Cytotoxic effect *in vivo* and *in vitro* of CHS 828 on human myeloma cell lines

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CHS 828 is a pyridyl cyanoguanidine with promising antitumor activity both *in vitro* and *in vivo*, and has previously been found especially active against tumor cells obtained from patients with B cell chronic lymphocytic leukemia. In the present study the cytotoxic effect *in vitro* of CHS 828 was investigated on a panel of 10 human myeloma cell lines using the fluorometric microculture cytotoxicity assay. CHS 828 induced a concentration-dependent, but variable decrease in tumor cell survival in the cell line panel with inhibitory concentrations 50% (IC_{50}) in the range 0.01–0.3 μM. These concentrations are below those achievable *in vivo*. There was no detectable dependence on P-glycoprotein-mediated or GSH-associated drug resistance and the drug showed low to moderate cross-resistance with standard drugs, including melphalan, vincristine and doxorubicin. Furthermore, sensitivity to CHS 828 showed no apparent relationship to growth factor dependence, tumor progression or phenotypic variability. CHS 828 was also tested *in vivo* using a hollow fiber model in rats with three of the cell lines. The results indicate a high cytotoxic activity of CHS 828. Overall, the results show a high cytotoxic activity of CHS 828 in the myeloma models, which might warrant its further development against myeloma.
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Introduction

Multiple myeloma (MM) is a hematopoietic malignancy characterized by a clonal expansion of malignant plasma cells located in the bone marrow. MM accounts for 10% of all malignant hematologic neoplasms [1]. 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% [2]. New drug combinations has not proved superior to this regimen [3]. With this regimen, the complete response rate is less than 10% and median survival is about 3 years [2]. The 5-year survival rate in patients treated with standard therapy for MM is 24% [2]. 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 [4]. However, for many patients conventional chemotherapy continues to be the only possibility. In this perspective, research on potential new cytotoxic drugs for MM is urgently needed.

In this study we have used the fluorometric microculture cytotoxicity assay (FMCA) in the *in vitro* evaluation of the candidate drug CHS 828 in myeloma cell lines followed by a hollow fiber *in vivo* model. The ability of the FMCA to detect disease-specific drug activity in primary cell cultures and provide important information on resistance mechanisms and cross-resistance patterns in a cell line panel has been indicated both with respect to standard and investigational anticancer agents [5–7]. NCI have since the 1990s used a hollow fiber method as a first *in vivo* assay before evaluating a new compound in traditional xenograft models [8]. We have established a similar hollow fiber model in immunocompetent rats, where both the drug activity and pharmacokinetics can be measured [9]. Although this model has uncertain predictive value for clinical tumor-type specific activity [10], the hollow fiber model provides quantitative indices of drug efficacy with a minimum expenditures of time and material.

CHS 828 [*N*-(6-chlorophenoxyhexyl)-*N''*-cyano-*N''*-4-pyridylguanidine] is a cyanoguanidine (with a molecular weight of 372 g/mol) which has shown interesting antitumor properties [11]. Using the FMCA, CHS 828 has shown a high *in vitro* activity against primary cell cultures from mainly hematological, but also against solid

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tumors [12]. *In vivo*, oral administration of CHS 828 has also shown significant antitumor activity in nude mouse models, especially in small cell lung cancer and breast cancer [13].

A phase I clinical trial of CHS 828 has recently been completed at the Department of Oncology, Uppsala University Hospital. The trial included 16 patients with solid tumors and the study was successful in establishing a recommended phase II dose [14]. An EORTC phase I trial has also been conducted and is currently being evaluated [15]. A phase II study in B cell chronic lymphocytic leukemia is ongoing.

In the present study the cytotoxic effect of CHS 828 and standard drugs for MM (melphalan, vincristine and doxorubicin) were investigated in a panel of 10 human myeloma cell lines using the FMCA and the hollow fiber *in vivo* model. The cell lines represent cells from MM patients both at diagnosis, treatment and relapse.

