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Report

Activity of CHS 828 in primary cultures of human hematological and solid tumors *in vitro*

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CHS 828 is a pyridyl cyanoguanidine that has shown promising preclinical anticancer activity against various experimental tumor models and is presently being tested in a phase II trial in man. In the present study the fluorometric microculture cytotoxicity assay was used for *in vitro* evaluation of CHS 828 activity in primary cell cultures from hematological and solid tumors. In total, 156 samples from various diagnoses were tested with 72-h continuous drug exposure. CHS 828 showed high relative *in vitro* activity against tumor cells from chronic lymphocytic leukemia as well as from acute leukemia and high-grade lymphoma. Activity was also observed in several solid tumor cell samples, although the group as a whole appeared less responsive. CHS 828 was significantly more active against hematological malignancies compared to normal lymphocytes. Correlation analysis with standard drugs revealed low to moderate correlation coefficients. The results show that CHS 828 has potent antitumor activity against primary cultures of human tumor cells from patients and might have a unique mechanism of action. [© 2001 Lippincott Williams & Wilkins.]

Key words: Anticancer drug, CHS 828, human tumor cells, chronic lymphocytic leukemia, cytotoxicity assays.

Introduction

Discovery and development of new anticancer drugs is a complex and largely empirical process, and selection of compounds for clinical trials depends on assays and models of uncertain predictive value. The current models for predicting clinical utility of new agents have relied on animal *in vivo* tumor models and established tumor cell lines from various types of tumors. Although a broad spectrum of activity in these models has indicated some relationship to clinical utility, the ability to reflect diagnosis-specific activity appears limited.[1,2] Preclinical models with the ability to predict the tumor-type specific activity of new cytotoxic drugs would thus be important and valuable tools in anticancer drug development process.

Non-clonogenic cytotoxicity assays using fresh or cryopreserved human tumor cells have previously been shown to reflect known clinical activity patterns of cytotoxic drugs and may be used for this purpose.[3–5] The ability of the fluorometric microculture cytotoxicity assay (FMCA) to detect disease-specific drug activity and provide important information on resistance mechanisms and cross-resistance patterns has been indicated both with respect to standard and investigational anticancer agents.[3,5]

CHS 828 [*N*-4-(6-chlorophenoxy)nexyl-*N*′′-cyano-*N*′′-4-pyridylguanidine] is a pyridyl cyanoguanidine that has shown interesting properties as a potential anticancer agent. CHS 828 was selected for preclinical development using a primary screening program based on human cell lines *in vitro* and rodent tumor cells *in vivo*. In follow-up studies CHS 828 was found even more active in human tumor cells compared to rodent cell lines with antiproliferative activity observed in the nM to μM range.[6]

Investigations using a panel of cell lines representing defined mechanisms of resistance revealed a differential pattern of antitumor activity with some cell lines sensitive in the subnanomolar range.[7] In addition there was a low cross-reactivity with clinically used standard agents and no significant influence of P-glycoprotein, multidrug resistance-associated protein, glutathione S-transferase, topoisomerase II or tubulin-associated multidrug resistance was apparent.[7]

In the subsequent pharmacodynamic evaluation *in vivo*, oral administration of CHS 828 has also shown

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significant antitumor activity in several tumor models, especially pronounced in a nude mouse model of small cell lung cancer (NYH) and in the MCF-7 breast carcinoma model.[7] Both of these models were found highly refractory in response to standard drugs, including paclitaxel.[7] *In vivo* activity was also observed in rat models with Yoshida hepatosarcoma and Walker 256 breast carcinoma as well as in chemically induced breast tumors in the rat.[7]

In the present study the FMCA was used on fresh and cryopreserved human tumor cells from patients and human normal cells in an attempt to further investigate the activity of CHS 828, and to identify promising targets for subsequent phase I and II trials.

