Development and characterization of two human tumor sublines expressing high-grade resistance to the cyanoguanidine CHS 828

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The cyanoguanidine CHS 828 has shown promising antitumor properties and is currently in early clinical trials, although the mechanism of action still is largely unknown. In this study, resistant sublines of the histiocytic lymphoma cell line U-937 GTB and the myeloma line RPMI 8226 were developed by culturing under gradually increasing concentrations of CHS 828 until reaching 25 times the parental line EC_{50}s. The new phenotypes demonstrate more than 400-fold resistance to CHS 828 and cross-resistance to six cyanoguanidine analogs, but no resistance to nine standard drugs of different mechanistic classes or to the cytotoxic guanidines *m*-iodobenzylguanidine and methylglyoxal-bis(guanylhydrazone). The resistant phenotypes were stable for several months even if cultivated in drug-free medium and no difference in proliferation, ultrastructural or morphologic appearance in the sublines could be detected. Neither was decreased accumulation of tritium-labeled CHS 828 observed. Furthermore, the new U-937 phenotype was not accompanied by changes in differentiation or an altered cell-cycle distribution. In the myeloma cell line, esterase activity was shown to be moderately enhanced. Two-dimensional protein electrophoresis was undertaken to unmask possible resistance-mediating proteins and/or the target molecule(s) for CHS 828. In the myeloma cell line, λ light chain immunoglobulin (down-regulated) and a fatty acid-binding protein (up-regulated) were identified. The findings presented here indicate that development of specific cellular alterations is responsible for the gained CHS 828 resistance. ***Anti-Cancer Drugs*** **15:45–54 © 2004 Lippincott Williams & Wilkins.**

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Introduction

Recently, Schou *et al.* presented a series of cyanoguanidines exerting potent antitumoral activity. A candidate drug, CHS 828 {*N*-[6-(4-chlorophenoxy)hexyl]-*N*′-cyano-*N*″-4-pyridylguanidine, was selected after studies of structure–activity relationships *in vitro* and preliminary evaluation *in vivo* [1]. CHS 828 possesses a high antiproliferative activity *in vitro* in human cancer cell lines and induces complete remissions in nude mice inoculated with human lung cancer NYH cells, and it seems that this new antineoplastic agent expresses a broad spectrum of activity against a variety of human tumors, both *in vitro* and *in vivo*, including those resistant to standard drugs [2]. The activity pattern in a human tumor cell line panel representing different types of resistance mechanisms indicates that CHS 828 activity is not affected by well-known resistance mechanisms, e.g. P-glycoprotein (P-gp), multidrug resistance protein (MRP), GSH and tubulin-associated multidrug resistance. Accordingly, the activity profile in this panel of 10 cell lines shows low correlation with standard chemotherapeutic agents [2].

Although the exact mechanism of action remains to be determined, it seems that CHS 828 exerts its activity through processes different from those of standard drugs. The dynamic process following CHS 828 exposure is characterized by an almost normal proliferation for the first 24 h followed by an abrupt shut-off of DNA and protein synthesis, a modest increase in caspase-3 activity and DNA fragmentation, and the first signs of cell death [3,4]. These processes seem dependent on an intact protein synthesis [3]. At high concentrations, CHS 828 may inhibit mitochondrial respiration similar to *m*-iodobenzylguanidine (MIBG). However, these effects are most probably neither necessary nor sufficient to fully

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explain the cytotoxic actions of CHS 828, and in context to MIBG, cells cultivated in glucose-free pyruvate-supplemented medium appear partly protected from the cytotoxic effects of CHS 828 [5]. CHS 828 also shares some pharmacological characteristics with another guanidine, the polyamine synthesis inhibitor methylglyoxal-bis(guanylhydrazone) (MGBG). Detailed studies do, however, indicate that these similarities does not originate from a shared mechanism of action [6]. Furthermore, the cyanoguanidine CHS 828 may act by two principally different mechanisms depending on drug concentration and exposure time [7].

In this study we present sublines of the histiocytic lymphoma cell line U-937 GTB and the multiple myeloma line RPMI 8226 expressing high-grade resistance towards CHS 828. The maternal cell lines are highly sensitive [2], whereas the sublines are more than 400-fold resistant with respect to their individual EC_{50}s. The overall aim was to investigate the phenotypic characteristics associated with CHS 828 resistance. Such information could provide insights into the mechanism of action and is of putative importance in the future clinical development of the drug.

Material and methods

Parental cell lines

The histiocytic lymphoma cell line U-937 GTB, a mycoplasma-free subline of U-937 [8], was from the Department of Genetics and Pathology, Uppsala University, Sweden. The multiple myeloma cell line RPMI 8226 [9] was a kind gift from W. S. Dalton (Department of Medicine, Arizona Cancer Center, University of Arizona). The cells were grown in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma), 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin, and maintained in the culture medium at 37°C in a humidified atmosphere with 5% carbon dioxide. Growth and morphology were monitored on a weekly basis, and the cells were passaged twice weekly. For experimental purposes, the cells were always harvested in log phase, i.e. 1 day after addition of fresh medium.