Materials and methods

Cell lines

The human myeloma cell lines (Table 1) RPMI 8226 and its doxorubicin-resistant 8226/Dox40 and melphalan-resistant 8226/LR5 sublines (kind gift from W. S. Dalton, Department of Medicine, Arizona Cancer Center, Tucson, AZ), U-266-1970, U-266-1984, U-1958, EJM, U-1996, LP-1 and Karpas 707 (kind gift from Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala, Sweden) were used, and their characteristics have been described elsewhere [16] and are summarized in Table 1

All MM cell lines were maintained in RPMI 1640 culture medium (Sigma, St Louis, MO) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), glutamine and antibiotics (100 IU/ml penicillin and 50 μg/ml streptomycin). 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 [17], and were routinely grown on a layer of IL-6-producing human AG1523 fibroblasts (The Human Mutant Genetic Cell Repository, Camden, NJ). 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 μg/ml of doxorubicin and the RPMI 8226/LR5 cells at each change of medium with 1.53 μg/ml of melphalan.

Reagents and drugs

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

For the *in vitro* experiments CHS 828 was tested at six different concentrations (1×10^{-4} to 10^{1} μM), whereas the drugs for comparison were tested at three concentrations (doxorubicin at 0.046, 0.46 and 4.6 μM, vincristine at 0.006, 0.06 and 0.6 μM, and melphalan at 0.33, 3.3 and 33 μM). V-shaped 96-well experimental microtiter plates (Nunc, Roskilde, Denmark) were prepared with 20 μl/well of drug solution in triplicates at 10 times the desired final concentration with the aid of a programmable pipetting robot (Propette; Perkin-Elmer, Norwalk, CT). The plates were stored frozen at –70°C until further use.

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 min and kept refrigerated in a dark glass bottle for a maximum of 1 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.

FMCA

The FMCA is based on the measurement of fluorescence generated from hydrolysis of FDA to fluorescein by cells with intact plasma membranes [18]. Briefly, on day 1, 180 μl of the tumor cell preparation was seeded into the wells (20 000 cells/well) of the microtiter plates, prepared as described above. 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% CO_2. 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 (Labsystems, Helsinki, Finland). Quality criteria for a successful assay included a fluorescence signal in control cultures of more than 5 times mean blank values and a mean coefficient of variation in control cultures of below 30%. Each cell line was analyzed 3–8 times.

Hollow fiber model

The hollow fiber model was modified from Hollingshead *et al.* [8]. Our model uses immunocompetent rats where the fibers are implanted s.c. only [19]. The study was approved by the Animal Ethics Committee of Uppsala University and is in accordance with the Helsinki Declaration of 1975. All animals received careful human care.

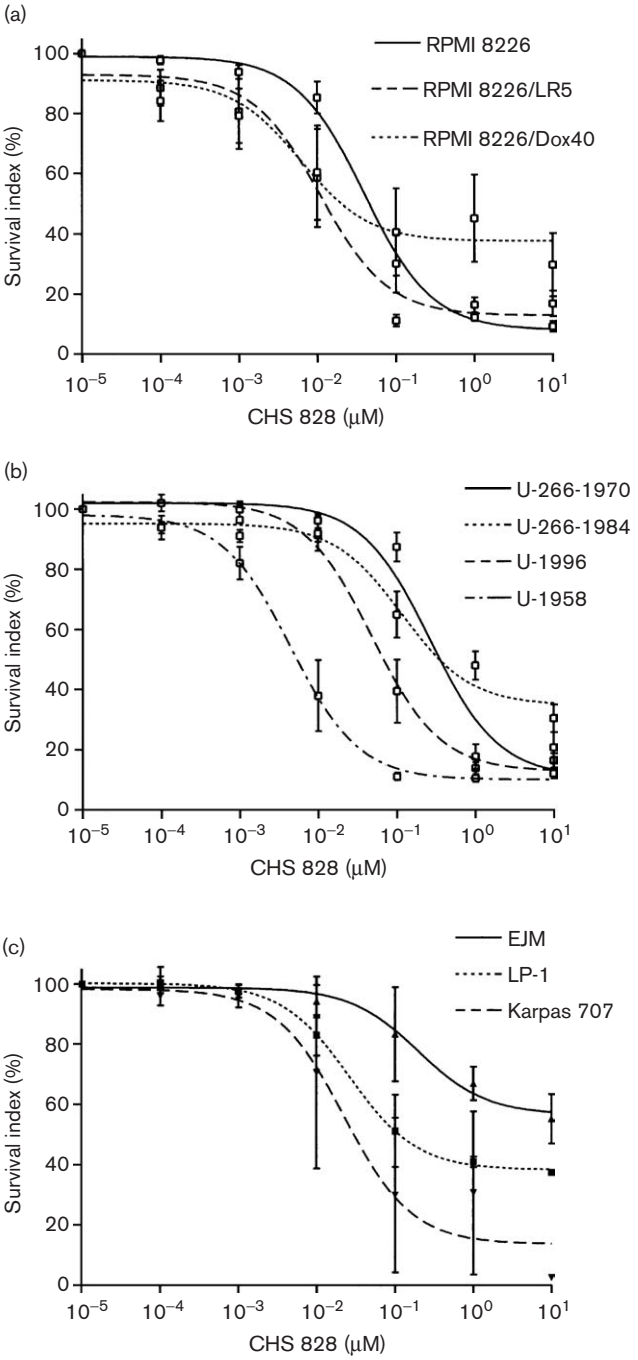
Cell suspensions were prepared from RPMI 8226, RPMI 8226/Dox40 and RPMI 8226/LR5 cells (1×10^{6} cells/ml),