Materials and methods

Tumor samples

A total of 156 consecutive successfully analyzed tumor cell samples were obtained from patients [median age 59 years (16–92), 78 males and 78 females] with different hematological and solid tumors. The histologic types of these samples are summarized in Table 1 The patients were undergoing routine surgery, diagnostic biopsy or bone marrow/peripheral blood sampling. The Research Ethical Committee at Uppsala University Hospital approved the FMCA method. One-fifth of the samples were collected from previously untreated patients. Tumor tissue from solid tumors was minced to 1 mm^3 size, and tumor cells were then isolated by collagenase dispersion and Percol (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation.[8] Leukemic cells were isolated from bone marrow or peripheral blood by 1.077 g/ml Ficoll-Isopaque (Pharmacia Biotech) density-gradient centrifugation.[9] Mononuclear cells from eight healthy donors were used in some experiments, and these cells were collected and processed in the same way as the leukemic samples. Viability was determined by the Trypan blue exclusion test and the proportion of tumor cells was judged by inspection of May–Grünwald–Giemsa-stained cytocentrifugate preparations by a trained cytopathologist. Culture medium RPMI 1640 (Hyclone, Cramlington, UK) supplemented with 10% heat-inactivated fetal calf serum (Sigma, St Louis, MO), 2 mM glutamine, 100 μg/ml streptomycin and 100 μg/ml penicillin was used throughout. Cells were cryopreserved in a medium containing 10% dimethylsulfoxide (DMSO; Sigma) and 90% FCS by initial freezing for 24 h at -70°C followed by storage in liquid nitrogen. Both fresh and cryopreserved samples were used in this study.

Table 1. Tumor and normal cell samples analyzed for *in vitro* activity of CHS 828

Tumor type	*N*
ALL	11
AML	17
CLL	26
NHL	7
Breast cancer	16
Colorectal cancer	6
Malignant melanoma	5
NSCLC	8
Ovarian cancer	34
Sarcoma	8
Assorted solid tumors[a]	18
Normal lymphocytes	8
Total	164

[a]Hepatocellular cancer (2), pheochromocytoma (2), ventricle cancer (1), thyroid cancer (2), renal cancer (1), adrenocortical cancer (1), small cell lung cancer (1), carcinoid (3), pancreatic cancer (1), schwanoma (1), mesothelioma (1) and unknown primary (2).

Reagents and drugs

Fluorescein diacetate (FDA) (Sigma) was dissolved in DMSO (10 μg/ml) and kept frozen (-20°C) as a stock solution (10 mg/ml) protected from light. CHS 828 was obtained from Leo Pharmaceutical Products (Copenhagen, Denmark) dissolved in DMSO (10 mM). Cisplatin 6.7 μM, cladribine 0.7 μM, cytarabine 1.6 μM, doxorubicin 0.9 μM, etoposide 8.5 μM, melphalan 8.2 μM, vincristine 0.54 μM and vinorelbine 2.3 μM were obtained from commercial sources. CHS 828 was in half of the samples tested at six concentrations, and in the rest of the samples tested at 0.1 and 1 μM only. The drugs for comparison were tested at empirically derived cut-off concentrations (EDCC) as previously described.[8,9] Experimental plates 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).[8] The plates were stored frozen at -70°C until further use. Repeated testing of normal lymphocytes controlled drug stability during storage conditions (data not shown). CHS 828 could be stored in this way for at least 2 months. All experiments were performed with continuous drug exposure.

Equipment

The drugs and cells suspended in culture medium were added to the wells by the pipetting robot, PROPETTE. The medium was removed by aspiration

using a 96 well microtiter plate washer (Dynatech, West Sussex, UK). Addition of buffer and FDA (Sigma) was performed with the aid of an automated 96-well dispenser, Multidrop (Labsystems, Helsinki, Finland). The 96-well scanning fluorometer (Fluoroscan II; Labsystems) was equipped with a xenon lamp and broadband interference filters exciting light at 485 nm. The emitted fluorescence from a vertical light path of each well was sequentially read at 538 nm.