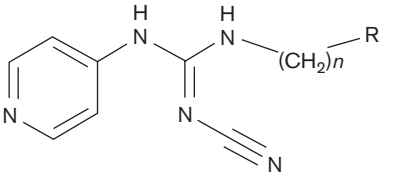
Development of resistant cell line

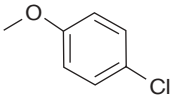
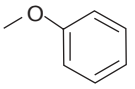
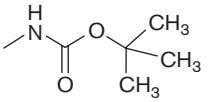
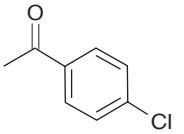
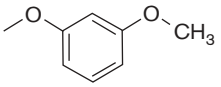
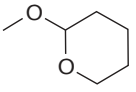
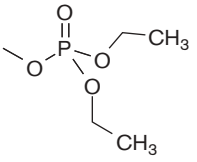
The resistant cell lines were established by culturing the cells under gradually increasing concentrations of CHS 828. The concentration of CHS 828 was increased every second week until a concentration of 10 times the parental EC_{50} was achieved and the cell lines were cultured in this concentration for more than 3 weeks. Subsequently, the cell lines were continuously cultured in the presence of 0.1 μM (U-937) and 0.5 μM (8226) CHS 828, respectively (approximately 25 times the parental EC_{50}). The resistant cell lines were designated as U-937/CHS and 8226/CHS, respectively. Both resistant cell lines retained their resistance to CHS 828 after culturing the cells in drug free medium for a period of several months.

Drugs and reagents

CHS 828 and analogs **1–6** (Fig. 1) were obtained from LEO Pharma (Ballerup, Denmark). A stock solution of 10 mM

Fig. 1



Compound	*n*	R
CHS 828	6	
1	7	
2	8	
3	6	
4	6	
5	8	
6	9	

Molecular structures of CHS 828 and the analogs tested.

CHS 828 (or analogs) in dimethyl sulfoxide (DMSO) was prepared and stored in the dark at −20°C. Dilutions to 1 mM were made with 30% DMSO and further with sterile phosphate-buffered saline (PBS). The [^{3}H]pyridine labeled CHS 828 used for accumulation experiments was produced by Amersham Pharmacia Biotech (Little Chalfont, UK) and obtained as a stock solution in ethanol with a specific activity of 57 Ci/mmol (1 mCi/ml).

MIBG (Sigma) was dissolved in 10% DMSO to yield a stock solution of 1.0 mg/ml and was stored at −70°C. Further dilutions with PBS were made prior to use. MGBG (Sigma) was dissolved directly in sterile water and stored at −70°C until use. All the standard drugs were obtained from commercial sources and dissolved according to the manufacturers' instructions.

[^{14}C]Thymidine (Amersham; 56 mCi/mmol, 50 μCi/ml) and [^{14}C]leucine (Amersham; 56 mCi/mmol, 50 μCi/ml) were handled according to the manufacturer's instruction, and stored in closed refrigerated containers until use.

Fluorescein diacetate (FDA; Sigma) was dissolved in DMSO to 10 mg/ml and kept frozen as a stock solution protected from light.

Measurement of cell survival

The fluorometric microculture cytotoxicity assay (FMCA) has been described in detail previously [10,11]. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were prepared with 20 μl drug solution at 10 times the desired concentration and stored for up to 2 months at −70 °C. Each drug concentration was tested in triplicate and six wells with PBS served as control. At start of the experiment, 2×10^4 cells in 180 μl fresh medium were added to the wells of the thawed plate and six blank wells received culture medium only. After 72 h incubation, the cells were washed once with PBS and 100 μl of FDA (10 μg/ml) in a physiological buffer was added. After 30 min incubation, fluorescence (ex. 485; em. 528 nm) was measured in a 96-well scanning fluorometer (Fluoroscan II; Labsystems, Helsinki, Finland). The fluorescence generated is proportional to the number of living cells and data are presented as survival index (fluorescence in test wells in percent of control wells with blank values subtracted). Data from three individual experiments were further processed with GraphPad Prism software (San Diego, CA) using non-linear regression and a standard sigmoidal concentration–response model to obtain EC_{50} (effective concentration 50%). The difference in sensitivity in the parental line versus subline was designated as the 'resistance factor' (RF) defined as EC_{50} in the subline divided by EC_{50} in the parental line.

Quality criteria for a successful assay included an initial cell viability of more than 90% as judged by the Trypan blue exclusion test, a mean coefficient of variation of less than 30% in blank, control and test wells, respectively, and a fluorescence signal in control cultures of more than 10 times the blank.

Morphology and ultrastructure

Cytospin preparations of cells cultured in microtiter plates were air-dried and stained with May–Grünewald–Giemsa (MGG) for morphologic evaluation in light microscopy. Cells for transmission electron microscopy were harvested in log phase, and processed for ultrastructural analysis and embedded in epoxy resin using a conventional protocol [12].

Proliferation: cell growth and macromolecular synthesis

Cell doubling time was estimated (according to the formula $c = c_0 \times 2^{t/t_d}$), by repeated Bürker chamber counting of cell density in suspensions during 72-h incubations.

