CHS 828, a Novel Pyridyl Cyanoguanidine with Potent Antitumor Activity *in Vitro* and *in Vivo*

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ABSTRACT

A new class of recently discovered antineoplastic agents, the pyridyl cyanoguanidines, exert a potent antitumor activity in rodents after oral administration. Optimization *in vitro* and *in vivo* has resulted in the selection of the lead candidate CHS 828 (*N*-(6-chlorophenoxyhexyl)-*N'*-cyano-*N''*-4-pyridylguanidine). CHS 828 was found to exert potent cytotoxic effects in human breast and lung cancer cell lines, with lesser effects on normal fibroblasts and endothelial cells. In a study using a panel of cell lines with different resistance patterns, the effects of CHS 828 showed a low correlation with the activity patterns of known anticancer agents, and no sensitivity to known mechanisms of multidrug resistance was observed. In nude mice bearing human tumor xenografts, CHS 828, at doses from 20 to 50 mg/kg/day p.o., inhibited the growth of MCF-7 breast cancer tumors and caused regression of NYH small cell lung cancer tumors. Oral administration of CHS 828 once weekly improved efficacy without increasing toxicity. CHS 828 was found to compare favorably with established chemotherapeutic agents such as cyclophosphamide, etoposide, methotrexate, and paclitaxel. In mice with NYH tumors, long-term survival (>6 months) was observed after treatment with CHS 828 was stopped. In conclusion, CHS 828 is an effective new antitumor agent, with a potentially new mechanism of action. CHS 828 is presently being tested in Phase I clinical trials in collaboration with the European Organization for Research and Treatment of Cancer.

INTRODUCTION

Chemotherapy is the mainstay of therapy for a large number of malignant tumors, especially in the metastatic setting. Although for certain tumor types this modality of treatment has been shown to produce impressive results including cures, the majority of human solid tumors is considerably less responsive. Today more than 50 established cytotoxic drugs are available for treatment of human malignant tumors in the Western world. The majority of these drugs act directly or indirectly on the DNA by inhibiting its synthesis, or they can also act at the level of microtubuli by interfering with mitosis, cell motility, or intracellular transport. They are mostly derived from natural products, they have to be parenterally administered, and they easily induce MDR[2] in the clinical setting.

In the search for new low molecular weight synthetic inhibitors of tumor cell growth, we unexpectedly discovered that a number of pyridyl cyanoguanidines showed antitumor activity after oral administration in a routine screening program in rats (1). Some pyridyl cyanoguanidines are known as potassium channel openers. Among these, pinacidil (*N*-1,2,2-trimethylpropyl-*N'*-cyano-*N''*-4-pyridylguanidine) is a structural prototype with potent antihypertensive activity

(2). Replacement of the side chain of pinacidil by longer aryl-containing side chains gave rise to compounds with increasing antitumor activity but without hypotensive activity. Monosubstitution in the terminal phenoxy group with a chloro group and optimization of the side chain length with regard to antitumor activity resulted in the selection of a drug candidate, CHS 828 (*N*-(6-chlorophenoxyhexyl)-*N'*-cyano-*N''*-4-pyridylguanidine; Ref. 1).

The data presented here comprise *in vitro* and *in vivo* studies with CHS 828. Cytotoxic activity and inhibition of cell proliferation were studied *in vitro*, using established cancer cell lines, cell lines resistant to known antineoplastic agents, and normal cells.

In vivo, CHS 828 was investigated in nude mice with various human tumor cell xenografts and in rats with transplanted tumors in a series of studies focusing on dose-response and schedule-dependent effects of CHS 828, on its antitumor activity in established tumors, and on comparison with reference chemotherapeutic agents.

MATERIALS AND METHODS

Compounds

In Vitro **Experiments.** For DNA synthesis and cytotoxicity experiments, CHS 828 (Department of Chemical Research, Leo Pharmaceutical Products), daunorubicin and paclitaxel (both from Sigma Chemical Co., St. Louis, MO) were dissolved at 10 mM in DMSO and stored at −20°C. Dilutions were made with DMSO.