Table 1. Characterization of the MM cell lines (from [16])

Cell line	Disease (status)[a]	Cell line characterization					
		Year of establishment	Overall profile[b]	Comments	Karotype (unique gene alterations)	Cytokine expression[c]	Cytokine response[d]
EJM	58 F, extramed, MM/PCL, IgGI, T, PE	1988	authentic CD56$^-$		48X, p53mut (ex5)	IGF$^+$, IGF-IR$^+$	IL-6$^+$, IGF-I (long R3)$^+$
Karpas 707	53 M, MM, IgGI, R, BM/PB	1981	authentic CD56$^+$		hypodiploid 45 Ph+	IL-6$^-$, IGF-I$^+$, IL-6R mRNA$^+$, IGF-IR$^+$	IL-6$^-$, IGF-I$^+$
LP-1	56 F, MM-PCL, IgGI, R, PB	1986	authentic CD56$^+$		73XX, altered expression of c-*myc* (RFLP[f]), t(4:14)	IL-6$^-$, IGF-I$^+$, IL-6R mRNA$^+$, IGF-IR$^+$	IL-6$^+$, IGF-I$^+$
U-1958	60 M, PCL, IgGκ, T, PE	1983	authentic	IL-6 dependent	46	IL-6$^-$, IL-6R mRNA$^+$	IL-6$^+$, IGF-I$^\pm$
U-1996	70 F, extramed, MM/PCL, Igκ, D, AF	1983	authentic		82	IL-6$^-$, IL-6R mRNA$^+$	IL-6$^+$
U-266-1970	53 M, MM/PCL, IgEI, T, PB	1968	authentic CD56$^-$	IL-6 dependent	43–44, 4-fold amplification of *bcl*-2	IL-6 mRNA$^+$, IL-6R$^+$, IGR-IR$^+$	IL-6$^+$, IGF-I$^-$
U-266-1984	53 M, MM/PCL, IgEI, T, PB	1968	authentic CD56$^-$	autocrine IL-6 loop	43–44, p53mut(ex5), translocation into IgH involving 11q13.3[g], biallelic loss of Rb1	IL-6$^+$, IL-6R$^+$, IGF-IR$^+$	IL-6$^-$, IGF-I$^-$
RPMI 8226	61 M, MM (BJP), IgGI, R, PB	1966	authentic CD56$^\pm$		58–67, p53mut(ex8), translocation into IgH (unidentified partner)[g], variant t(16:22)	IL-6$^-$, IL-6R$^+$	IL-6$^-$
Subline LR5 (melphalan resistant, GSH-associated MDR)							
Subline Dox40 (doxorubicin resistant, P-gp expressing)							