The synthesis of DNA and proteins were measured in a Cytostar-T plate (*In situ* mRNA Cytostar-T assay kit; Amersham) using ^{14}C-labeled substrate (thymidine and leucine, respectively). The Cytostar-T plate is a 96-well microtiter plate with scintillants molded into the transparent polystyrene bottom. When labeled drug is absorbed to the intracellular compartment of the cells adherent to the bottom of the wells, the radioisotope is brought into proximity with the scintillant, thereby generating a detectable signal. Free radiolabeled drug in the supernatant is unable to stimulate the scintillant [13,14].

Cells were suspended in fresh media containing 111 nCi/ml [^{14}C]thymidine for DNA synthesis experiments, yielding a final radioactivity per well of approximately 20 nCi. For the protein synthesis experiments, fresh medium was supplemented with 222 nCi/ml [^{14}C]leucine, yielding a final radioactivity of 40 nCi/well. Addition of 180 μl labeled cell suspension (5×10^4 cells) to each well started the experiment. Blank wells received isotope and medium only. Radioactivity was measured with a computer controlled Wallac 1450 MicroBeta trilux liquid scintillation counter (Wallac, Turku, Finland), using MicroBeta Windows Workstation software (Wallac). After addition of the cell suspension the plate was read immediately and at different time points up to 10 h. During measurement the plate was covered with a plate sealer to avoid any bacterial contamination. The plate was stored in a regular cell-cultivating incubator between measurements. The experiment was performed two (U-937) or three (8226) times.

Differentiation analysis

In the U-937 cell lines the expression of monocytic differentiation markers, CD11c/p150.95, CD14 and CD49f/α_6 integrin, were measured using indirect

immunofluorescence and flow cytometry (FACSort; Becton Dickinson, Mountain View, CA). Primary antibodies used were Leu-M5 (CD11c) and Leu-M3 (CD14) (Becton Dickinson, Mountain View, CA), and GoH3 (CD49f) (CLB, Amsterdam, Netherlands), and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse $F(ab')_2$ fragments (Janssen, Beerse, Belgium) was used as the secondary antibody.

Cell-cycle phase distribution

Analysis of the U-937 cell-cycle phase distribution of the cells was performed according to Vindelöv [15]. Briefly, cells were washed once in cold PBS, and nuclei were prepared by exposing the cells to 0.03 mg/ml of trypsin (Sigma) for 10 min at room temperature followed by RNase A (Sigma; 0.08 mg/ml) for 10 min at room temperature and, finally, stained by propidium iodide (PI; Sigma, 0.2 mg/ml). The stained nuclei were subsequently analyzed by the CellQuest program using a FACScan (Becton Dickinson) and MacCycle software (Phoenix Flow Systems, San Diego, CA) to calculate the percentage of cells in each cell cycle phase.

Drug uptake

The accumulation of CHS 828 in cells was measured using ^{3}H-labeled drug. Cells were washed and resuspended in fresh room temperature medium to a cell density of 2.0×10^6 cells/ml. Tritium-labeled drug was added (0.01 μM, 0.57 μCi/ml) and at the defined time points (10 s, 5 and 15 min, 1 and 4 h) aliquots of 1000 μl (2.0×10^6 cells; 0.57 μCi) were transferred to 40 ml ice-cold PBS to inhibit further drug transport [16,17]. Cells were centrifuged at 500 *g* for 5 min and washed once with another 40 ml ice-cold PBS. After centrifugation the tubes were air-dried for at least 2.5 h. Naphthalene-based scintillating fluid (7.0 ml per sample, Quicksafe N; Zinsser Analytic, Frankfurt am Main, Germany) was added and the pellet was solubilized for at least 30 min, after which the fluid was mixed vigorously and then transferred to a scintillating vial (20 ml; Packard, Wellesley, MA) and the radioactivity was measured in a Wallac 1414 Win Spectral counter (1 min counting period). The values were normalized to the 4-h value (index 100) to minimize interexperimental variations, further incubation did not increase the signal (tested up to 22 h).

Calcein acethoxymethyl ester (calcein/AM) accumulation—functional analysis of P-gp- and MRP-like efflux proteins

Previous studies [2] have shown a moderately decreased cytotoxic activity of CHS 828 in a P-gp-expressing cell line. Even though it was shown that this probably did not correlate, we performed additional studies with calcein/AM as a functional probe for P-gp- and MRP-mediated drug resistance. The assay was performed as described by Liminga *et al.* [18] with minor modifications. Briefly, cells were washed and resuspended to 350 000 cells/ml in PBS containing 5 mM glucose. The cell suspensions were then distributed into four wells of a microtiter plates and exposed to calcein/AM at 5 μg/ml. After 30 min incubation at 37°C the generated fluorescence (ex. 485; em. 538) was measured. As a positive control in the myeloma RPMI 8226 cell line pair the calcein uptake was compared to a strongly P-gp-expressing doxorubicin-resistant subline, 8226/Dox40 [19,20]. The experiment was repeated 3 times. Washing the cells prior to measurement of generated fluorescence did not decrease the signal (one experiment, not shown).

Two-dimensional (2-D) protein gel electrophoresis

For each gel 5×10^6 cells were collected 24 h after cell split. The cell pellet was washed once with PBS, and the cells were lysed in 300 μl buffer containing 8 M urea, 2% CHAPS, 0.001% bromophenol blue and 20 mM DTT.