Cytotoxicity assay procedure

The FMCA is based on the measurement of fluorescence generated from hydrolysis of FDA to fluorescein by cells with intact plasma membrane. On day 1, 180 μl of the tumor cell preparation ($0.8–6 \times 10^5$ cells/ml culture medium) was seeded into the wells of V-shaped 96-well experimental microtiter plates (Nunc, Roskilde, Denmark), prepared as described above. Six blank wells received only culture medium 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 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. Quality criteria for a successful assay included 70% or more tumor cells in the cell preparation prior to and in the control wells after incubation, a fluorescence signal in control cultures of more than 5 times mean blank values, and mean coefficient of variation (CV) in control cultures of greater than 30%.

Definitions and statistical analysis

The cell survival in each well was presented as survival index (SI), calculated as the fluoresence in drug-treated wells as a percentage of the fluorescence in control wells with blank values subtracted, i.e. lower numerical values indicates a higher cytotoxic effect. The leukemias and lymphomas were included in the hematological tumor group and the remaining diagnoses in the solid tumor group. SI values at different concentrations for the various diagnoses were compared using the Student's *t*-test for unpaired comparisons. Activity of CHS 828 was, in those samples tested at all six concentrations, expressed as IC_{30} (concentration reducing SI to 70%) and IC_{50} (concentration reducing SI to 50%). The values were calculated from the mean concentration–response curve for each group. From these curves the maximal effect (lowest cell survival=SI_{min}) was also estimated, which usually was the SI at 10 μM.

A 50% or greater decrease in SI was arbitrarily defined as *in vitro* response. *In vitro* response rate was defined as the fraction of samples showing a more than 50% decrease in SI. The *in vitro* response rates for the hematological and solid tumor groups, respectively, at different concentrations of CHS 828 were compared by using a χ^2 test. To evaluate the solid of hematological tumor specificity of CHS 828, a solid/hematological (S/H) activity ratio was calculated, defined as the fraction of the solid tumor samples showing a SI < 50% to the drug divided by the fraction of hematological samples showing a SI < 50%. Thus, high and low ratios indicate a relatively high activity in solid and hematological tumors, respectively. Pearson's correlation coefficient between SI values was calculated to compare the activity of CHS 828 with standard chemotherapeutic drugs.

Results

A total of 156+8 samples were successfully analyzed. The histologic types of these samples are summarized in Table 1. In Figure 1 the relative activity of CHS 828 in different diagnoses was investigated to detect disease-specific activity. Most samples from CLL and NHL patients showed a 50% or greater decrease in SI. In the solid group response was observed among others in samples from ovarian cancer and non-small cell lung cancer (NSCLC). Response was observed in none of the colorectal cancer and malignant melanoma samples. To evaluate whether the SI obtained in response to CHS 828 in the solid tumor group would decrease further with prolonged assay time, this was tested in ovarian carcinoma cell samples. The results showed a tendency towards lower SI values with prolonged exposure time (data not shown).

A concentration-dependent decrease in SI followed by a plateau could be discerned for all the hematological tumor types and for normal lymphocytes whereas solid tumors as a group appeared less responsive (data not shown). Chronic lymphocytic leukemia (CLL) was most sensitive in terms of IC_{50} and SI_{min} followed by high-grade non-Hodgkin's lymphoma (NHL), acute myelocytic leukemia (AML), acute lymphocytic leukemia (ALL) and normal lymphocytes (Table 2). The solid tumor group was the least sensitive (Table 2). The differences between the cell survival of the solid tumor group and the hematological tumors, were highly significant ($p < 0.001$) at all concentrations. The effect

