial ApoGlow™ kit. This bioluminescent method utilises an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin. Cells were incubated with desired drugs in an opaque MTP. After addition of a nucleotide monitoring and releasing agent, relative light units (RLU) were measured in a luminometer.

Extracellular acidification rate, as a measure of metabolic activity in the cells, was investigated in a Cytosensor Microphysiometer as previously described in detail (Ekelund et al. 1998). U-937 GTB cells were immobilised in an agarose mixture in a perfusion cup. The cups were placed in sensor chambers and perfused with low buffering-capacity medium. Drugs were diluted in medium and perfused through a second channel after 1 h of baseline establishment. A repeated 120 s pump cycle was used, where the rate of acidification was measured as -µV/s for 30 s, followed by 90 s perfusion. The change in voltage is measured by a light-addressable potentiometric sensor (LAPS). The results are presented as percent change of acidification rate compared to baseline that was set to 100%.
experimental design & results

The experiments are presented as they were performed, one leading to the next. Roman numerals in the subheadings refer to the individual papers in this thesis. Previously unpublished observations are labelled (u).

It was already known that CHS 828 induces a high degree of cell kill in different cell types, as assessed by the conventional FMCA with an exposure time of 72 h (Vig Hjarnaa et al. 1999). Based on this information, a modified FMCA was used to investigate the exposure time dependent cytotoxic effect for CHS 828. Total viability was assessed after 8, 24, 32, 48, 56 and 72 h of continuous exposure to 0.1 μM, revealing an exposure time dependent cytotoxic effect, starting after the first two measuring points. The same pattern was evident in vivo, as investigated by the hollow fibre model. In the set of fibres retrieved 24 hours after drug administration, cells showed full viability, while fibres retrieved after 120 h exhibited a 75% reduction in viable cells than on implantation day.

To explore the degree of active participation by the cells, the effects on DNA and protein synthesis as a function of exposure time was determined with Cytostar® technology. Like viability, these parameters remained largely unaffected during the first 24 h of CHS 828 exposure. Shortly past this time point, the synthesis of both macromolecules was sharply inhibited.

To determine whether CHS 828 induced cytotoxicity is dependent on these 24 h of intact macromolecular synthesis, a new FMCA series of experiments was performed. This time cytotoxicity of 72 h CHS 828 exposure was assessed in the presence of specific inhibitors of protein (0.1 or 1.0 μg/ml cycloheximide) and DNA (0.1 or 1.0 μg/ml aphidicholin) synthesis. Concentrations of the inhibitors were chosen to provide maximum specific inhibition, and minimum effect on viability. Co-exposure with aphidicholin did not protect cells from CHS 828 induced cell death, except at low concentrations (0.001-0.01 μM) of CHS 828. In contrast, co-exposing cells to CHS 828 and cycloheximide resulted in major increases in viability compared to all concentrations of CHS 828 exposure as single agent, indicating a potential need for de novo synthesis of certain proteins by the cell, to assist in its death.
The MiCK assay is an easy and robust method for receiving a real time, visual representation of morphological events in whole cell cultures. This was utilised to further explore the kinetics and the mode of CHS 828 action on U-937 GTB. To challenge the possibility of the previously observed effects being cell type related, CEM/S and RPMI 8226/s cell lines were also included. U-937 GTB cultures exhibited an OD curve that initially did not divert from that of control cultures. However, after 24-32 h the absorbance did not only increase at a slower rate, it started to decrease. Similar patterns were observed for the other cell lines (Fig 7). Having this information on crude morphology, cytospin slides of U-937 GTB cells continuously exposed to 0.1 $\mu$M CHS 828 for 8, 24, 32, 44, 56 and 72 h were prepared. These preparations revealed that CHS 828 exposure for 44-72 h induce extensive and increasing disintegration of the cytoplasmic compartment and eventually total cellular collapse, but with little or no nuclear condensation or formation of apoptotic bodies. Slides prepared from shorter exposure times revealed unaffected cells, not distinguishable from untreated controls. This was also reproducible in other cell lines. To further explore the details of CHS 828 effects on cellular appearance, ultra structure was investigated by EM after 24, 48 and 64 h of continuous exposure. This investigation confirmed the notion that CHS 828 leave cells unaffected for 24 h. However, after 48-64 h of exposure (0.1 $\mu$M) cells exhibited swollen mitochondria with an inner membrane structure under decomposition and dilated endoplasmic reticule enclosed by an apparently normal plasma membrane. Occasional cells showed apoptotic features of the nuclei, however most were found largely unaffected.