An aliquot of 50 μl of the sample was applied to 18-cm Immobiline dry strips with an immobilized pH non-linear gradient, pH 3–10. The first dimension was performed on an IPGphor isoelectric focusing unit (Amersham Pharmacia). For the second dimension, the strips were equilibrated for 15 min in 10 ml 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.001% bromophenol blue and 65 mM DTT followed by a 15-min alkylation step in 10 ml of the same solution, but with 135 mM iodoacetamide instead of DTT. The second dimension was performed with 12.5% polyacrylamide gels (18×18 cm) at constant current of 24 mA until the dye front reached the bottom of the gel. Molecular weight markers (97, 66, 45, 31, 21 and 14 kDa from the top) were applied on a small piece of filter paper at the anodic end of the strip before running the second dimension SDS–polyacrylamide gel. The gels were stained with silver according to Shevchenko *et al.* [21]. The experiment was performed twice for each cell line pair.

Gels of sensitive and resistant cell lines were compared visually [22], and spots that were considerably and reproducibly up- or down-regulated in the resistant cell lines were characterized according to p*I* (by comparison with reference gels) and molecular weight (by use of molecular weight markers).

Proteins of interest appearing in sufficient amounts were excised from 2-D gels and cleaved with trypsin by in-gel digestion. Peptide analysis was performed by electrospray ionization mass spectrometry (ES-MS) according to Wilm *et al.* [23]on a Q-tof using Masslynx software (Micromass, Manchester, UK). The peptide masses were compared to the NCBI nr database using the Mascot search engine [24].

Determination of immunoglobulin λ light chain

For the confirmation of one of the 2-D gel hits (see Results and Discussion), cell culture medium was taken

from the supernatant of the cell cultures and analyzed in the hospital routine analysis for immunoglobulin in urine.

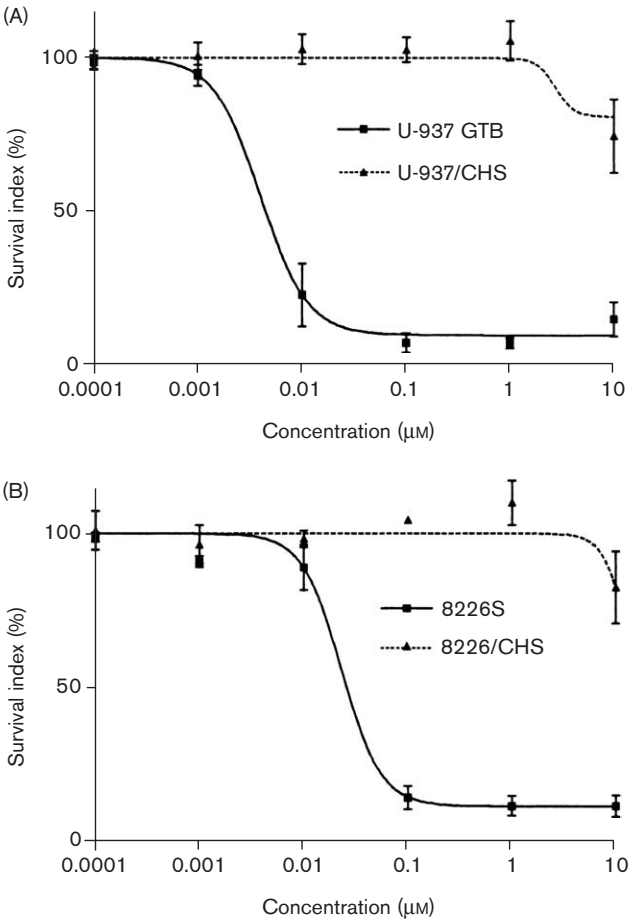
Results

General characteristics including proliferation and ultrastructure

Both resistant cell lines exhibited similar growth patterns as their respective parental line with an estimated doubling time of approximately 24 (U-937) and 30 (8226) h, respectively. Furthermore, incorporation of radiolabeled thymidine and leucine monitored for up to 10 h to reflect the levels of DNA and protein synthesis revealed no significant differences between parental and sublines (not shown).

No morphological differences could be detected by examination of MGG-stained cytospin preparations in the light microscope (not shown). Ultrastructurally, the cell line pairs appeared similar with spherical or rounded cells with short clumsy protrusions. The nuclei were invaginated with one or two nucleoli and a spotty pattern of heterochromatin. Mitochondria, ribosomes, Golgi apparatus, vesicles and slightly dilated endoplasmic reticulum filled the cytoplasmic compartment (not shown). Despite the similarities in organelle appearance, some mitochondria in the U-937/CHS cells appeared enlarged, sometimes with a disrupted inner membrane. It is not clear whether this mitochondrial abnormality is associated with the development of resistance or is just an experimental artifact, since a similar change has been observed as a toxic insult to U-937 cells dying from CHS 828 exposure [3]. In the myeloma 8226 cells, some signs of high activity in the cytoplasm with swollen electron-dense endoplasmic reticulum could be observed.

Fig. 2



Concentration–response curves for CHS 828 in the cell lines. Error bars indicate SEM; three separate experiments.

