Preclinical and Clinical Development of the Novel Cyanoguanidine CHS 828 for Cancer Treatment

PETER HOVSTADIUS
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Abstract

CHS 828 is a cyanoguanidine with anti-tumour properties which has shown promising effects in several preclinical models. This thesis describes both preclinical and clinical studies aiming to investigate disease specific activity, clinical tolerability and efficacy of CHS 828.

In paper I we investigated CHS 828 activity in a cell line panel with human myeloma cells, three of these cell-lines were also tested in vivo using a hollow fibre rat-model. In paper II we investigated CHS 828 activity in primary human tumour samples from patients. CHS 828 showed an effect on all tumour cell types tested both the primary human tumour samples and the myeloma cell lines. Notably, CHS 828 showed a high relative in vitro activity against tumour cells from chronic lymphocytic leukaemia and high-grade lymphoma.

In a phase I trial we determined the maximum tolerated dose (MTD) of CHS 828. Haematological toxicity was generally mild and dominated by transient thrombocytopenia and lymphocytopenia. Non-haematological toxicity was mostly of gastrointestinal origin. The recommended phase two dose (RPTD) of CHS 828 was estimated to be 20 mg once daily for five days in cycles of 28 days duration.

In a phase II trial we investigated the effect of CHS 828 on patients diagnosed with B-CLL. In total 12 patients were enrolled. CHS 828 was found to be well tolerated and the most common haematological toxicity was thrombocytopenia. Non-haematological toxicities were generally mild. Transient decreases in lymphocyte counts could be discerned coinciding with drug dosing, but no sustained clinical responses could be achieved.

In conclusion, CHS 828 demonstrated marked effects in the preclinical investigations suggesting haematological malignancies as the main target. The clinical phase I study established a safe dose and the subsequent phase II trial in B-CLL patients showed biological effect but with no clinical disease response.

Keywords: CHS 828, Cyanoguanidines, Oncology, Clinical Drug Development

Peter Hovstadius, Department of Medical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

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I think I could, if I only knew how to begin. For, you see, so many out-of-the-way things had happened lately that Alice had begun to think that very few things indeed were really impossible.

Lewis Carroll
(From: Alice's Adventures in Wonderland)

To my beloved children, Sara and David
List of Papers

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


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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>B-CLL</td>
<td>B-cell Chronic Lymphocytic Leukaemia</td>
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<tr>
<td>CHS 828</td>
<td>N-(6-chlorophenoxyhexyl)-N'´-cyano-N'´-4-pyridylguanidine</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum plasma concentration</td>
</tr>
<tr>
<td>CR</td>
<td>Complete remission</td>
</tr>
<tr>
<td>CTC</td>
<td>Common Toxicity Criteria</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochromes P450</td>
</tr>
<tr>
<td>DLT</td>
<td>Dose-limiting toxicity</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>GCP</td>
<td>Good Clinical Practice</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Concentration resulting in 50 % cell survival</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonisation</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitory kappa B kinase</td>
</tr>
<tr>
<td>LD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>The dose that kills 10% off the experimental animals</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>MPA</td>
<td>Medical Product Agency</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance associated protein</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximal Tolerated Dose</td>
</tr>
<tr>
<td>MTT</td>
<td>([3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide)</td>
</tr>
<tr>
<td>NCI</td>
<td>The National Cancer Institute</td>
</tr>
<tr>
<td>NF-kappa B</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pgp</td>
<td>Permeability glycoprotein 170</td>
</tr>
<tr>
<td>PR</td>
<td>Partial remission</td>
</tr>
<tr>
<td>R</td>
<td>Pearson’s correlation coefficient</td>
</tr>
<tr>
<td>RPTD</td>
<td>Recommended Phase two Dose</td>
</tr>
<tr>
<td>STD&lt;sub&gt;10&lt;/sub&gt;</td>
<td>the dose that causes severe toxicity (or death) in 10% of the experimental animals</td>
</tr>
<tr>
<td>TDL</td>
<td>Toxic Dose Level</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Time to reach C&lt;sub&gt;max&lt;/sub&gt;</td>
</tr>
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</table>
Introduction

CHS 828

CHS 828 is a cyanoguanidine with a molecular weight of 371.87 g/mol which has shown interesting properties as a potential anticancer agent (1). CHS 828 was selected via a primary in vitro screening program based on human cell lines and rodent cell lines. Investigations using a panel of cell lines, representing defined mechanisms of resistance, revealed a differential pattern of anti-tumour activity with some cell lines sensitive in the subnanomolar range. In addition there was a low cross-reactivity with clinically used standard agents indicating a novel mechanism of action (2).

![Chemical structure of CHS 828](image)

*Figure 1 The chemical structure of N-(6-(4-chlorophenoxy)hexyl)-N’-cyano-N’’-4-pyridylguanidine, CHS 828.*

In the subsequent pharmacodynamic evaluation, oral administration of CHS 828 has also shown significant anti-tumour activity in several tumour models, especially pronounced in a nude mouse model of small cell lung cancer (NYH) and in the MCF-7L breast carcinoma model (2).

Studies investigating the mode of cell death caused by CHS 828 have indicated an active process, but it did not conform to a typical morphology of classical apoptosis (3). Furthermore, inhibition of mitochondrial respiration leading to extracellular acidification has been noted (4). Although the exact mechanism of action is not completely understood, CHS 828 has recently been shown to inhibit NF-kappa B activity trough down regulation of IKK activity (5).
Oncology Drug Development

In order to advance the quality of care for cancer patients and to prevent, treat and potentially cure this group of diseases, new therapies must continually be developed. Every year, the Cancer Therapeutic Evaluation Program (CTEP) at the national Cancer Institute (NCI) screens over 20,000 potential new anticancer compounds using tumour cell assays. The pharmaceutical industry screens in all probability even more that this number every year. After selection of a candidate compound with *in vitro* and preliminary *in vivo* testing, extensive preclinical pharmacological and toxicological studies need to be performed to assess the possible therapeutic potential. The toxicological profile and the selectivity of effect play a crucial role in determining the success or failure of a drug development. For every 10,000 compounds screened, approximately 500 may show anti-tumour activity *in vitro*. Among these 100 may be worthy of testing on animals. Of these 100 compounds, 10 may meet the criteria to perform extended pharmacological and toxicological studies to obtain enough information to warrant clinical trials in humans (6).