In the activity profile study, fluorescein diacetate (Sigma Chemical Co.) was dissolved in DMSO and stored at −20°C as a stock solution protected from light. Reference drugs were obtained from commercial sources and were dissolved according to the guidelines from the manufacturers. The Pgp-blocker SDZ PSC 833 was obtained from Novartis, Stockholm, Sweden, MIBG from Sigma Chemical Co., and MGBG from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD).

In Vivo **Experiments.** CHS 828 was formulated as a suspension in 2% carboxymethyl cellulose in 0.9% NaCl solution. Cyclophosphamide (Sendoxan, Asta Medica, Frankfurt am Main, Germany) was dissolved in distilled water. Etoposide (Vepesid, Bristol-Myers Squibb, Syracuse, NY) and paclitaxel (Taxol, Bristol-Myers Squibb) were dissolved in 0.9% NaCl solution.

Cells. The human cancer cell lines used for the studies of DNA synthesis and cytotoxicity included NYH SCLC cells (Rigshospitalet, Copenhagen, Denmark) and MCF-7 breast cancer cells (ATCC, Rockville, MD). MRC-5 fetal lung fibroblasts (European Collection of Cell Cultures, Salisbury, United Kingdom) and HUVEC cells (ATCC) were used as normal human reference cells.

NYH cells were grown as partly floating cultures. Floating and loosely adherent cells were passaged once a week in RPMI 1640 with the addition of 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Fresh growth medium was added every 2nd or 3rd day.

MCF-7 cells were cultured as monolayer cultures. Monolayers were passaged once a week in DMEM deprived of phenol red and supplemented with 5% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The growth medium was changed every 2nd or 3rd day.

MRC-5 cells were cultured as monolayer cultures. Monolayers were passaged once a week in DMEM with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The growth medium was changed every 2nd or 3rd day.

HUVEC cells were cultured as monolayer cultures. Monolayers were pas-

saged once a week in M199 medium supplemented with 10% FCS, 0.1 mg/ml heparin, 30 μg/ml endothelial cell growth supplement, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The growth medium was changed every 2nd or 3rd day.

For the activity profile studies, a human tumor cell line panel consisting of four sensitive parental cell lines, five drug resistant sublines, representing different mechanisms of resistance, and one cell line with primary resistance was used (3). The cell lines included the myeloma cell line RPMI 8226/S and its sublines 8226/Dox40 and 8226/LR-5 (W. S. Dalton, University of Arizona, Tucson, AZ), the lymphoma cell lines U-937 GTB and U-937-Vcr (4, 5), the SCLC cell line NCI-H69 and its subline H69AR (ATCC, Rockville, MD), the renal adenocarcinoma cell line ACHN (ATCC), and the leukemic cell line CCRF-CEM and its subline CEM/VM-1 (W. T. Beck, University of Tennessee, Memphis, TN).

The 8226/Dox40 was selected for doxorubicin resistance and shows the classical MDR phenotype with overexpression of Pgp 170 (6). The 8226/LR-5 was selected for melphalan resistance, proposed to be associated with increased levels of GSH (7, 8). The U-937-Vcr was selected for vincristine resistance, proposed to be tubulin-associated (5). The H69AR, selected for doxorubicin resistance, expresses a MDR phenotype proposed to be mediated by MRP (9, 10). The CEM/VM-1, selected for teniposide resistance, expresses an atypical MDR, which is proposed to be topoisomerase II (11, 12). The exact mechanism of resistance for the primary resistant ACHN cell line is not known and may be multifactorial (13).

The cell lines were grown in RPMI 1640 with 10% FCS, 2 mM glutamine, 50 μg/ml streptomycin, and 60 μg/ml penicillin. The 8226/Dox40 was treated once a month with doxorubicin at 0.24 μg/ml and the 8226/LR-5 with melphalan at 1.53 μg/ml at each change of medium. The U-937-Vcr was continuously cultured in the presence of 10 ng/ml of vincristine, and the H69AR was alternately fed with drug-free medium and medium containing 0.46 μg/ml of doxorubicin. The CEM/VM-1 cell line was cultured in drug-free medium without any loss of resistance for a period of 6–8 months. The resistance patterns of the cell lines were routinely confirmed in control experiments.