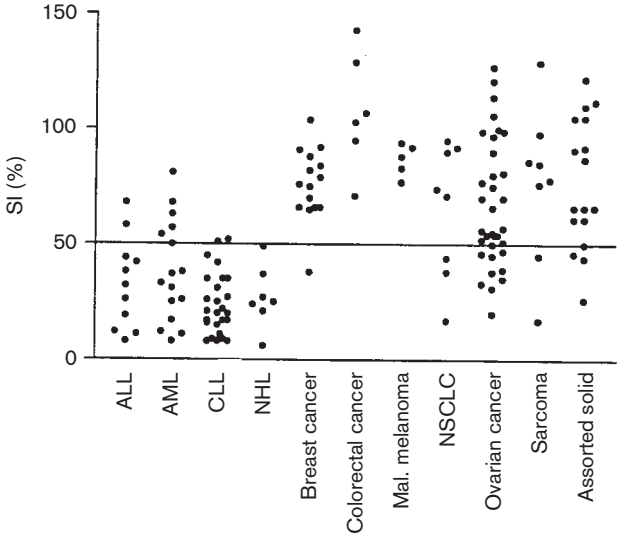

Figure 1. Cell survival after exposure to 1 μM CHS 828 in 156 tumor samples. A 50% survival is indicated; samples showing lower cell survival arbitrarily defined as responders.

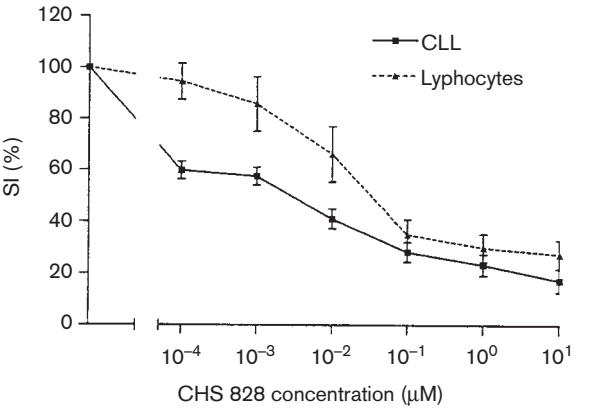

Figure 2. Effect of CHS 828 on SI for CLL (n=24) and normal lymphocytes (n=8). SI=fluorescence in test wells/fluorescence in control wells with blank values subtracted. The bars indicate SEM.

Table 2. Activity of CHS 828 expressed as inhibitory concentration 30% (IC_{30}), 50% (IC_{50}) and effect maximum (SI_{min}) for the diagnoses tested for dose–response

Diagnosis	IC_{30}[a] (μM)	IC_{50}[a] (μM)	SI_{min}[a] (%)
ALL (4)[b]	1×10^{-3}	2×10^{-2}	38
AML (9)	7×10^{-4}	9×10^{-3}	22
CLL (24)	3×10^{-5}	3×10^{-3}	17
Colorectal cancer (5)	>10	>10	100
Malignant melanoma (3)	>10	>10	76
NSCLC (5)	7×10^{-2}	>10	69
Ovarian cancer (18)	1×10^{-1}	>10	65
Assorted solid tumors[c] (12)	8	>10	69
Normal lymphocytes (8)	7×10^{-3}	4×10^{-2}	28

[a]Calculated from the mean concentration–response curve for each group. IC_{30} = concentration reducing SI to 70%. IC_{50} = concentration reducing SI to 50%. SI_{min} = lowest observed SI.
[b]Values within brackets = number of samples.
[c]Sarcoma (2), ventricle cancer (1), thyroid cancer (1), renal cancer (1), adrenocortical cancer (1), small cell lung cancer (1), carcinoid (1), schwanoma (1), mesothelioma (1) and unknown primary (2).

of CHS 828 on cell survival of CLL cells and normal lymphocytes is shown in Figure 2. The difference in SI values between malignant CLL lymphocytes and normal lymphocytes was statistically significant at concentration of 0.0001–0.01 μM ($p<0.05$).