The primary objective of clinical phase I studies is to evaluate the safety of the drug and to define the Recommended Phase Two Dose (RPTD). The efficacy of the drug is first studied in phase II trials in a well defined patient population and is subsequently validated in larger phase III trials.

Pre-clinical testing

Prior to Phase I studies, preliminary *in vitro* characterisation of the mechanism(s) of action, resistance, and schedule dependencies as well as anti-tumour activity *in vivo* should have been made. The primary aims of the *in vitro* studies are to obtain mechanistic information about the test substance and to characterise the activity profile (7).

The use of well-characterised cell lines as regards genotype and biochemistry is essential for obtaining information regarding general drug sensitivity and general resistance. New compounds are often tested in cell line panels representing a variety of different human solid tumours. The best known and most widely used panel is developed by the National Cancer Institute (NCI) in the USA. Today the NCI are using a panel of 60 cell lines representing both solid and haematological malignancies (8). This *in vitro* screen is technically simple, relatively fast, cheap, reproducible, and provides valuable indicative data of mechanistic activity and target interaction. Yet it is not without its limitations since established cell lines may have lost some of their original tumour characteristics. *In vitro* assays based on cells prepared from patients’ tumours might be an alternative. Fresh tumour samples from patients representing different diagnostic groups can be utilized to investi-
gate disease-specific activity (7). The clonogenic assay has been used for this purpose, but the correlation between in vitro activity and clinical efficacy has not been conclusively demonstrated (9). The use of in vitro assays based on the concept of a total cell-kill is another alternative. The FMCA assay used in our laboratory has been evaluated with regards to tumour type specific activity with promising results (10, 11).

The primary aims of in vivo studies are to obtain further information with respect to anti-tumour activity, therapeutic index (ratio of the toxic dose to the therapeutic dose) and schedule dependency. Studies in animals are usually carried out in rodents, mainly in mice, giving due consideration, when possible, to likely differences to man in pharmacokinetics/dynamics. The hollow fiber method can be used for preliminary in vivo characterization. The method uses tumour cells that are cultured in semipermeable hollow fibres, which are subsequently implanted into rats. This is a relatively rapid and cost effective demonstration of in vivo activity as described by Hollingshead et al. (12). The National Cancer Institute (NCI) started using this method in 1995 as a routine in vivo screening assay. Presumably, this assay would prioritize compounds for secondary xenograft screening and help reduce the large number of active compounds generated by large cell line screening (7). Looking retrospectively, it has been shown that the hollow fibre method can, to a certain extent, predict some level of xenograft activity (13).

Advancing a compound from preclinical testing to phase I require an estimation of the starting dose in humans. A conservative low-dose approach will result in a subtherapeutic or ineffective dose, whereas an aggressive dose escalation may produce toxicity. Traditionally for anticancer agents, one tenth of the LD$_{10}$ (the dose that kills 10% off the experimental animals) in mice or one third of the toxic dose level (TDL) in the dog (in mg/m$^2$) is used as the starting dose in phase I clinical trials (14). Studies that actually measure death as an endpoint, however, are now not required as long as the dose range studied includes doses that cause severe, life-threatening toxicity. Thus, the starting dose is generally now chosen as one-tenth of the dose that causes severe toxicity (or death) in 10% of the rodents (STD$_{10}$) on a milligrams per meter squared basis, provided that this starting dose, i.e. one-tenth the STD$_{10}$, does not cause serious irreversible toxicity in a nonrodent species (15). If irreversible toxicities are produced at the proposed starting dose in nonrodents (usually dogs) or if the non-rodent is known to be the more appropriate animal model, then the starting dose would generally be one sixth of the highest dose tested in nonrodents that does not cause severe, irreversible toxicity.
Phase I studies

There are two goals in phase I cancer trials: precise definition of an optimal recommended phase II dose (RPTD) and safe treatment of the individual patient at doses that are close to therapeutic levels. These studies also explore pharmacological parameters, pharmacokinetic parameters and the toxicological profile of the drug (16).

Anticancer agents selected for further testing in man must have shown evidence of activity against tumours in one or more animal models. However, the predictive value of these preclinical tests are very uncertain (13). Drugs which do not have activity in such models are not tested in man; thereby making the true negative rate unknown. In general, only one of every ten anticancer compounds who have entered in clinical trials will have significant activity in man (16). Phase I trials are therefore designed to expose as few patient as possible to ineffective agents while at the same time gathering the data required to determine optimal drug dosing and efficacy for phase II studies.

Phase I studies of cancer drugs are carried out in patients with malignant disease which have proved unresponsive to treatment with established treatment schedules or for which there are no accepted therapies. Patients with a variety of malignancies may be entered in phase I trials, mostly solid tumours. Most patients entered in phase I trials have had several courses of prior therapies, often including surgery and/or radiotherapy. This particular patient population might suffer from progressive disease burden during the trial, and sometimes die before the trial has been completed.