Pharmacology

The resistant cell lines expressed high-grade resistance to CHS 828 (Fig. 2) and six other closely related cyanoguanidines (Table 1A), with more than 1000- and 400-fold difference in EC_{50}, respectively.

In U-937, cross-resistance with known compounds was in general low to moderate (Table 1B); sensitivity to the vinca alkaloid vincristine differed most, with a RF of 6.1. The two other mitosis inhibiting agents tested, paclitaxel (Taxol, taxane) and etoposide (podophyllotoxin derivative) did not show signs of altered activity in the resistant cell line (RF of 1.8 and 1.6, respectively). The RF values for the antimetabolites cladribin (a purine analog) and cytarabine (pyrimidine analog) were 3.3 and 1.6, respectively. The RFs for the other standard drugs in the panel—a topoisomerase I inhibitor (topotecan), an alkylating agent (melphalan), a platinum compound (cisplatin) and the guanidines (MIBG and MGBG)—were low (below 1.4). Interestingly, a tendency for cross-resistance to the protein synthesis inhibitor cycloheximide (RF 4.3) was observed. The U-937/CHS cell line retained its resistance even after growth in drug-free medium for up to 3.5 months (not shown).

Also in the myeloma lines, cross-resistance with known compounds was in general low (Table 1B). Again, the vinca alkaloid vincristine gained the highest RF (2.4) among the standard drugs. Collateral sensitivity was observed for some of the drugs including both antimetabolites, cladribin (RF $<$ 0.65) and cytarabine (RF $<$ 0.20). The sensitivity toward the guanidine MGBG did, however, indicate some degree of cross-resistance, the dose–response curve for this compound had a very shallow shape, slowly declining to 59% survival at 100 μg/ml (versus 23% in parental 8226S cells, not shown) yielding RF $>$ 300. The 8226/CHS cell line retained its resistance even after growth in drug-free medium for up to 3.5 months (not shown).

Table 1 **Individual EC_{50} values for the cyanoguanidines (A) and standard chemotherapeutic agents (B)**

Drug	U-937 GTB	U-937/CHS	RF	8226S	8226/CHS	RF
(A)						
CHS 828	4.0 nM	10 μM	$>10^3$	23 nM	>10 μM	$>10^2$
1	<0.1 nM	>10 μM	$>10^5$	3.6 nM	>10 μM	$>10^3$
2	<0.1 nM	6.2 μM	$>10^4$	0.45 nM	4.6 μM	$>10^4$
3	<0.1 nM	4.6 μM	$>10^4$	1.8 nM	>10 μM	$>10^3$
4	<0.1 nM	>10 μM	$>10^5$	1.9 nM	>10 μM	$>10^3$
5	<0.1 nM	6.3 μM	$>10^4$	1.4 nM	>10 μM	$>10^3$
6	<0.1 nM	0.80 μM	$>10^3$	<0.1 nM	4.5 μM	$>10^3$
(B)						
Cisplatin	0.49 μg/ml	0.64 μg/ml	1.3	1.7 μg/ml	1.2 μg/ml	0.71
Cladribin	4.3 ng/ml	14 ng/ml	3.3	>0.20 μg/ml	0.13 μg/ml	<0.65
Cycloheximide	0.20 μg/ml	0.86 μg/ml	4.3	0.17 μg/ml	0.49 μg/ml	2.9
Cytarabine	30 ng/ml	49 ng/ml	1.6	>0.50 μg/ml	0.10 μg/ml	<0.20
Doxorubicin	87 ng/ml	92 ng/ml	1.1	0.13 μg/ml	0.27 μg/ml	2.1
Etoposide	0.13 μg/ml	0.21 μg/ml	1.6	1.1 μg/ml	0.88 μg/ml	0.80
Melphalan	0.67 μg/ml	0.92 μg/ml	1.4	2.9 μg/ml	1.3 μg/ml	0.45
MGBG	0.34 μg/ml	0.47 μg/ml	1.4	0.32 μg/ml	>100 μg/ml	>300
MIBG	11 μg/ml	11 μg/ml	1.0	7.0 μg/ml	15 μg/ml	2.1
Paclitaxel	2.7 ng/ml	4.8 ng/ml	1.8	4.9 ng/ml	12 ng/ml	2.4
Topotecan	19 ng/ml	17 ng/ml	0.89	0.31 μg/ml	0.034 μg/ml	0.11
Vincristine	0.46 ng/ml	2.8 ng/ml	6.1	1.4 ng/ml	3.3 ng/ml	2.4

Differentiation and cell-cycle analysis (U-937 only)

The resistant U-937/CHS cells did not show enhanced levels of differentiation markers in comparison with normal parental cells as judged by measurement of CD11, CD14 and CD49 expression [25] (Fig. 3A). Furthermore, cell-cycle distribution was similar in both U-937 GTB and the resistant subline, U-937/CHS (Fig. 3B), and there was no evidence for accumulation of cells in the G_0/G_1 phase of the cell cycle.

Drug uptake

The level and kinetics of [^{3}H]CHS 828 uptake is shown in Figure 4. During the 4-h assay period, all cell lines accumulated drug into the intracellular compartment(s) reaching equilibrium. Thus, increasing the observation period up to 22 h did not result in any further increase (not shown). There was no difference in the U-937 pair, whereas paradoxically in the 8226 pair the resistant cell line appeared to accumulate almost 2 times more drug.