When the frequency of samples showing a 50% or greater decrease in SI was determined for all six CHS 828 concentrations, a similar pattern, with hematological samples more sensitive than solid samples, was apparent (Table 3). Hematological tumors showed a higher frequency of responders with increasing concentrations of CHS 828. However, an *in vitro* response rate in the range of 12–26% was observed for solid tumors at 0.1–10 μM CHS 828. The tumor types responding at 1 μM CHS 828 were nine of 34 ovarian cancers, three of eight NSCLCs, two of eight sarcomas, one of 16 breast cancers, one of one mesothelioma, one of one small cell lung cancer and one of two adenocarcinomas of unknown origin. The difference in *in vitro* response rate between hematological and solid tumors was statistically significant at all concentrations tested ($p<0.01$) (Table 3). In Table 4 the S/H ratio for CHS 828 is compared with that of standard drugs, for which S/H ratios have been previously published.[10,11] This demonstrates a ratio for CHS 828 higher or in the same range as for hematological-specific agents like cytarabine and vincristine, but slightly lower than the ratio for doxorubicin and etoposide for which clinical solid tumor activity has been demonstrated.

Correlation between SI values for CHS 828 (1 μM) and standard drugs (at their EDCC) in samples tested for multiple drugs are shown in Table 5. SI values for CHS 828 correlated weakly to moderately with the standard drugs. The highest correlation coefficient (R=0.70) was obtained with vincristine. When the analysis was based only on hematological tumors the correlations with most drugs became weaker to a variable extent, including vincristine (R=0.47). In Figure 3 the correlation plot between SI values for vincristine and the tubuline-active vinca alkaloid vinorelbine (R=0.93) observed in the present material of hematological samples is compared with that of vincristine and CHS 828 (R=0.47).

Table 3. *In vitro* response rate (%)[a] of CHS 828

Tumor type	CHS 828 concentration (μM)					
	1×10^{-4}	1×10^{-3}	1×10^{-2}	0.1	1	10
Hematological tumors	33 (12/36)	41 (15/37)	64 (25/39)	84 (47/56)	83 (50/60)	94 (33/35)
Solid tumors	3 (1/39)	5 (2/40)	11 (5/45)	12 (10/81)	19[b] (18/94)	26 (9/34)

[a]*In vitro* response rate was defined as the fraction of samples showing a more than 50% decrease in SI.
[b]The tumor types responding at 1 μM were nine ovarian cancer, three NSCLCs, two sarcomas, one breast cancer, one mesothelioma, one small cell lung cancer and one unknown primary.

Table 4. *In vitro* S/H ratios of CHS 828 and standard drugs[a]

Drug	S/H ratio
CHS (0.1 μM)	0.14
CHS (1 μM)	0.22
Cisplatin	1.28
Cladribine	0.01
Cytarabine	0.07
Doxorubicin	0.32
Etoposide	0.70
Vincristine	0.13

[a]Data from standard drugs were taken from Fridbord *et al.*[5] (n=91–128 for solid tumors and n=60 for hematological tumors).

Table 5. Correlation coefficients between the resulting SI values for CHS 828 (1 μM) and standard drugs in all samples reported

Drugs	Hematological samples		All samples	
	R[a]	(n)[b]	R	(n)
Cisplatin	0.36	(40)	0.46	(57)
Cladribine	0.50	(51)	0.62	(63)
Cytarabine	0.56	(48)	0.62	(78)
Doxorubicin	0.35	(40)	0.56	(75)
Etoposide	0.05	(43)	0.25	(119)
Melphalan	0.15	(43)	0.60	(73)
Vincristine	0.47	(50)	0.70	(73)

[a]R= Pearson's correlation coefficient.
[b]n= number of samples.

Discussion

In the present study the concentration–response curves, obtained using normal lymphocytes and patient tumor cells, were typically plateau-shaped, which is similar to what was previously reported in human tumor cell lines.[7] Since the patient tumor cells have a low proliferative rate under the present assay conditions the plateau does not appear to be dependent on cell proliferation. Studies in established cell lines have shown that the rate of proliferation remains intact for the first 24 h of CHS 828 exposure