[a]Diagnosis (MM, multiple myeloma; PCL, plasma cell leukemia), sex (M, male; F, female), treatment (D, at diagnosis; T, during therapy; R, at relapse) and specimen site (BM, bone marrow; PB, peripheral blood; PE, pleural effusion; AF, ascitic fluid) as indicated in the original literature.
[b]Overall profile refers to the authentication of the MM cell line determined by the following criteria: EBV negativity, *Ig rearrangement, structural abnormalities or immunophenotype consistent with the MM profile CD19$^-$, CD20$^-$, CD138$^+$, CD28$^+$, CD11a$^-$, CD49e$^-$, CD56$^\pm$.
[c]Cytokine or cytokine receptor expression at mRNA (RT-PCR, Northern) or at protein level (binding assay or flow cytometry analysis): + = expression, − = no expression, ± = reported + or −.
[d]Effects of cytokines on proliferation or growth as measured by [^{3}H]thymidine incorporation or cell counting: + = induction, − = no effect.
[e]Expression lost with aggressive disseminating disease.
[f]Restriction fragment length polymorphism; mut, mutation; ampl, amplification.
[g]Illegitimate switch recombination fragments (ISRF) have frequently been demonstrated in MM and a translocation to the IgH locus is therefore considered a universal event.

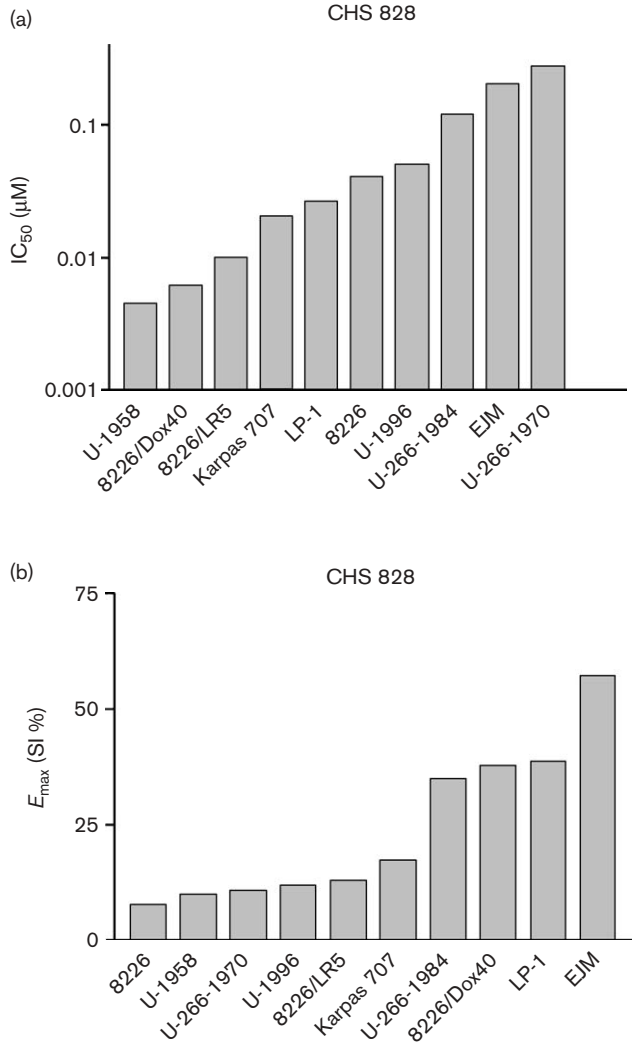
Fig. 1



Cytotoxic activity of increasing concentrations of CHS 828 in a panel of 10 myeloma cell lines. Activity is shown as SI. Curve fitting and parameter estimation were performed with a standard sigmoidal concentration–response model with variable slope. The results are presented as mean values ± SEM (n = 3–8).