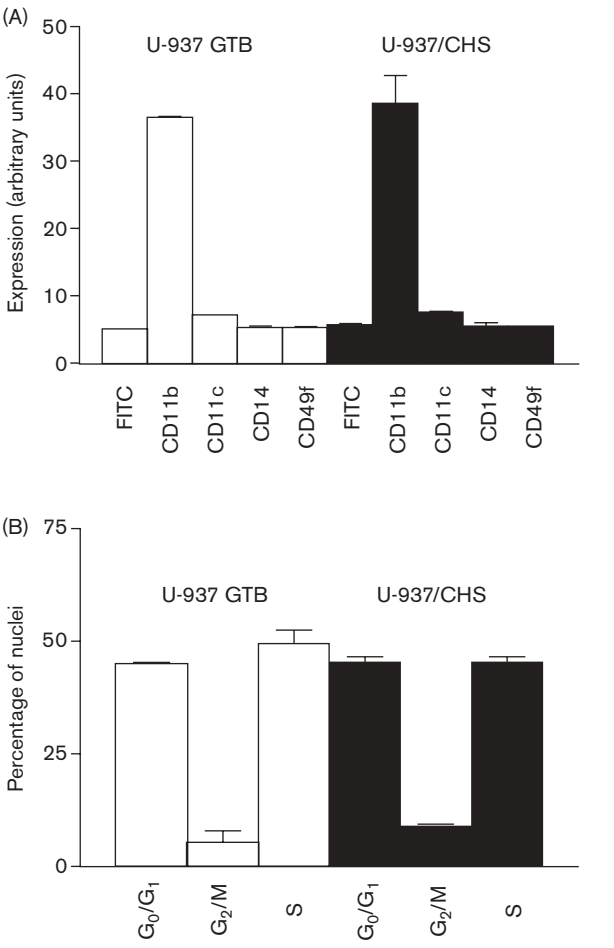
Calcein/AM accumulation

The accumulation of fluorescent calcein generated from hydrolysis (and subsequent ion trapping) of the ester was similar in cells from the two lymphoma cell lines (Fig. 5A). However, resistant 8226/CHS cells yielded a significantly ($p < 0.01$) higher signal than 8226/S cells (Fig. 5B). This unexpected observation, together with the increased [^{3}H]CHS 828 accumulation, remains to be clarified, but may be explained by the approximately 50% higher esterase activity in the resistant cells as measured by FDA hydrolysis (not shown). As expected, the P-gp-expressing 8226/Dox40 cells accumulated considerably less calcein than 8226/S cells.

2-D protein gel electrophoresis

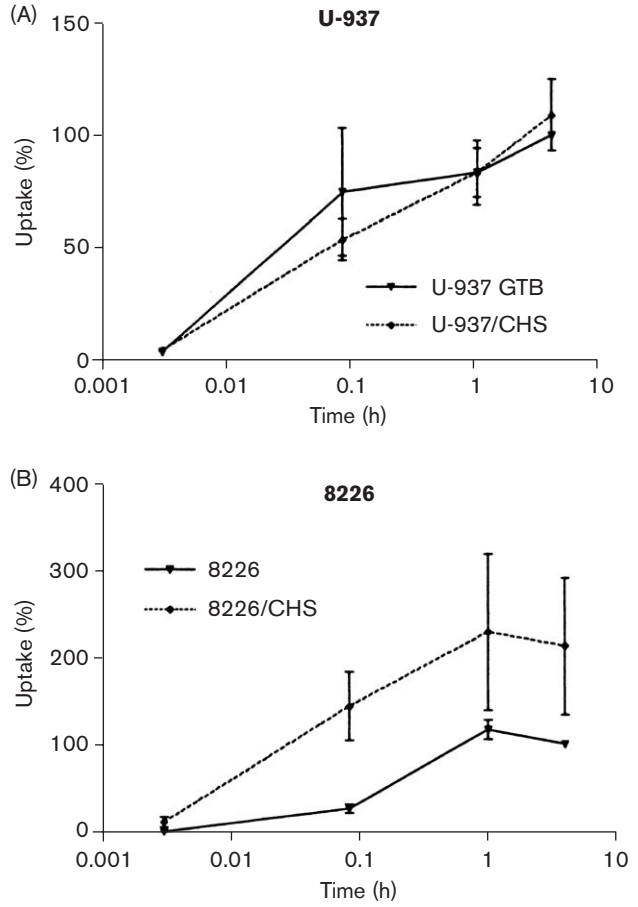
Two-dimensional gels of sensitive and resistant cell lines were compared visually and reproducible, and major