The primary goal of phase I studies is to determine the appropriate dose for phase II evaluation. In the case of cytotoxic agents, an assumption is made that the higher the dose, the greater the likelihood of efficacy. Because most cytotoxic agents exhibit a dose-toxicity relationship, dose-related toxicity is regarded, in general, as a surrogate for efficacy: the highest safe dose is assumed to be the one most likely to be efficacious. This view creates a situation where the achievement of significant, but reversible, toxicity is desirable. Those toxic effects that by nature of their severity limit further dose escalation (dose-limiting toxicity; DLT) are defined in advance in phase I trials, and the maximum-tolerated dose (MTD) is defined as that dose producing a certain frequency of DLT within the treated patient population. At the same time, the investigators conducting these trials have a responsibility to limit the risk of individual patients to unacceptable levels of toxicity. Historically this has been accomplished using a "conventional" phase I trial design conducted by selecting a safe starting dose of one tenth of the mouse equivalent to LD10 (MELD10) or lower, accruing patients in cohorts of three, and escalating the dose according to a modified Fibonacci sequence with decreasing increment schemes (eg, dose increases of 100%, 65%, 50%, 40%,
and 30% to 35% thereafter) (17). The dose escalation is continued in cohorts of three patients until the MTD is reached. The next lower dose level is the recommended phase II dose (RPTD). The major problems raised with respect to the "standard" phase I approach described above have been the following:

- **Ethical**: With three patients entered per dose level, substantial numbers of patients are treated at doses that are retrospectively predicted to be nontherapeutic.
- **Efficiency**: The Fibonacci escalation scheme may result in fairly extensive trials in which dozens of patients and many months are required to determine the phase II dose.

To address these shortcomings new designs have been proposed (18). These designs have evaluated higher starting doses, fewer patients per dose level and more rapid dose escalations. The ultimate goal of these efforts is to construct a design which shorten the duration of phase I trials and limit the number of patients who are treated at very low, subtherapeutic, doses. One design that meet these criterias was proposed by Simons et al (19). This design is based on the concept of two stages; one stage with an accelerated dose titration until the first instance of a grade two CTC, and a second stage where dose titration reverts to a classic Fibonacci scheme.

**Phase II studies**

The primary objective of a phase II cancer clinical trial of a new drug or regimen is to determine whether it has sufficient biological activity against the disease under study to justify more extensive development. Some phase II studies evaluate combinations of agents, although most consider single agent therapy. Treatments that are acceptably promising go through further evaluation in either follow-up phase II studies or large-scale phase III trials. Viewing phase II studies as means of screening new agents helps motivate the choice of short-term clinical endpoints for evaluating efficacy. While the final goal of cancer chemotherapy is cure, few cytotoxic drugs have had an obvious and dramatic impact on survival in phase II studies which are most often designed to detect tumour shrinkage and not improved survival. Trials designed to detect increased survival require large numbers of patients, prolonged follow-up, and are carried out in phase III. It is generally accepted that tumour shrinkage is required for improved survival, and that the higher the response rate, the more likely it is that these responses will result in prolonged life. Therefore, response is used in phase II as a surrogate endpoint. Even if not associated with improved survival, reducing tumour burden can often relieve symptoms and improve quality of life (20).

In designing phase II studies, it is desirable to achieve the goals of the study with a minimal number of patients, especially if the treatment turns out
to be inefficacious. To achieve this goal, multistage sequential designs in which a fixed number of patients are accrued in each stage have been proposed, and the study is stopped or continued depending on the observed number of treatment successes and failures (21, 22). In the 1960s Gehan (23) developed one of the best-known statistical designs based on the concept of two stages. This design was established in order to reject a drug candidate if no responses were observed in a first cohort of 14 patients. The study stops if no responders are observed, or proceeds to stage 2 to accrue another cohort of patients if one or more responders are observed. Fleming (24) developed, in 1982, a more efficient analytical designs allowing for the trials to be ended prematurely in case of sufficient efficacy in terms of response. Since then, there has been an increase in the number of articles devoted to the development of statistical methods in this field (25-28). Each of these recent methods allows the establishment of designs that ensure an ethical dimension by minimising the number of subjects exposed to an insufficiently active drug.

Phase III studies

Phase III studies are performed after preliminary evidence suggesting effectiveness of the drug has been obtained in Phase II, and are intended to gather the additional information about effectiveness and safety that is needed to evaluate the overall benefit-risk relationship of the drug. Phase III studies also provide an adequate basis for extrapolating the results to the general population and transmitting that information in the physician labeling. For most oncology studies this will be the first trial in which the patients are randomised between treatment with the experimental drug and standard therapy, usually the best standard treatment. Occasionally oncology compounds may be compared to the best supportive care. Phase III studies usually include several hundred to several thousand people.

Ethical aspects of oncology drug development

Drug development programs differ because the risk-benefit ratio for a drug depends on the target disease and the patient population. Cancer is an aggressive and potentially fatal disease for which the patients receive therapy conventionally administered at near maximally tolerated doses. The life-threatening nature of advanced cancer allows the acceptance of considerably more risk than would be accepted in other circumstances. Even within oncology drug development, differences exist for drugs being developed for chemoprevention compared with drugs being developed for the treatment of refractory disease because both the potential benefit of therapy and the acceptability of risk vary in different cancer settings. Oncology chemo preven-
tive drug development more closely resembles that of drug development for non-life-threatening disease (16).

Anticancer drugs are not tested in healthy volunteers because of their small therapeutic index, serious side effects, and significant potential for damage to DNA. Although some biological agents might be considered safe enough to test in healthy volunteers, unknown risks make such testing rare.

Regardless of the serious toxicities of many anticancer drugs, careful dosing, frequent clinical monitoring and rapid treatment of toxicity should make the side effects less threatening to a patient than their disease.