and were flushed into polyvinylidene fluoride (PVDF) hollow fibers (500 kDa molecular weight cut-off, 1-mm inner diameter; Spectrum, Laguna Hills, CA). The fibers were transferred to Petri dishes with complete RPMI medium and incubated for 48 h prior to implantation in rats. Anesthesia was induced in male Sprague Dawley rats (284 ± 10 g; Charles River, Uppsala, Sweden) by inhalation of anesthetic gas. The fibers were inserted s.c. between two skin incisions on the back [19]. Two separate experiments were carried out with a total of 12 (6 + 6) rats. Each rat had eight fibers 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

Fig. 2



Activity of CHS 828 expressed as (a) IC_{50} (concentration reducing SI to 50% of the maximal effect) and (b) E_{max} (minimum cell survival). Parameters obtained of concentrations showing the largest distributions in SI values were used for this analysis, see Fig. 1. A low IC_{50} and a low E_{max} suggests high drug potency and a large drug effect at high drug concentrations, respectively.

animals in each group for both experiments. One group received vehicle and one group received CHS 828. The day after surgery and the 4 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 fibers were retrieved, put in six-well plates filled with culture medium and kept at 37°C until cell staining.

The cell suspension from the fibers from one treated and one untreated rat was also used to examine morphological changes of the tumor cells. Cytospin slides were May–Grünwald–Giemsa stained according to standard procedures.

Definitions and statistical analysis

The results obtained are presented as survival index (SI) defined as fluorescence of experimental in percent of control cultures with blank values subtracted. Activity of CHS was expressed as IC_{50}, i.e. concentration reducing SI to 50% of the maximal inhibitory effect (lowest cell survival; E_{max}). The values were calculated from the mean concentration–response curve for each cell line. Concentration–response curves were produced using non-linear regression to a sigmoid E_{max} model with variable slope (GraphPad Prism version 3.02 for Windows; GraphPad Software, San Diego, CA).

Pearson's correlation coefficient was used to compare the activity pattern of CHS 828 with that of the standard chemotherapeutic drugs. SI values obtained of concentrations showing the largest distributions in SI values were used for this analysis (CHS 828 0.1 μM, doxorubicin 0.46 μM, vincristine 0.06 μM and melphalan 33 μM).

In the animal model, living cell density was determined for triplicate fiber 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]. Data from both experiments were pooled. The extreme studentized deviate or Grubbs' test were used to identify outliers, three of 78 values were omitted [20].

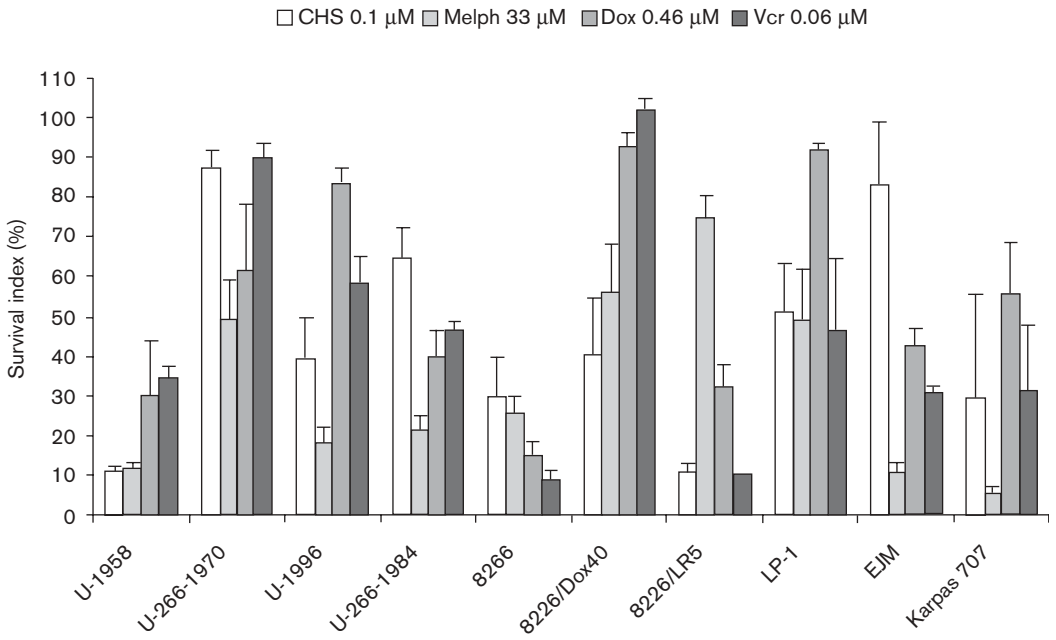
The cell densities in the fibers from the animals were expressed as net growth, defined as: (mean absorbance on retrieval day–mean absorbance on implantation day)/mean absorbance on implantation day.