Fig. 3



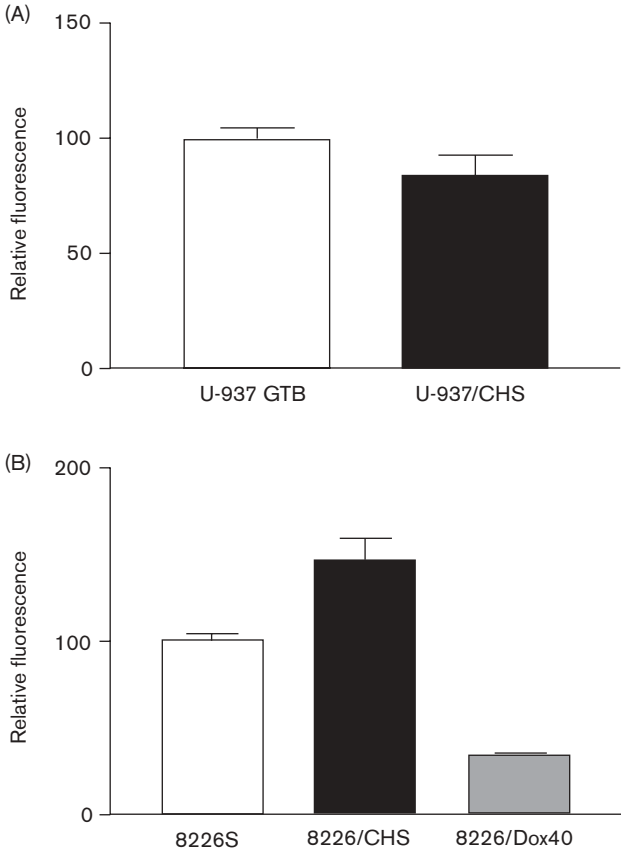
Differentiation analysis (A) and cell-cycle phase distribution (B) in the U-937 cell lines. Error bars indicate SEM.

Fig. 4



Accumulation of radiolabeled CHS 828 over time. Error bars indicate SEM; three separate experiments.

Fig. 5



Accumulation of fluorescent calcein as described in the text. Error bars indicate SEM; three separate experiments.

alternations are presented in Table 2 with estimated molecular weights and p*I*. Spots of adequate intensity were further identified by ES-MS as described in Material and methods.

In the resistant U-937 lymphoma cell line only one protein was up-regulated and two down-regulated (Table 2, top). None of these proteins were present in sufficient amounts for identification by mass spectrometry.

In the resistant myeloma cell line four proteins were identified as up-regulated and six as down-regulated (Table 2, bottom). In the 8226/S cell line, spot 9 with an estimated molecular weight of 25 kDa and p*I* of 6.7 was completely absent in the resistant 8226/CHS cell line. The protein was identified as immunoglobulin λ light chain. We confirmed this observation by qualitatively measuring immunoglobulin λ light chain in cell-free culture medium from the two myeloma cell lines (not shown). The two other identifiable down-regulated proteins were heat shock protein 27 and parvalbumin α (multiple peaks in mass spectrum, probably multiple peptides). Only one of the up-regulated proteins could be identified, epidermal fatty acid binding protein (E-FABP, spot 1). This protein was virtually absent in the parental 8226 line, but highly expressed in the resistant subline.

Discussion

The novel cyanoguanidine CHS 828 has shown unusual features and promising activity both *in vitro* and *in vivo* [1,2,26,27] and is now in phase I/II trials [28]. Broad spectrum of activity, favorable *in vitro* therapeutic indices [1,2,29] and IC_{50}s in the nanomolar range have prompted the extensive study in elucidating the mechanism of action.

In this study we investigated the biological characteristics and potential mechanism of resistance in two human tumor cell lines selected for resistance to CHS 828. Resistance levels of at least several 100-fold was achieved in both resistant sublines. Although the U-937/CHS and 8226/CHS cell lines were highly cross-resistant to other

Table 2 **Results of the 2-D protein gel electrophoresis**

(A)

Spot	M_r (kDa)[a]	p*I*[a]	U-937 GTB	U-937/CHS	Regulation	Identification[b]
1	21	6.3			↑	–
2	26	6.6			↓	–
3	40	5.3			↓	–

(B)

Spot	M_r (kDa)[a]	p*I*[a]	RPMI 8226	8226/CHS	Regulation	Identification[b]
1	14	6.3			↑	epidermal fatty acid binding protein
2	22	7.1			↑	–
3	31	7.0			↑	–
4	23	5.3			↑	–
5	12	4.9			↓	parvalbumin α (multiple peptides)
6	22	5.2			↓	–
7	31	5.3			↓	–
8	31	5.7			↓	heat shock protein 27
9	25	6.7			↓	immunoglobulin λ light chain
10	21	6.3			↓	–

[a]Estimated values by comparison with reference gels (p*I*) and molecular weight markers (M_r).

[b]Identification by ES-MS.

cyanoguanidine analogs, they showed low cross-resistance or rather collateral sensitivity to the established drugs of different classes examined. Vig Hjarnaa *et al.* have previously shown that CHS 828 activity is independent of common resistance mechanisms, like expression of MRP, GSH, topoisomerase II or tubulin-associated MDR [2]. However, lower activity of CHS 828 in the P-gp-expressing cell line RPMI 8226/Dox40 compared to that in the parental RPMI 8226/S line was reported, thereby suggesting a possible role for P-gp in protecting the cells from the CHS 828 effect. However, P-gp blockers were not able to reverse resistance and the differential activity probably relies upon some other unknown feature of this doxorubicin-selected subline. A slight tendency to lower proliferative capacity, thereby generating a slower cell kill, might contribute [2].