Myeloma

Multiple myeloma (MM) is a haematopoietic malignancy characterized by a clonal expansion of malignant plasma cells located in the bone marrow. MM accounts for 10% of all malignant haematological neoplasms (29). MM has a high initial response to chemotherapeutics but virtually no cures due to inherent or acquired drug resistance. The overall response rate with standard melphalan and prednisone therapy is approximately 50%, with a CR rate of less than 10%, and a median survival of about 3 years (30). The 5-year survival rate in patients treated with standard therapy for MM is only 24% (30). More recently, new drugs, such as thalidomide and the proteasome inhibitor bortezomib, have shown promising results. However, none of these drugs, alone or in combination with glucocorticoids, have yet been shown to prolong survival in myeloma patients. High-dose therapy supported by autologous bone marrow transplantation represents a significant improvement over conventional therapy and may be the treatment of choice for good prognostic patients up to 65 years old (31). However, for many patients conventional chemotherapy continues to be the only possibility. In this perspective, research on potential new therapeutic agents for MM is urgently needed.

B-cell Chronic Lymphocytic Leukaemia

B-cell chronic lymphocytic leukaemia (B-CLL) is a clonal disorder of morphologically mature but immunologically less mature lymphocytes that express high levels of the BCL-2 protein (32, 33). The disease is characterised by progressive accumulation of these cells in the blood, bone marrow, and lymphatic tissue (34). The overall 5-year survival of B-CLL patients approximates 60%, but large differences between patients exist depending on stage of disease. B-CLL is the most common adult leukaemia in Western society, yet the aetiology remains unknown. At time of diagnosis most patients are over 60 years of age, and 90% are over age 50. The prevalence of B-CLL is 2:1 male to female, and the annual incidence approximates 3.9 and
2.0 per 100,000 males and females, respectively. With the possible exception of allogeneic stem cell transplantation, no curative treatment exists. As instituting therapy in B-CLL patients with indolent disease has not proved to prolong survival (35), therapy is normally withheld until the disease has become active or is progressing. Chlorambucil (with or without prednisone) has been the most frequently used first-line drug in the treatment of B-CLL for several decades (36). Fludarabine has proved to be an effective treatment with approximately 57% partial remissions in previously treated B-CLL patients (37). In the last decades important advances has been made in the treatment of B-CLL with development of new cytotoxic drugs (mainly fludarabine) and monoclonal antibodies (i.e. rituximab and alemtuzumab). Although these new regimens have resulted in higher response rate and longer duration of remission, no effect of the overall survival of patients with B-CLL has so far been observed (38). All this considered, clinical trials with novel therapies in B-CLL are warranted.
NF-κB inhibition – a new target in cancer drug therapy

Although chemotherapeutic agents have been successfully used in treating patients with many different types of cancer, acquisition of resistance to the cytotoxic effects of chemotherapy has emerged as a significant impediment to effective cancer treatment. One feature that cytotoxic treatments of cancer have in common is their activation of the transcription factor nuclear factor-κB (NF-κB), which regulates cell survival. NF-κB activation suppresses the apoptotic potential of chemotherapeutic agents and contributes to resistance (39). Cytotoxic stimuli activate a signal transduction pathway that induces the translocation of NF-κB to the nucleus (figure 1). The process triggers the nuclear translocation of NF-κB via activation of the inhibitor-of-NF-κB (IκB) kinase complex (IKK). IKK phosphorylates IκB bound to NF-κB. This phosphorylation is the signal for ubiquitination of IκB (i.e. the attachment of a chain of the protein ubiquitin) by a ubiquitin ligase. This marks IκB for degradation by the proteasome, which then results in the release of NF-κB. The transcription factor is now free to become translocated to the nucleus where it binds to specific DNA elements and activates the transcription of NF-κB-dependent genes (40). In vivo models of ovarian cancer, colorectal cancer and pancreatic cancer have shown that NF-κB inhibition increases the efficacy of anticancer drugs (41-44). It is thought that NF-κB inhibition prevents tumours from becoming resistant to chemotherapeutic agents. Therefore, development of NF-κB inhibitors could increase the efficacy of many anticancer drugs. Several approaches to inhibit NF-κB activation are now being explored and tested (39). Examples of these are:

- proteasome inhibitors
- inhibitors of IκBα phosphorylation
- inhibitors of TNFα
- IKK inhibitors

CHS 828 has been shown to suppress NF-κB activity in cancer cells through down regulation of IKK activity (5)
Anticancer drugs induce nuclear translocation of NF-κB and activation of NF-κB target genes through the direct activation of the NF-κB pathway and/or the secondary production of NF-κB activators such as reactive oxygen species (ROS), interleukin-1 (IL-1) and tumour-necrosis factor-α (TNFα). All of these effects can lead to enhanced resistance to anti-cancer drugs. By inhibiting different cellular targets, such as IKK, ULS and the proteasome, this biological cascade can be stopped and the resistance reversed. (MEKK, MAPK/ERK kinase kinase; ULS, ubiquitin ligase system; P, phosphor; U, ubiquitin.)
The general aim of this doctoral project was to investigate the cytotoxic activity of CHS 828 in cell-lines and in primary human tumour samples to find a target phase II diagnosis. Using this information the aim was to establish proof of concept in a phase II study for further testing in larger patient populations.

The specific aims were to:

I To use \textit{in vitro} methods and the \textit{in vivo} hollow fibre model in rats to assess CHS 828 activity in a panel of human myeloma cell-lines.

II To use primary human tumour samples from patients to investigate tumour type disease specific activity of CHS 828 for choice of phase II diagnose.

III To establish a recommended phase two dose (RPTD) for CHS 828 in a phase I trial.

IV To investigate the clinical efficacy of CHS 828 in chronic lymphocytic leukaemia.
Materials and methods

Reagents and drugs

Fluorescein diacetate (FDA; Sigma) was dissolved in dimethylsulfoxide (DMSO) and kept frozen (-20o C) as a stock solution (10 mg/ml) protected from light. CHS 828 was obtained from LEO Pharma, Ballerup, Denmark, dissolved in DMSO 10mM. Vincristine, melphalan, doxorubicin, cisplatin, cladribine, cytarabine, etoposide and vinorelbine were obtained from commercial sources.