The EM results were followed by TUNEL on CHS 828 (0.1 $\mu$M)
pharmacological studies of CHS 828 & etoposide induced tumour cell death

exposed cells, since DNA fragmentation is an apoptotic marker even in the absence of classical apoptotic morphology of the nucleus. In accordance with previously investigated factors no DNA fragmentation was detected during the first 24 h of continuous exposure, or even 32 h. After this, the number of TUNEL positive cells increased gradually to 30% at 72 h. This increase paralleled the appearance of cells with collapsed cellular integrity, as judged from MGG stained morphological preparations.

Another lead from the EM investigation was the visually obvious effects on mitochondria. Would this be linked to a specific disruption of the $\Delta \psi_m$, typical of apoptosis, before or around 24 h, as with the other investigated parameters? On the contrary, it was revealed that an initial hyperpolarisation of mitochondria at 24 and 32 h of whole cell CHS 828 exposure was evident. Loss of transmembrane potential followed at 48-72 h.

An essential role for mitochondria in the course of apoptosis is the release of pro-apoptotic factors, e.g. cytochrome C and Apaf-1. One theory is that this occurs through the opening of a PT pore, which can be blocked by cyclosporin A (CsA) (Zamzami et al. 1996). Although mitochondria of CHS 828 exposed cells were shown to hyperpolarise, we investigated the effect on cell viability of co-incubation with CHS 828 and CsA by FMCA (u). At non-toxic concentrations of CsA, no protective effect on CHS 828 induced cell kill was observed (Fig 8).

Continuing the search for factors to explain the unusual kinetics of CHS 828 induced cell death, coupled with a seemingly active participation by the cell, without apoptotic features, the caspases were next in line for exploration. Instead of the common detection of Western blotted cleavage products, we used a commercial kit to investigate specific caspase activity. Caspase 3 was picked for general effector activity (commonly attributed to caspase 3, 6 and 7), and caspase 8 and 9, for activity in the two separate initiator pathways, respectively. U-937 GTB cells were continuously exposed to 0.1 $\mu$M CHS 828 for 8, 24, 48 or 72 h. No detectable effects were found, with the exception of a twofold increase in caspase 3 activity 48 h after start of exposure. The importance of this modest increase was studied by a new series of

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**Fig 8.** FMCA investigation of viability of U-937 GTB cells exposed to CHS 828, CsA or a combination thereof. Concentrations in $\mu$M. Presented as mean SI%, error bars = SEM.
FMCA, MiCK and morphology experiments. U-937 GTB cells were co-exposed to 1.0 μM CHS 828 and either one of the specific caspase 3 inhibitor DEVD-fmk or the pan-caspase inhibitor Z-Asp-DCB. Separate FMCA experiments were performed on the inhibitors as single agents, to enable a choice of non-toxic concentrations (not shown). All three investigations showed that caspase inhibitors lacked influence on the shape and kinetics of CHS 828 induced cell death. Thus far it was impossible to distinguish CHS 828 induced cell death as apoptotic or necrotic. We resigned to the fact that we were probably facing the possibility of a combined or dual effect of CHS 828. Maybe the molecule itself, or products of its initial effects, inhibits pathways necessary for the completion of a clear cut death mode. Even so, there had to be identifiable triggers, signalling factors and events involved. Investigating and manipulating factors that ordinarily determines the mode of cell death seemed a plausible route to find further answers. One possible switch in cell death mode, is decreasing ATP levels, and depleted intracellular energy stores would explain the absence of apoptotic features in cells exposed to CHS 828. Intracellular ATP content was measured and found lowered at 24 h and virtually abolished by 32 h. In order to find the source for this ATP decrease, the possible involvement of PARP was investigated. Co-exposure to the known PARP-inhibitor 3-aba postponed the CHS 828 induced drop in ATP, with intact levels at 24 h and complete depletion at 48 h. Metabolic activity, measured as extracellular acidification by microphysiometry revealed that the CHS 828 induced early increase in metabolic rate, was prolonged by co-exposure to 3-aba. Viability at 72 h, as measured by FMCA, was increased by 3-aba at lower concentrations (1 nM – 0.1 μM) of CHS 828. May-Grünwald/Giemsa stained morphology preparations revealed that CHS 828 alone induced cell death with a predominantly necrotic appearance, while co-incubation with 3-aba resulted in a significant proportion of cells with apoptotic morphology. However, measurements of both direct PARP activity and its product, PAR, testified against PARP as the ATP depletor, both being negative. The other main explanation for ATP depletion is lowered mitochondrial production. This is commonly triggered by NO and/or ROS. However, the levels of reactive oxygen intermediates were investigated fluorometrically and were found non-existing to low for all timepoints from 15 min to 48 h of continuous exposure. FMCA experiments on
cells co-exposed to CHS 828 and the antioxidant vitamin C 100 nM supported the negative ROS results by producing the exact same SI values as CHS 828 exposure alone. Also, the protein kinase C (PKC) activator, PMA, also effective as a NOS inducer, and NOS inhibitor L-NMMA in combination with CHS 828 produced no differential effects on cell survival as assessed by the FMCA (Fig 9).