Results

A concentration-dependent decrease in SI followed by a plateau could be discerned for all 10 cell lines (Fig. 1a–c). The three RPMI 8226 cell lines showed fairly similar CHS 828 sensitivity, but a lower maximum effect could be observed for the P-glycoprotein (P-gp)-expressing subline RPMI 8226/Dox40 (Fig. 1a). U-266-1970 and U-266-1984, both originating from the same patient, also showed similar drug sensitivity and were among the most CHS 828-resistant cell lines in the panel (Fig. 1b). EJM was the only cell line where 10 μM CHS 828 could not induce 50% cell kill (Fig. 1c).

Clear differences in the potency of CHS 828 were observed among the 10 cell lines with respect to both IC_{50} (Fig. 2a) and E_{max} (Fig. 2b). IC_{50} varied approximately 100-fold among the cell lines with U-1958 being the most sensitive and U-266-1970 the most resistant. The EJM cell line had the highest E_{max} and RPMI 8226 the lowest, representing a 6-fold difference in E_{max}.

Fig. 3



Comparison of drug sensitivity expressed as cell survival (SI) after exposure to fixed concentrations of the indicated drugs. The figure shows SI values at drug concentrations used for the calculation of the correlation coefficient. Data are presented as mean values ± SEM (n = 3–8).

Table 2 Relationship between the activity of CHS 828 and the standard drugs (CHS 828 0.1 μM, doxorubicin 0.46 μM and melphalan 33 μM)

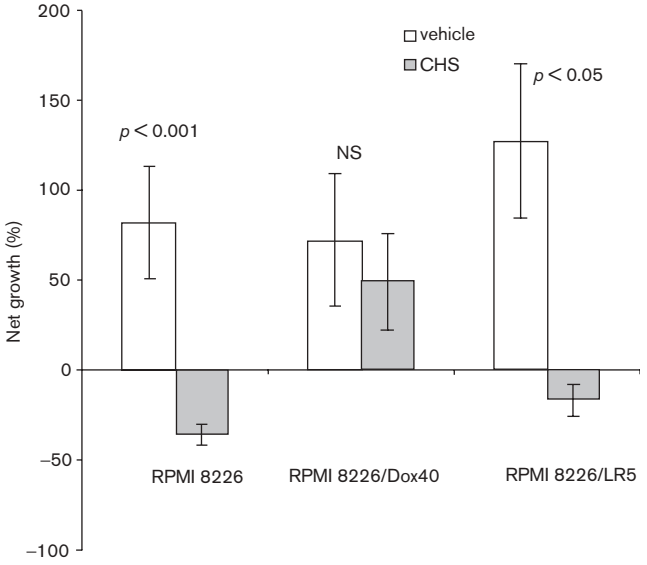
Drugs	Mechanistic class	r [a]
Vincristine	tubulin active	0.46
Melphalane	alkylator	−0.08
Doxorubicin	topo II inhibitor	0.24

[a]Pearson's correlation coefficient.

The activity pattern of CHS 828 was fairly different from that of three standard drugs used in the treatment of MM (vincristine, melphalan and doxorubicin) (Fig. 3). EJM, which was most resistant to CHS 828, was relatively sensitive to the standard drugs. On the other hand, RPMI 8226/Dox40, which was resistant to the standard drugs, was among the most sensitive to CHS 828. When the cell survival for CHS 828 and the standard drugs in all cell lines were correlated, low to moderate correlation coefficients were observed (Table 2). The highest correlation (0.46; NS) was observed with vincristine.