For the **in vitro** experiments CHS 828 was tested at six different concentrations (1x10^{-4} - 10^3 μM), whereas the drugs for comparison in paper I were tested at three concentrations (doxorubicin at 0.046, 0.46, 4.6 μM, vincristine at 0.006, 0.06, 0.6 μM and melphalan at 0.33, 3.3, 33 μM). In paper II the drugs for comparison were tested at empirically derived cut-off concentrations (EDCC) as previously described (45, 46). V-shaped 96 well experimental microtiter plates were prepared with 20 µl/well of drug solution in triplicates at 10 x the desired final concentration with the aid of a programmable pipetting robot.

For the **in vivo** experiments CHS 828 was formulated as a 10 mg/ml suspension with 2 % methylcellulose in Millipore water. The suspension was ultrasonicated for 30–60 minutes and kept refrigerated in a dark glass bottle for a maximum of one week. Before each administration, the suspension was mixed thoroughly with a magnetic stirrer. A vehicle formulation was made in the same way but without drug.

For the phase I trial CHS 828 was manufactured by Nova Laboratories Ltd and was certified and supplied by Leo Pharmaceutical Products, Denmark. The dosage form was 10 mg gelatin capsules for oral administration.
Human myeloma cell lines


All MM cell lines were maintained in RPMI 1640 culture medium containing 10% fetal bovine serum, glutamine and antibiotics. The U-1958, U-1996 and U-266-1970 cell lines grew partly adherent and partly in suspension. These cell lines were dependent of exogenous IL-6 for survival and/or growth (47) and were routinely grown on a layer of IL-6 producing human AG1523 fibroblasts. The IL-6 independent cell lines (RPMI 8226, LP-1, EJM, Karpas 707 and U-266-1984) all grew in suspension. Medium was replenished twice a week. The RPMI 8226/Dox40 cells were treated once a month with 0.24 $\mu$g/ml of doxorubicin and the RPMI 8226/LR5 cells at each change of medium with 1.53 $\mu$g/ml of melphalan.

Tumour samples

Tumour samples from cancer patients were obtained from surgery, diagnostic biopsy or bone marrow/peripheral blood sampling. A total of 156 consecutive successfully analyzed tumour cell samples were obtained from patients (78 males and 78 females) with different haematological and solid tumours. The research Ethics Committee at Uppsala University Hospital approved the sampling. Normal peripheral blood mononuclear cells (predominantly lymphocytes) from eight healthy donors, were also used in this investigation.

Mononuclear cells from bone marrow or peripheral blood were isolated by density gradient centrifugation (46). Solid tumour tissue was minced to a size of 1 mm$^3$ and the cells were then isolated by collagenase dispersion and density gradient centrifugation (45). Viability was determined by the trypan blue exclusion test and the proportion of tumour cells was judged by inspection of May-Grünwald-Giemsa stained cytocentrifugate preparations on day 0 and day 3. All experiments were performed in culture medium, RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 $\mu$g/ml streptomycin and 100 $\mu$g/ml penicillin. Cells were cryopreserved in culture medium containing 10 % DMSO and 90 % fetal calf serum and stored at -150°C. Before use, these cells were thawed and washed twice with culture medium.
The fluorometric microculture cytotoxicity assay

The fluorometric microculture cytotoxicity assay (FMCA) is based on the measurement of fluorescence generated from hydrolysis of FDA to fluorescein by cells with intact plasma membranes (46). Briefly, day one 180 µl of the tumour cell preparation was seeded into 96-well microtitre plates (5-100 x 10^3 cells for patient cells, 20 x 10^3 cells for cell lines). Six blank wells received culture medium only and six wells with cells but without drugs served as control. The culture plates were then incubated at 37° C in humidified atmosphere containing 95% air and 5% CO2. After 72 h of incubation, the plates were centrifuged (200 g, 5 min) and the medium was removed. After one wash with phosphate buffered saline (PBS), 100 µl of hepes buffered saline containing FDA (10 µg/ml) was added to control, experimental and blank wells. Subsequently the plates were incubated for 40 min before reading the fluorescence in a Fluoroscan II. Quality criteria for a successful assay included a fluorescence signal in control cultures of > 5 x mean blank values and a mean coefficient of variation (CV) in control cultures of < 30%. Each cell line was analyzed 3 – 8 times. The results obtained are presented as a survival index (SI) defined as fluorescence in test wells in per cent of that in control cultures, with blank values subtracted. Low numerical values indicate a high cytotoxic effect.

In study II activity of CHS 828 was expressed as IC30 (concentration reducing survival index to 70 %) and IC50 (concentration reducing survival index to 50 %). The values were calculated from the mean concentration-response curve in each diagnosis. From these curves the maximum effect (lowest survival = SImin) was also estimated. To mirror the relative activity in solid tumours and haematological malignancies, an S/H ratio was introduced. This was defined as the ratio between the fraction of responders (showing a SI<50%) among the solid tumour samples and the corresponding fraction among the haematological samples. Thus, high and low ratios indicate relatively high activity in solid tumours and haematological malignancies, respectively.

Hollow fiber method

The hollow fiber method is an in vivo-system where tumour cells are cultured in semipermeable hollow fibers, which are implanted into rats. The hollow fiber model was modified from Hollingshead and co-workers (12). Our model used immunocompetent rats where the fibers were implanted subcutaneously only (48).

Male Sprague Dawly rats obtained from Charles River, Uppsala, Sweden were used in paper I. They were acclimatized for at least one week before randomization and start of the experiments. The animals had free access to
food and water and were kept in a room lighted up for 12h (7 a.m. to 7 p.m.) throughout the study. Anaesthesia was induced by inhalation of anaesthetic gas. The study was approved by the Animal Ethics Committee in Uppsala University and was in accordance with the Declaration of Helsinki.