Fig 9. Total survival of U-937 GTB cells exposed to indicated concentrations of CHS 828, alone or in combination with Vit C, the NOS inducer L-NMMA or the NOS inhibitor PMA. Each point is a mean of 2-4 experiments with S.E.M indicated.

As one investigated parameter after the other in cells exposed to CHS 828 exhibited patterns not typical of apoptotic cell death, the need for a clear-cut positive control in our methods arose. The well-characterised substance etoposide was picked for this purpose, and all investigations were performed in the same manner as for CHS 828. However, time frames were adjusted to catch the much faster process of etoposide induced cell death (2-8 h). To complement previously published observations on etoposide induced cell death (Garrido et al. 1999, Sun et al. 1999, Shrivastava et al. 2000) and to relate our own findings on CHS 828 induced cell death, a series of experiments were performed on U-937 GTB cells exposed to 25 µM etoposide: TUNEL assay, detection of DNA and protein synthesis, detection of the activity of caspase 3, 8 and 9, investigation of caspase dependency including morphology and MiCK assay. It has been shown previously by agarose gel electrophoresis that DNA is fragmented in an organised manner in this setting (Ghibelli et al. 1995), and this was confirmed in our TUNEL experiments. The extent of cell aggregation, chromatin condensation, nuclear fragmentation and TUNEL positivity was time dependent over 8 h of exposure. DNA synthesis was instantly inhibited, while protein synthesis remained intact for 4 h of continuous exposure before
tapering.
Caspases 3 and 8 were massively activated after 4 h of continuous exposure (530% and 260% compared to untreated controls, respectively), while caspase 9 remained at low levels of activity. The striking features of apoptotic morphology were evident in etoposide exposed cells, both in morphological preparations (Fig 10) and in the MiCK patterns. Equally evident was the dependence on intact caspase activation for the development of these features, in both these systems. Morphological preparations showed an inhibitor concentration dependent (12.5 to 50 µM) extinction of morphological apoptosis features, while the MiCK assay produced more subtle changes. Investigating the influence of these inhibitor induced changes on the total viability by FMCA, revealed that 25 µM etoposide cause near complete cell kill in U-937 GTB cells (SI 1%). Co-incubation with the selective caspase 3 inhibitor did significantly increase the survival, however, cell kill was still near complete (SI 7%).

Thus far, CHS 828 and etoposide seem to induce vastly different modes of cell death. CHS 828 exhibited prolonged, yet tightly controlled kinetics with a sharp break point in several factors around 24 h of continuous exposure, and both features and viability seemed independent of caspases. There is an active component involved, however, not resulting in classical apoptosis. Etoposide, on the other hand, induce rapid apoptosis, with striking features and an obvious dependency on caspases. Notably, both substances effectively kill U-937 GTB cells in vitro.