Compared to the parent cell lines, the resistant sublines showed similar proliferative rate and unaltered morphology in light microscopy. Also, ultrastructurally, the cells appeared similar, although some mitochondria appeared slightly enlarged with a disrupted inner membrane in the U-937/CHS cells. At this point it is not clear whether these alterations are associated with the resistance or just coincidental.

According to Ekelund *et al.* [5], high concentrations (1 and 10 μM) of CHS 828 share the same inhibitive effects on mitochondrial respiration as MIBG with a subsequent increase in glycolysis to cover the energy need and do not seem to explain the cytotoxic effect at lower concentration. Interestingly, this high concentration effect is similar in U-937/CHS and U-937 GTB cells [5]. Hence, the resistant phenotype does not seem protected from mitochondrial effects of CHS 828, although more than 1000-fold higher EC_{50} as seen in the cytotoxicity assay. This supports the theory of dual mechanisms of actions for this drug as suggested by Hassan *et al.* [7]. In addition, it was shown that cells grown in pyruvate-supplemented glucose-free medium are, at least partly, protected from CHS 828 toxicity, thereby indicating that the extent of glycolysis may be an important determinant for optimal CHS 828 activity [5].

Few other changes in the phenotypes were observed. The expression of monocytic differentiation markers in the U-937 lymphoma cell lines remained reasonably unchanged and there was no difference in cell cycle phase distribution between the cell lines. Moreover, the accumulation of radioactive CHS 828 was not decreased in the resistant sublines; in contrast, the 8226/CHS cells showed a paradoxical increase in drug accumulation. Yet to be explained, the results at least suggest that the mechanism of resistance is not dependent on an active efflux mechanism or a decreased uptake by an altered system for active transport. In addition, the 8226/CHS cell line proved to accumulate excessive amounts of calcein/AM, possibly indicating an increased capacity of loading lipophilic or acidic byproducts.

Another dependent factor seems to be protein synthesis—exposure to the peptidyl transferase inhibitor cycloheximide (1 μM, which completely inhibits protein synthesis) protects U-937 GTB against CHS 828 [3]. The rationale for the dependence on active protein synthesis remains to be explained. Interestingly, the antiproliferative effect of this compound is somewhat decreased in the resistant cell lines (RF 4.3 and 2.9, respectively, as presented in Results).

The resistant phenotypes were stable even after a few months of cultivation in drug-free medium. This indicates that specific genetic alterations rather than momentary induction of detoxifying genes confer the resistance. Furthermore, the low level of cross-resistance to known standard drugs indicates that these alterations are specific for cyanoguanidine resistance and may in fact be associated with the mechanism of action, e.g. as an altered primary target, more or less specific for the cyanoguanidine group of drugs. This has been evaluated in the 2-D protein gel electrophoresis. No common resistance modifying protein alteration could be detected, but the expression of several proteins, some of which are yet to be identified, was increased or decreased.

When the results from 2-D protein gels electrophoresis were analyzed there was unfortunately no match between the hits in the two cell line pairs. In addition, the U-937 cell lines did not express any changes in spots strong enough to allow ES-MS identification. However, in the myeloma pair, some altered proteins could be further characterized and identification suggested. In the resistant 8226/CHS line, immunoglobulin synthesis was almost totally shut down, whereas the parental cell line is characterized by very active production of free λ light chains [9]. Furthermore, the elevated level of E-FABP in the resistant cell line is notable. Several members of the FABP families have been characterized, e.g. liver (L-FABP), epidermal (E-FABP) or intestinal (I-FABP), and the expression has also been suggested to be associated with atypical MDR in mitoxantrone-selected EPP85-181RNOV pancreatic adenocarcinoma [30]. Preliminary studies of mitoxantrone sensitivity in the 8226 cell lines did not show any cross-resistance (not shown). The up-regulation of E-FABP could perhaps explain the increased uptake of CHS 828 in 8226/CHS. E-FABP has been shown to bind fatty acids [31] and the chemical structure of CHS 828 indicates that the drug might be a ligand for this protein. Binding of the drug might alter the subcellular distribution, thus altering the toxic effect. Further investigation of the roles of these proteins for

development of CHS 828 resistance and their relevance for explaining the mechanism of action is needed. A recent study indicates that CHS 828 exerts its cytotoxic effect by inhibiting the NF-κB signaling in tumor cells. *In vitro*, I-κB phosphorylation is inhibited by CHS 828 at very low concentrations [32]. However, the status of the NF-κB signaling system in the resistant sublines is not yet known.

In summary, the new phenotypes of the CHS 828-resistant cells display one major new feature—high-grade resistance to CHS 828 and other cyanoguanidines, with essentially unaltered sensitivity to other cytotoxic drugs. The difference between the sublines and the parental cell lines is not accompanied by alterations in morphology or ultrastructure, nor by an altered cell-cycle phase distribution or expression of monocytic differentiation markers (U-937). Neither are the differences due to a decreased intracellular accumulation of the drug nor a slower proliferation rate. The specific resistance is stable over time and remains even in drug-free medium, indicating that specific genetic alterations account for the developed resistance. Further investigations of such alterations may contribute to reveal the mechanism of action for this new class of compounds.

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