Cell suspensions were prepared from cell lines RPMI 8226, RPMI 8226/Dox40 and RPMI 8226/LR5 cells (1x10^6 cells/ml) and were flushed into polyvinylidene fluoride (PVDF) hollow fibers (500 kDa molecular weight cut-off, 1 mm inner diameter; Spectrum, Laguna Hills, CA). The fibres were transferred to petri dishes with complete RPMI medium and incubated for 48 h prior to implantation in rats. Anaesthesia was induced in male Sprangue Dawley rats (284 ±10 g) by inhalation of anaesthetic gas. The fibres were inserted subcutaneously between two skin incisions on the back (48). Two separate experiments were carried out with a total of 12 rats (6 + 6). Each rat had eight fibres implanted, four each containing RPMI 8226 and/or RPMI 8226/Dox40 and/or RPMI 8226/LR5 cells. In each experiment the animals were randomly allocated in two groups, three animals in each group for both experiments. One group received vehicle and one group received CHS 828. The day after surgery and the four subsequent days, the rats received once daily 75 mg/kg CHS 828 or the corresponding volume of vehicle by oral gavages. The day after the last dose the fibres were retrieved and put in 6-well plates filled with culture medium and kept at 37°C until cell staining.

The cell suspension from the fibres from one treated and one untreated rat was also used to examine morphological changes of the tumour cells. Cyto- spin slides were May-Grünwald-Giemsa stained according to standard procedures.

In the animal model, living cell density was determined for triplicate fibre samples from in vitro incubation on filling day, implantation day and day of retrieval to assess the growth of the cells. The cell density was evaluated by staining with MTT ([3-4,5-dimethylthiaxol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO). Data from both experiments were pooled. The extreme studentized deviate (ESD) or Grubbs' test were used to identify outliers, 3 of 78 values were omitted (49).

Phase I methodology

The main objective of this trial was to establish a Recommended Phase Two Dose (RPTD) for CHS 828 employing once daily dosing for five consecutive days in cycles of 28 days duration. The study was an open label, one armed, phase I safety study, involving dose escalation until MTD was reached.

In order to minimize the number of patients treated at inactive concentrations and increase the probability for the individual patient to receive bioac-
tive concentrations, a design with an initial accelerated titration stage was employed (19) (table 1) followed by a modified Fibonacci procedure. The study was approved by the Medical Product Agency in Sweden and by the Uppsala University ethics committee.

Table 1.

<table>
<thead>
<tr>
<th>Accelerated phase</th>
<th>Switch</th>
<th>Standard design stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohorts of 1 new subject</td>
<td>Second instance of grade 2 toxicity or first</td>
<td>Cohorts of 3-6 subjects per dose level</td>
</tr>
<tr>
<td>per dose level</td>
<td>instance of DLT at any course</td>
<td>Dose increments of 30-40 %</td>
</tr>
<tr>
<td>Dose increments of 100 %</td>
<td></td>
<td>No intra-patient dose escalation</td>
</tr>
<tr>
<td>Intrapatient dose escalation</td>
<td></td>
<td>Two cycles per treatment</td>
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</table>

Except for the first dose level where patients received 10 mg for three consecutive days, each patient was allocated a dose of CHS 828, which was given orally every 24 hours for the first five days of each 28-day cycle. During the standard stage of the study each patient received two cycles of treatment on the allocated dose level. Dose escalation steps were 30-40% and no intra-individual dose escalation was permitted. Cohorts of three to six patients were entered on each dose level until the MTD was reached.

Phase II methodology

The primary objective of this study was to determine the frequency of responses, partial or complete, as measured by the National Cancer Institute Response Criteria in Chronic Lymphocytic Leukaemia (50). The study was designed as a multi centre, open label, non-comparative study using Ensign’s three stage design for phase II oncology trials (25).

The starting dose, as established in the phase I study, was 20 mg once daily for five days. All patients who received treatment were to be considered with regard to efficacy. New patients was to be included according to the response data obtained after three treatment cycles on the first 9 evaluable patients. The study was to be stopped if there was no response (complete or partial) among the first 9 evaluable patients (i.e. patients having completed all three cycles of treatment at 20 mg once daily for five days or higher dosages). If there were one or more responses among the first 9 patients, the study was to proceed to stage two. If less than five responses were reported among the first 23 evaluable patients then the study was to be stopped at stage two, otherwise the study was to proceed to stage three. A maximum of 44 evaluable patients were to be included in the study.
Phase I and II patients

In paper III patients were required to have a histologically proven solid tumour malignancy for which no satisfactory therapy was available or had failed.

In paper IV patients were required to have a diagnosis of B-CLL according to NCI criteria and treated with \( \geq 1 \) chemotherapy regimen. The patients were also required to be in need for chemotherapy, and not candidate for chlorambucil.

Upon study entry, patients were informed of the investigational nature of the treatment and potential side effects. All patients were required to give written informed consent to participate in the trials. The studies were carried out according to the ICH guidelines for GCP. The studies were approved by the Medical Product Agency in Sweden and by the Uppsala University ethics committee.
Results and discussion

Cytotoxic effect *in vivo* and *in vitro* of CHS 828 on human myeloma cell lines (paper I)

This study was undertaken to investigate the effect of CHS 828 and standard drugs for multiple myeloma (MM) on human myeloma cell lines, both *in vitro* and *in vivo*.

The study showed that CHS 828 had an antiproliferative or cytotoxic effect on all 10 human myeloma cell lines but with different IC$_{50}$s (figure 2) and $E_{\text{max}}$ values. All concentration-response curves were plateau-shaped, which is typical for CHS 828 (2). The maximum effect varied between an almost complete cell kill for some cell lines to less than 50% for others.