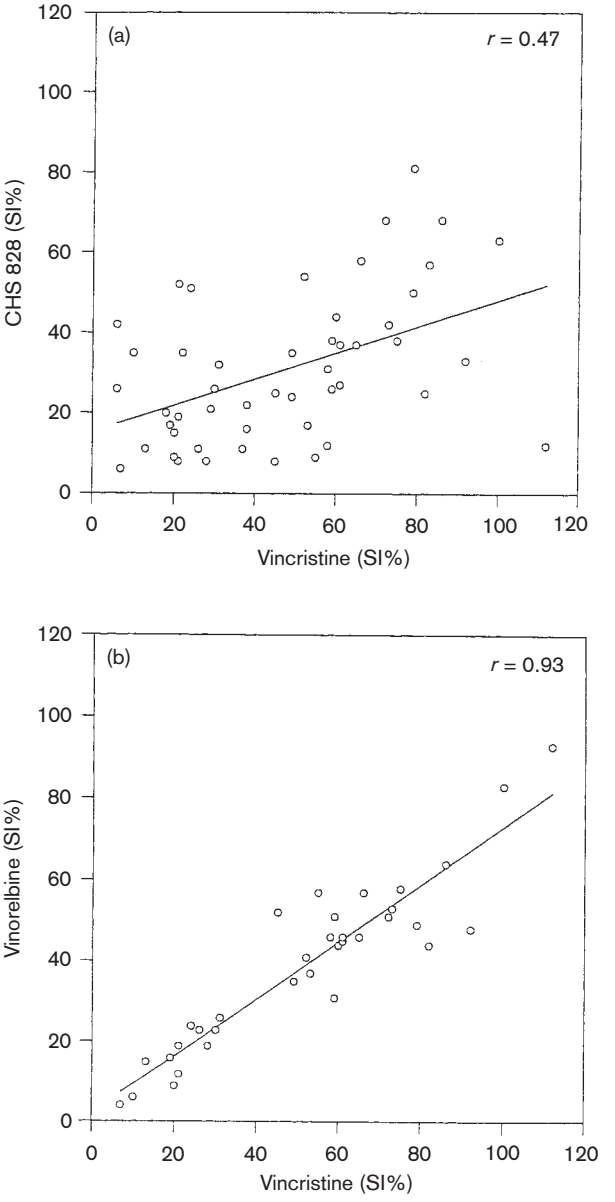

Figure 3. Relationship between SI values for vincristine (0.54 μM) and CHS 828 (1 μM) (n=50) (a) and for vincristine (0.54 μM) and vinorelbine (2.3 μM) (n=35) (b) in hematological tumor samples. Each dot represents one patient tumor sample.

followed by an abrupt and complete inhibition of DNA and protein synthesis. A gradual cellular disintegration for the next 48 h is responsible for progressively decreasing survival during the incubation period.[12] The mechanism of induction of delayed and asynchronous cell kill by CHS 828 remains to be defined.

Information on tumor-type specific activity *in vitro* may help targeting suitable diagnoses for phase II clinical trials. Indeed, the ability of the FMCA to detect disease-specific drug activity has been indicated both with respect to standard and investigational anticancer agents.[5] However, it should be noted that the drug exposures used for many comparisons in the present study (0.1 and 1 μM) were 3–30 times higher than the achievable exposure recently reported from the first phase I trial in man.[13] In this study CHS 828 demonstrated high *in vitro* activity against hematological tumors, especially those of the CLL type at very low (nanomolar) concentrations. The activity of CHS 828 was less marked in the solid tumors as a group. However, a fraction of samples in the solid tumor group showed a decrease of SI below 50%. The S/H ratio was in the same range as for vincristine and melphalan that are well-known hematological tumor active agents. Despite this activity pattern with higher activity in hematological tumors than in solid tumors, the fact that activity was observed in a fraction of solid tumors together with the *in vivo* activity observed in several animal models of solid tumors may indicate a potential role for CHS 828 also in the treatment of solid tumors. Further evaluation of CHS 828 in an expanded panel of solid tumor types using the present approach seems warranted and is underway.