Why do these apparently apoptosis prone cells not respond with launching of its apoptotic machinery upon ultimately lethal CHS 828 stimulus? Two aspects of these observations were investigated; 1) Does CHS 828 have a dual mechanism of action, which initially induce active cell death, but subsequently inhibits its own effector pathways, rendering the cells unable to execute their cell death program? 2) Could the vast differences in kinetics and cellular events as a response to the two drugs be combined in a beneficial way?

To shed light on the theory of CHS 828 exerting a dual mechanism of action, a series of experiments were set up to investigate etoposide induced apoptotic factors in cells pre-exposed to CHS 828. Different temporal U-937 GTB culture varieties were used: continuous co-
exposure (25 µM etoposide, 1.0 µM CHS 828), or different time periods of pre-exposure to CHS 828 followed by 4 h co-exposure with etoposide. These cultures were then investigated with respect to crude morphology by MiCK, conventional morphology, total viability by FMCA, and activation of caspase 3, 8 and 9.

Exposure to only etoposide, continuous co-exposure for 4 h, and 4 h pre-exposure followed by 4h co-incubation resulted in similar morphologies. May-Grünwald/Giemsa stained slides revealed massively apoptotic cells, and this response was also evident in the MiCK assay. Co- and/or pre-exposure for up to 12 h generally potentiated caspase activity (statistically significant for caspase 3). In paper i, ii and v, most measured parameters remained unaffected by up to 24 h of CHS 828 exposure, and apparently the drug has only minor effects on the pathways utilised by etoposide, during the first part of this period. However, in this experimental set-up, pre-exposure to CHS 828 for the full 24 h period had major effects. Etoposide now completely failed to induce apoptotic morphology, evident both microscopically and by the MiCK assay, and also failed to activate the caspases (statistically significant for all three caspases). The same set-up of the MiCK and the FMCA was used to observe the extent of these effects in the CHS 828 resistant sub-line U-937 GTB (u). Strikingly, this proved that the resistant cells are resistant not only to the cell kill property of CHS 828, but also to the effect on the apoptotic machinery. No period of pre-exposure to 1.0 µM CHS 828 rendered these cells unable to respond with apoptotic gross morphology to etoposide as visualised by MiCK (Fig 11).

Fig 11. MiCK curves of U-937 GTB and U-937/CHS cells pre-exposed to CHS 828 for indicated times and etoposide added at start of MiCK. Total time for registration: 24 h. One representative experiment.
stimuli, despite the presence of CHS 828. Adding etoposide for 4 h after 18 h of pre-incubation with CHS 828 plus 3-aba doubled the caspase activation (p<0.05). Six hours later, cultures with 3-aba still activated all three caspases upon etoposide stimuli, while cultures without 3-aba failed to do so (statistically significant differences in activity levels for all three caspases). After 48 h both cultures exhibited equal and low activity levels for all caspases, with or without 3-aba (Fig 12).

To investigate common interaction between CHS 828 and etoposide in vitro, FMCA experimental results were studied by means of the isobole method and the additive model. First, single drug dose-response curves were produced and concentrations of the two drugs were extracted from the midpoint of each resulting isobole, where both drugs are expected to contribute equally to the total effect. New FMCA combination experiments performed at concentrations with expected effect levels (100 - %SI) of 30%, 40%, 50%, 60% and 70% all produced observed effect levels of 76-79% in U-937 GTB cells. Using either interaction calculation model, this represents a high degree of synergistic interaction.

Fig 12. Apoptosis induced by 4 h exposure to 25 µM etoposide (dashed line). Caspase activities shown as mean of three separate experiments ±SEM. Asterixes indicate statistically significant differences between cultures pre-exposed to only CHS 828 and cultures pre-exposed to CHS 828 and 3-aba (*p< 0.05, **p< 0.001)

cytotoxic synergy between CHS 828 & etoposide
CHS 828 exhibits attractive features compared to standard cytotoxic drugs, and is already in phase II clinical trials, 6 years after the discovery of its antitumoural properties. The mechanism of action does most likely not fall into one of the common categories and it does not induce cross-resistance to other commonly used cancer chemotherapeutic drugs.