In the *in vivo* experiments, the three cell lines showed a positive net growth in the range 82–127% in the fibers from animals exposed to vehicle (Fig. 4). In the animals exposed to CHS 828, a net cell kill was observed for RPMI 8226 and RPMI 8226/LR5 (–36%, $p < 0.001$ and –17%, $p < 0.05$, respectively), whereas there was only a tendency to growth inhibition for RPMI 8226/Dox40 (49%, NS).

Fig. 4



Effect of CHS 828 on three different cell lines in the *in vivo* hollow fiber 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 fiber during the week of the experiment; negative net growth indicates a cell kill.

When the morphology of the cells from the *in vivo* experiment was studied at the day of fiber retrieval (not shown), cells with necrotic features dominated from

animals exposed to CHS 828. A small proportion of the cells displayed apoptotic morphology. Cells from animals exposed to vehicle were predominantly intact with only a few necrotic cells.

Discussion

CHS 828 is a new candidate drug, which has shown interesting preclinical properties, and *in vitro* studies have provided evidence of an effect on cells from hematological tumors. The molecular events leading to cell death caused by CHS 828 are not yet fully understood. Recent studies suggests an early metabolic effect leading to an increased extracellular acidification, followed by an abruptly inhibited DNA and protein synthesis after approximately 24 h [21,22]. CHS 828 exposure eventually results in a delayed cell death with both apoptotic and necrotic features [21].

The present study was undertaken to investigate the effect of CHS 828 on human myeloma cell lines both *in vitro* and *in vivo*. 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 [10]. For prediction of tumor-type specific activity, the use of fresh primary cultures of human tumor cells from patients may be a better model [6]. 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 [16].

CHS 828 showed an antiproliferative or cytotoxic effect on all 10 human myeloma cell lines, but with different IC_{50}s and E_{max} values. IC_{50}s were between 0.01 and 0.3 μM, which are below the peak concentrations observed in the first phase I trial. [14]

All concentration–response curves were plateau-shaped, which is typical for CHS 828 [13]. The maximum effect varied between an almost complete cell kill for some cell lines to less than 50% for others. The plateau may indicate a slow cell death process and is probably dependent on the assay time used.

CHS 828 had a somewhat smaller effect on the P-gp-expressing subline RPMI 8226/Dox40 than on the parental cell line RPMI 8226. The difference was, however, of a much lower magnitude than for the classical P-gp substrates doxorubicin and vincristine. Previous studies have shown that P-gp probably does not influence the effect of CHS 828 to any major extent [13]. CHS 828 has also been shown not to be affected by other common resistance mechanisms such as multidrug resistance (MDR) protein, glutathione and tubulin-associated MDR [13], a useful characteristic in treating tumors resistant to standard anticancer agents.

In general, the activity of CHS 828 in the myeloma cell line panel does not show an obvious dependence on cell line characteristics regarding growth factors, tumor progression or phenotypic variables. 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 tumor progression [23]. Furthermore, both EJM and U-266-1984 have mutations in the *p53* gene, which can result in genomic instability and drug resistance [24]. 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 as well as studies in primary tumor cells from patients where the effect of established drugs correlated poorly with the effect of CHS 828 [12,13]. Drugs with new mechanisms of action are attractive as complements to the standard agents used today.

This study also shows a high *in vivo* activity of CHS 828 on the myeloma cell lines RPMI 8226, RPMI 8226/Dox40 and RPMI 8226/LR5 tested in the hollow fiber model. Also other cell lines and primary cultures of human tumor cells have previously been shown to be sensitive to CHS 828 in the hollow fiber model at this dose level, causing no detectable toxicity in the animals [9,19]. However, when interpreting these *in vivo* data, one must keep in mind that there seems to be a species difference in tolerance to CHS 828 (Lindhagen *et al.*, manuscript in preparation).

Overall, the results showed an antiproliferative or cytotoxic effect on all 10 human myeloma cell lines and in the *in vivo* hollow fiber model. Thus, MM could be regarded as a potential target diagnosis for CHS 828.

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