The three most resistant cell lines in terms of IC$_{50}$ (U-266-1970, EJM and U-266-1984) all lack expression of CD56, an adhesion molecule that might be down regulated during tumour progression (51). Furthermore, both EJM and U-266-1984 have mutations in the p53 gene, which can result in genomic instability and drug resistance (52). Hypothetically, these two factors may contribute to CHS 828 resistance, but this has to be investigated further.

The cytotoxic effect of CHS 828 in the myeloma cell line panel correlated poorly to moderately with the effect of standard drugs used in MM, which suggests a different mechanism of action. This result is in accordance with studies in another cell line panel where the effect of established drugs correlated poorly with the effect of CHS 828 (2). Drugs with new mechanisms of action are attractive as complements to the standard agents used today.

To investigate three of the cell lines in an *in vivo* system we used the hollow fiber model. Although this model has uncertain predictive value for clinical tumour-type specific activity (53) the hollow fibre model provides quantitative indices of drug efficacy with a minimum expenditures of time and material. The outcome of this experiment showed a high *in vivo* activity of CHS 828 on two cell lines (figure 3). However, one should be careful to extrapolate these animal data to man since there seems to be a species difference in tolerance to CHS 828 (54).
Figure 2. Activity of CHS 828 expressed as IC₅₀ (concentration reducing SI to 50 % of the maximal effect). A low IC₅₀ suggests high drug potency.

Figure 3. Effect of CHS 828 on three different cell lines in the *in vivo* hollow fibre model. Data from two experiments were pooled and presented together as percent net growth ± SEM (n = 11-14). Positive net growth indicated a growth of the cells in the fibre during the week of the experiment, negative net growth indicates a cell kill.
Overall, the results showed that CHS 828 had an antiproliferative or cytotoxic effect on all 10 human myeloma cell lines using the FMCA and in three cell lines using the in vivo hollow fibre model. Thus, MM could be regarded as a potential target diagnosis for CHS 828.

In should be emphasised that, in general, established human tumor cell lines do not predict well for tumor type specific activity probably due to the gradual in vitro growth-dependent loss of phenotypic characteristics of the tissue of origin (13). For prediction of tumor-type specific activity the use of fresh primary cultures of human tumor cells from patients may be a better model (10). However, the present myeloma cell line panel may be a reasonably valid alternative model system since myeloma specific characteristics are well retained and the panel includes different stages of myeloma tumor progression (55).

Activity of CHS 828 in primary cultures of human hematological and solid tumours in vitro (paper II)

This study was undertaken to study the in vitro relative activity of CHS 828 in different malignant diagnosis to detect disease-specific activity. Correlation analysis between standard drugs and CHS 828 in terms of activity was also made. Moreover, cytotoxic assays may be used for preclinical evaluation of dose-limiting toxicities by comparing tumour cell response with those of normal cells (56). For this purpose we used normal lymphocytes from healthy donors.

A total of 156 samples were successfully analyzed. The activity of CHS 828 was high in all haematological malignancies, in particular in samples from patients with chronic lymphocytic leukaemia (CLL) and NHL, where most samples showed a 50 % or greater decrease in SI (figure 4). A concentration-dependent decrease in SI followed by a plateau could be discerned for all the haematological tumour types and for normal lymphocytes whereas solid tumours as a group appeared less responsive. CLL was most sensitive in terms of IC50 and SImin followed by high-grade non-Hodgkin's lymphoma (NHL), acute myelocytic leukaemia (AML), acute lymphocytic leukaemia (ALL) and normal lymphocytes. SI values for CHS 828 correlated weakly to moderately with the standard drugs.
Exposing normal lymphocytes to CHS 828 showed that CLL cells are more sensitive; CLL cells showed 10 times higher sensitivity in terms of IC₅₀ and 100 times higher sensitivity in terms of IC₃₀. This result indicates that there could be a therapeutic window for CHS 828 in CLL.

It should be emphasised that indication of activity in vitro in a specific tumour type is not a guaranteed prediction of clinical efficacy in the same tumour type. In vivo induction of tumour cells death by a drug is a complex phenomenon, depending not only on the attained serum drug concentration, but also i.e. on the distribution of the drug to the tumour cell, heterogenicity and cell re-growth rate. Still, the FMCA assay used in our laboratory has been evaluated with regards to tumour type specific activity with promising results (10, 11). The results from this study were used in the development of the clinical phase II trial protocol where CLL was used as the disease inclusion criteria.
Phase I study of CHS 828 in patients with solid tumour malignancy (paper III).

Based on the preclinical observations of the cytotoxic effect of CHS 828 we decided to perform a phase I study with the main objective to confirm a recommended phase II dose (RPTD) of CHS 828. Secondary objectives included characterisation of drug related toxicities, pharmacokinetics and to assess tumour response.

The study included 16 patients (6 males and 10 females), mean age was 58 years and all patients had received previous treatment with chemotherapy and/or radiotherapy and/or surgery and/or hormonal therapy. The total numbers of evaluable cycles of CHS 828 were 49.

The RPTD of CHS 828 in this study was determined to be 20 mg/day for five days in cycles of 28 days duration. The main DLTs when employing the present dosing schedule seemed to be of gastrointestinal origin. Diarrhoea, nausea and vomiting were frequently observed and there was a tendency towards an increase in incidence with higher doses.

Localized mucositis of the genital tract, generally presented as a reddish erosion of the mucosa surrounding the urethral orifice, was a relatively frequent adverse reaction. The mechanism behind this atypical reaction is not clear but may involve high local concentrations of active drug or metabolite from residual urine around the orifice. This problem may be at least partly avoided by simply instructing the patients to carefully clean the urethral orifice after passing urine.