CHS 828 induces a process in exposed cells which leads to their death 72 h later. The first sign of response is an increased metabolic activity starting only minutes after start of exposure and remaining elevated for approximately 15 h. However, this is possibly unrelated to the lethal effect of CHS 828, as the same response is detected in U-937/CHS cells (Ekelund et al. 2000). Next, most investigated parameters deviate from control values around 24 h after start of exposure, or later as with the decrease in viability. The results of this study could easily be interpreted as CHS 828 inducing exposure time dependent responses. However recent data reveal that 1 h of exposure suffices to induce similar responses at equal times after start of exposure (Saadia Bashir Hassan, submitted manuscript). This suggests an early drug-target interaction, which induces a cascade of events, including synthesis of certain proteins in turn causing cell death.

Apparently, de novo protein synthesis during these initial 24 h is essential for the cytotoxic properties of CHS 828, indicative of active cell death commonly intimately associated with apoptosis. It was therefore a surprise to find very few apoptotic cells in morphological preparations, EM preparations, and no sign of apoptosis on the MiCK curves. In addition, caspases seem largely unimportant for the development of CHS 828 induced cell death. Although significant activity of caspase 3 could be detected, it was modest and activity inhibitors had no effect. The cell death patterns of necrosis, apoptosis and CHS 828 induced cell death are summarised in Table 1. The classification of CHS 828 induced cell death into either of these two models would clearly be difficult. It is no secret that the rigidity surrounding the concept of apoptosis, as well as extensive confusion of terminology has caused classification problems before (Hockenbery 1995, Blagosklonny 2000, Häcker 2000). In addition, vastly different other types of active cell death have been described, although the majority in metamorphosing or involutional cell systems, rather than in cancer chemotherapy exposed cells (Zakeri et al. 1996, Jochová et al.)
1997, Kitanaka et al. 1999). However, at least some of this confusion is alleviated by the increased understanding of the molecular pathways involved, as well as by the “switch” theory (Depraetere et al. 1998, Lockshin et al. 2000).

There is a risk for underestimation of the commonness of these phenomena. Investigators may be reluctant to publish seemingly negative findings, or results not compatible with the accepted theories and early contributions describing atypical cell death phenomena may be suppressed by editors. For example, the hyperpolarisation of mitochondria in CHS 828 exposed cells, which much surprised us, has recently been described by others as a priming phase preceding the commonly reported depolarisation (Scarlett et al. 2000).

However, the probability of CHS 828 inducing a known active molecular pathway, which is then inhibited or “switched” after ~24 h, vastly outweighs the probability of a previously undiscovered molecular pathway and type of cell death. After all, U-937 GTB is a widely used cell line, and the observed phenomenon seem to encompass also other common cell lines.

A plausible switch in the case of CHS 828 is ATP. The cells run out of energy and stop synthesising proteins, become unable to activate caspases and start to die. The maintenance of near original levels of intracellular ATP partly protects the cells from cell death in general, but also shifts it over to apoptosis. However, it remains unclear whether the main effect of CHS 828 is interrupted by depleted energy stores, or if the main effect directly leads to disrupted energy metabolism hence killing the cells. The involvement of ROS and NO was certainly expected at this stage of the study, but was surprisingly missing. The

<table>
<thead>
<tr>
<th>Table 1</th>
<th>apoptosis</th>
<th>necrosis</th>
<th>CHS 828 induced cell death</th>
</tr>
</thead>
<tbody>
<tr>
<td>timing</td>
<td>Tight, short process</td>
<td>Dose dependent</td>
<td>Tight, long process</td>
</tr>
<tr>
<td>morphology by MGG</td>
<td>Cellular and nuclear condensation and fragmentation</td>
<td>Cellular and nuclear disintegration</td>
<td>Cellular disintegration with preserved nuclear structure</td>
</tr>
<tr>
<td>gross morphology by MICK</td>
<td>Rapid and steep increase of OD</td>
<td>Stable or decreasing OD</td>
<td>OD as control → decreasing OD</td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td>Pre-death</td>
<td>Post-death</td>
<td>Post-death?</td>
</tr>
<tr>
<td>dependency on protein synthesis</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>caspase activation</td>
<td>Yes</td>
<td>No</td>
<td>Slight</td>
</tr>
<tr>
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<tr>
<td>ATP level</td>
<td>~75%</td>
<td>&lt;50-25%</td>
<td>&lt;25%</td>
</tr>
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possibly unusual hyperpolarisation of mitochondria in CHS 828 cells may prove intimately related to the energy patterns. The mitochondria seem to build up an electrochemical membrane gradient, without being able to extract the potential energy in the usual manner. Indeed inhibition of mitochondrial F0F1-ATPase with specific probes has been shown to result in mitochondrial hyperpolarisation (Leist et al. 1997).