In many samples not reaching 50% SI there was nevertheless evidence for a SI plateau at a higher level (50–80% SI). Whether this plateau would be reduced beyond the 72-h incubation needed to be addressed, especially in view of delayed activity previously shown in cell lines.[12] When this was experimentally tested in different ovarian carcinoma samples a tendency towards lower SI levels with increasing incubation time was observed. Overall, this effect was considered to be modest and did not differ from what was observed for standard drugs. The 72-h incubation time may thus be considered reasonably adequate for comparison of CHS 828 activity with other cytotoxic drugs and between different diagnoses.

To some extent, cytotoxicity assays may be used for preclinical evaluation of dose-limiting toxicity by comparing tumor cell responses with those of normal cells.[14] In this study the efficacy of CHS 828 in CLL samples compared with the normal counterpart (normal lymphocytes from healthy donors) revealed a large difference. CLL cells showed a 10-fold higher sensitivity in terms of mean IC_{50} and a 100-fold higher sensitivity in terms of mean IC_{30}. It is especially interesting to note that these effects were obtained at concentrations 100- to 1000-fold lower than those achievable *in vivo* in animals (unpublished data). Thus, based on these results CLL appears to be a suitable target for initial phase II evaluation. However, if these *in vitro* predictions and indicated therapeutic window will translate into the *in vivo* situation remains to be established in preclinical studies of CHS 828 activity in leukemia models *in vivo* as well as in clinical trials. Results from studies using hollow fiber cultures of CLL cells in a rat model have indeed demonstrated high *in vivo* activity.[15] A phase II trial with CHS 828 in CLL is presently underway.

Analyzing cross-resistance patterns with some standard drugs, we found the highest correlation with vincristine. This may indicate some mechanistic similarity to this class of tubulin-active drugs. However, these results contrast to some extent with results in cell lines where correlations were low.[7] Furthermore, removing the unresponsive solid tumors from the analysis clearly lowered the correlation coefficient. The correlation between the tubulin-active vinca alkaloid derivative vinorelbine and vincristine in the present material of hematological tumors was considerably higher than that between CHS 828 and vincristine. These facts clearly argue against a direct mechanistic link between CHS 828 and this group of compounds.

The cross-resistance analysis may also have implications when combination therapy is considered. A desirable feature of the component drugs of a combination regimen may be low levels of cross-resistance when development and influence of drug resistance is to be minimized. Direct testing of interactions between CHS 828 and other classes of chemotherapeutic compounds may provide useful information on these matters in addition to the mechanistic information. Such interaction studies may be performed using the FMCA[16–18] and is presently in progress.

Valid *in vitro* models for prediction of tumor type-specific activity of new cytotoxic drugs is of great value in the preclinical phase of drug development. Performing this type of studies with the FMCA is feasible and fairly easy to perform. A large number of samples from patients with different diagnoses, disease progression and treatment history may be tested, and the results may target diagnoses and patients to clinical studies. The type of phase II *in vitro* trial described here may not be applicable to all

types of anticancer drugs. Since the FMCA measures cell damage in the whole tumor population, consisting of largely non-dividing cells, purely antiproliferative drugs may not be accurately detected by this assay.[4] It should also be emphasized that *in vitro* indication of high drug activity in a specific tumor type is not the same as prediction of clinical utility, which also depends on the achievable plasma concentrations of the particular drug. *In vivo*, it may not be possible to achieve cytotoxic concentrations of the drug either in plasma or in the tumor, due to dose-limiting toxicity, pharmacokinetic factors or factors associated with the biology of the tumor itself.[19]

Conclusion

The FMCA was used for *in vitro* evaluation of CHS 828 activity in primary cell cultures from hematological and solid tumors. We showed that CHS 828 had a high relative *in vitro* activity against tumor cells from CLL as well as from acute leukemia and high-grade lymphoma. Activity was also observed in several solid tumor cell samples, although the group as a whole appeared less responsive. Correlation analysis with standard drugs revealed low to moderate correlation coefficients. The results from this study indicate that CHS 828 is active *in vitro* against hematological but also some of the solid tumors and the drug appears non-cross-resistant with standard drugs.

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