There was a large variation in pharmacokinetics of CHS 828 both between and within patients. The large inter-individual variation may in part be explained by the fact that CHS 828 has been shown to be a substrate for CYP 3A4, a drug metabolizing enzyme known to produce large variation in systemic exposure of many drugs. The intra-individual variation, on the other hand, may reflect the impact of local factors in the gastrointestinal tract such as unpredictable fluctuations in gastric pH and/or inherent properties of the present drug formulation. There is an uncertainty to the attained RPTD in view of the large intra-individual variation in systemic exposure.

There was an apparent inverse relationship between AUC and platelet nadir. If this observation is confirmed, this relationship may form the basis for pharmacologically guided dosing strategies of CHS 828. Furthermore, these results also demonstrate that the attained RPTD in the present study was able to produce detectable systemic biological effects in all patients receiving this dose.

The present study was performed using an accelerated design combined with a modified Fibonacci titration. The intention was to make the study less time- and resource consuming, and to increase the chance for a clinical benefit for the first patients in the study. However, due to encountered toxicity at relative low dose levels, only six patients were included in the accelerated
phase and only four patients was individually dose-escalated. Thus, the benefit of the novel study design applied in this study was lower than expected. No objective tumour responses were observed.

A phase II study of the IkB kinase inhibitor CHS 828 in patients with chronic lymphocytic leukemia (paper IV)

The preclinical observations of CHS 828 induced lymphocytopenia, in conjunction with the finding in the earlier phase I trial that dosing of CHS 828 induced a decreased lymphocyte count in solid tumour patients, suggested that CLL might be a well-suited disease for CHS 828. The primary objective of this study was thus to determine the frequency of responses, partial or complete, as measured by the National Cancer Institute Response Criteria in Chronic Lymphocytic Leukaemia.

The study included 12 patients (8 males and 4 females) in three centers. Mean age was 62 years. The total numbers of evaluable cycles of CHS 828 were 31. No objective tumour response was seen according to NCI Response Criteria. Due to this fact, the study was stopped at the first stage. 6 patients had stable disease and 3 patients had progressive disease.

The haematological toxicity seen in this study was thrombocytopenia, anaemia and neutropenia, which is in accordance with the findings in the phase I trial. These toxicities, however, seemed to be severer than those observed in the phase I trial. Seven of nine thrombocytopenias had CTC-grade 3-4 and resulted in dose adjustment. Seven patients received platelet transfusion. One case of neutropenia had CTC-grade 4. The increased intensity of haematological toxicity seen in the present study may be associated with the primary disease (i.e., bone marrow infiltration). Furthermore, the incidence of thrombocytopenia appeared to increase over the duration of treatment and total dose of CHS 828 administered. The most frequent non-haematological toxicity encountered was gastrointestinal disorders with manifestations of diarrhoea, nausea, abdominal pain, constipation and vomiting. The gastrointestinal disorders were general mild. Apart from one case of diarrhoea CTC-grade 3, the majority of the events had CTC-grade 1-2, and thus, did not cause dose adjustment. This is in contrast to the findings of the phase I trial, where the gastrointestinal toxicity appeared to be the major dose limiting toxicity for CHS 828.

Some patients responded with decreased lymphocyte counts during CHS 828 dosing within individual courses, exemplified by patient 3 (figure 5), but the overall treatment effect of CHS 828 did not show a decline in total lymphocyte count in CLL patients.

A slight decrease in the size of spleen and lymph nodes size was noticed after the treatment with CHS 828, which may indicate a biological activity of
CHS 828. This biological activity, however, did not translate into a clinical benefit. The lack of efficacy of CHS 828 in the CLL trial might be that the patients had been heavily treated by other chemotherapies and therefore less sensitive to a new cytotoxic agent like CHS 828.

The major mechanism of action for CHS 828 is probably inhibition of I kappa B kinase (IKK) leading to reduced NF-kappa B mediated transcription (5). The lack of efficacy as a single drug is not completely surprising since NF-kappa B inhibition is known to modulate the balance between cell death and survival signals induced by other drugs rather than induction of apoptosis by itself (39). Indeed, CHS 828 has shown promising activity in combination with other chemotherapeutic drugs (57, 58) and future studies should investigate the potential role of CHS 828 in combination therapy.

Figure 5. Individual plot of patient number 3, lymphocyte count (x10⁹/L)
Summary and Conclusions

Referring to the aims (page 19) the results may be summarised as:

I CHS 828 had an antiproliferative or cytotoxic effect on 10 human myeloma cell lines using the FMCA. In the in vivo hollow fibre method two out of three cell lines responded with a net cell kill. Thus, MM could be regarded as a potential target diagnosis for CHS 828.

II In primary human tumour samples CHS 828 had a high relative activity in haematological malignancies, especially CLL. CHS 828 appeared not to be cross-resistant with standard drugs.

III The phase I study enrolled a total of 16 patients. The RPTD of CHS 828 in the phase I study was determined to be 20 mg/day for five days in cycles of 28 days duration. The main dose limiting toxicities were of gastrointestinal origin.

IV The phase II study enrolled a total of 12 B-CLL patients. Transient decreases in lymphocyte counts could be discerned for some patients coinciding with drug dosing, but no sustained clinical responses were achieved. The study was halted after 9 evaluable patients due to lack of response. The most common haematological toxicity seen was thrombocytopenia. Non-haematological toxicities were generally mild and of gastrointestinal origin.

In conclusion, CHS 828 demonstrated marked effects in the preclinical investigations suggesting haematological malignancies as the main target. The clinical phase I study established a safe dose and the subsequent phase II trial in CLL patients showed biological effect but with no clinical disease response. Future studies should be directed towards combining CHS 828 with other apoptosis inducing agents, possibly also using other schedules/modes of administration.
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