The main purpose of the etoposide study was to get a reliable apoptosis control in all the experimental settings utilised for investigating CHS 828 induced cell death. Maximal apoptotic response was observed after 4 h etoposide exposure, confirming the specificity of our methods.

Cell death induced by etoposide in U-937 GTB cells is heavily reliant on caspases and exhibits a striking apoptotic morphology. The results also support those investigators suggesting a caspase 8 mediated pathway for etoposide induced apoptosis (Wesselborg et al. 1999).

The effort to provoke apoptosis development in CHS 828 exposed cells, by 4 h etoposide treatment revealed some surprising results. The expected outcome was a complete inhibition of caspase activity by CHS 828, and hence no apoptotic effect of etoposide. However, the immediate presence of CHS 828 does not seem to have any major effect on the actions of etoposide. Twelve hours after the addition of CHS 828 to the cultures, etoposide actually produce a significantly enhanced effect on caspase activity in these cells. Another 12 h after the start of CHS 828 exposure however, the cells are unable to undergo etoposide induced apoptosis. Rather expectedly, the cells still die, although not to the same extent as by etoposide alone. At least morphologically, this apoptosis inhibitory effect of CHS 828 does not develop in the CHS 828 resistant subline, suggesting a connection between the apoptosis inhibition and cytotoxicity. This connection could be the lowering of intracellular ATP levels.

To confuse these matters further, the interaction analysis of the basic combination of CHS 828 and etoposide from time zero, revealed impressive cytotoxic synergy of the two substances. The implications of this information in the context of treatment of cancer needs to be carefully considered in the light of the previously discussed inhibitory effects of this combination, with respect to schedule and sequence of administration of the two drugs.
The ATP level conserving effects of 3-aba was impressive, in cells exposed to CHS 828. It was a great surprise to find that this effect was not mediated by the well studied PARP inhibitory effect of 3-aba. Other effects of 3-aba in the literature include indirect PKC inhibition and effects on the cytoskeleton level, however, no connection to ATP is described here (Tiozzo et al. 1996, Ricciarelli et al. 1998).
conclusions

- Characteristic for the kinetics of CHS 828 induced cell death is the general breakpoint at 24-30 h. Before this the cells seem strikingly unaffected except for a very early metabolic stimulation. Complete cell kill is achieved 72 h after start of exposure.

- The observed CHS 828 induced cytotoxicity does not conform to the typical morphology and biochemistry of classical apoptosis, even though U-937 GTB cells evidently have an intact apoptotic machinery, as shown by the etoposide study. Etoposide initiates complete apoptosis in U-937 GTB cells within 4 h of exposure, seemingly mediated by a caspase 8 dependent pathway. CHS 828 induced cell death, on the other hand, is caspase-independent.

- Combining CHS 828 and etoposide results in cytotoxic synergy. However, pre-exposure to CHS 828 before etoposide treatment renders U-937 GTB cells unable to launch the apoptotic program. Interestingly, CHS 828 resistant U-937 cells are still responsive to the apoptotic stimulus of etoposide, despite the presence of CHS 828, suggesting that the cytotoxic and the apoptosis inhibitory effects of CHS 828 are closely related.

- The MiCK assay proved valuable in capturing kinetics of cell death morphology, and able to visualise more subtle changes than end point morphology. Monitoring CHS 828 induced cell death proved difficult, in the respect that the shape of the OD curve produced by exposed cultures did not conform to the distinct shapes of apoptosis and necrosis previously described (Kravtsov et al. 1996, Kravtsov et al. 1998, Kravtsov et al. 1999).

- The ATP depletion as a consequence of CHS 828 exposure is not due to consumption by PARP or inhibition of mitochondria by ROS. The protective effects of 3-aminobenzamide must thus be other than PARP-inhibition.
This thesis did not provide the Rosetta stone to the actions of CHS 828. There are still major question marks to be straightened out. In order to better the use of CHS 828 as an anti-cancer drug, we need to find out the exact signalling pathways, the exact target molecule and the exact death event of affected cells. Only then can the optimal changes be made to the molecule and the most effective drug combinations be engineered. Further, more studies are warranted on formulations, administration, dosing schedules, pharmacokinetics and dynamics in man. The laboratories at Clinical Pharmacology in Uppsala and at Leo Pharmaceutical Products in Denmark are still pursuing the mechanism of action and the fourth clinical study has begun to include patients. There is reason to believe that CHS 828 will continue to elude its pursuers for some time yet, but also that there may be a pot of gold at the end of the rainbow.
Det är mycket fikonspråk i den här boken, vilket tyvärr gör den helt obegriplig för den som inte rotar i samma hörn av okunskapen som jag. Detta språk är dock nödvändigt för att med tillräcklig precision beskriva nya rön inom forskningen. Dessvärre är den dessutom på engelska, vilket gör den ytterligare obegriplig för oss svenskar. Det här kapitlet är därför tillägnat de tappra som kommit så här långt genom boken utan att förstå vad den handlar om, och fortfarande vill veta!


Först studerade och beskrev jag tidsförloppet för celldödsprocessen orsakad av CHS 828, med olika metoder. Det visade sig att förloppet är ovanligt långdraget och oberoende av hur mycket CHS 828 man behandlar cellerna med (i).
Vidare undersökte jag vilken variant av celldöd behandlingen med CHS 828 orsakar, med utgångspunkten att det är antingen aktiv celldöd, apoptos, eller passiv celldöd, nekros. Resultatet blev att CHS 828 inte inducerar en renodlad form av någon av dessa två klassiska celldödstyper, utan cellerna uppvisar komponenter från båda (ii). Eftersom detta är ovanligt ville vi utreda det vidare (iv).

Ett arbete ägnades alltså helt åt att beskriva den apoptos etoposid orsakar, med samma metoder som jag använder för att beskriva celldöden orsakad av CHS 828, för att ha en invändningsfri kontroll för dessa metoder (iii).


Det avslutande arbetet går vidare med möjliga förklaringar till de tidigare beskrivna händelserna kring celldöden orsakad av CHS 828, med tyngdpunkten på cellens energiläge. En möjlig förklaring till det vi observerat visade sig vara en tidig förlust av energi i cellerna (v).

Sammanfattningsvis ger den aktuella avhandlingen detaljerade beskrivningar av olika delar av celldödsprocessen hos U-937 GTB celler behandlade med CHS 828 och/eller etoposid. En slutgiltig verkningsmekanism återstår dock att beskriva, även om ett flertal alternativ i och med dessa studier har kunnat uteslutas.
expressions of gratitude

No false modesty – this is my book, and I wrote it! However, there are so many without whom it would have never happened, not been such an experience and not a fraction as much fun. To each and every one of these individuals, I owe my sincerest thanks, and I would like to use this space to acknowledge you.

My best friends
Anna Carolina
Johanna Karin
Linus

Anders       Anna
Britt-Marie   Elin
Fredrik      Gunnar
Helena       Henrik
Jocke        Katalin
Manuel       Niklas
Saadia       Sara
Sumeer

As a PhD student your supervisor is your guiding star, your inspiration, your source of wisdom, your work provider, your wallet, your mentor, your disillusion, your scientific parent. I’m truly grateful to have been under the wings of Rolf Larsson.

Co-supervisor
Peter Nygren
who lets nothing pass.

My PhD colleagues and friends at Foll
Agneta Lise
Jörgen

The “oldies”; Mormor, Farmor and Farfar. My idol brother Jonas and his Annika. The best parents ever, Ann-Margrethe and Leif. Most of all for encouraging me to embark on these “mission impossibles”, for unwithering love, friendship and support through thick and thin.
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