In the xenograft studies, MCF-7 breast cancer cells (Clone L, N. Brünner, Rigshospitalet, Copenhagen, Denmark), NYH SCLC cells (P. Buhl Jensen, Rigshospitalet, Copenhagen, Denmark), and H-460 non-SCLC cells (ATCC, Rockville, MD) were used. The cells used in the rodent tumor studies included rat Yoshida hepatosarcoma cells (Finsen Institutet, Copenhagen, Denmark), and rat Walker 256 mammary carcinoma cells (Deutsche Krebsforschungzentrum, Heidelberg, Germany).

Cell Line Experiments

DNA Synthesis. The ability of CHS 828 to inhibit DNA synthesis was determined by the incorporation of tritiated thymidine. Paclitaxel and daunorubicin were used as reference compounds.

The MCF-7 and NYH cells were seeded in tissue culture vessels at the concentration of 7.5×10^3 cells/ml, the test compounds were added 2 h after plating, and the cells were cultured with the test compounds for 96 h (MCF-7 cells) or 144 h (NYH cells). MRC-5 fibroblasts were seeded in tissue culture vessels at the concentration of 2.5×10^3 cells/ml, the test compounds were added 2 h after plating, and the cells were cultured with the test compounds for 144 h. Endothelial cells were seeded at the concentration of 25×10^3 cells/ml in multidish plates in M199 medium without heparin and endothelial cell growth supplement and were incubated for 24 h. Then the test compounds were added, and the cells were incubated for an additional 96 h with 1 ng/ml vascular endothelial growth factor and with 2% FCS.

Tritiated thymidine (5 Ci/mmol, Amersham, Denmark) was added to the cultures at the concentration of 1 μCi/ml, and the cells were incubated for an additional 4 h. The incorporated thymidine was measured with a β-counter. Each drug concentration was tested in triplicate.

Cytotoxicity. The ability of CHS 828 to induce cytotoxic effects was determined by the conversion of MTT to formazan by mitochondrial dehydrogenases. The cells were seeded in tissue culture vessels at the concentration of 2.5×10^3 cells/ml, were incubated for 2 h, and were then exposed to the test compounds for 96 h (MCF-7 cells) or 120 h (NYH cells). Then, MTT was added at the concentration of 5 mg/ml, and the cells were incubated for 4 h. To solubilize the formazan product, 5% SDS was added to the cultures overnight.

The absorbance of the solubilized formazan was then determined with an ELISA reader. Each drug concentration was tested in triplicate.

Activity Profile Studies. The FMCA is based on measurement of fluorescence generated from the hydrolysis of fluorescein diacetate to fluorescein by cells with intact plasma membranes and has been described in detail previously (14). Briefly, 180 μl of cell suspension were seeded into the wells of 96-well microtiter plates prepared with drugs. Cell density ranged from 5 to 20×10^3 cells/well. Each drug concentration was tested in triplicate. Six wells with cells but without drugs served as control and six wells with only culture medium as blank.

The plates were incubated for 72 h. At the end of the incubation period, the plates were centrifuged, and the medium was removed. The cells were washed once and 100 μl/well of fluorescein diacetate (10 μg/ml) was added. The plates were incubated for 45 min, and the generated fluorescence from each well was measured at 538 nm in a 96-well scanning fluorometer (Fluoroscan II, Labsystems OY, Helsinki, Finland).

To evaluate the schedule dependency of drug activity, RPMI 8226S cells were exposed to the drug for 2 or 72 h followed by washing and the addition of new culture medium, after which FMCA was performed at 72 h.

Animal Models

Animals and Animal Welfare. All of the animal experiments were conducted according to the guidelines and ethical standards of the Danish Committee for Animal Experiments. Female NMRI nu/nu mice 6 weeks of age were purchased from M&B (Ry, Denmark). Female outbred Sprague Dawley rats, 4–6 weeks old, and female inbred Lewis rats, 9–11 weeks old, were obtained from M&B (Ejby, Denmark). The nude mice were housed under specific pathogen-free conditions, whereas the rats were maintained under standard laboratory conditions.

MCF-7 Xenografts. Oophorectomized 17β-estradiol-substituted NMRI nu/nu mice were inoculated with $1–1.5 \times 10^7$ MCF-7 cells in both flanks, and the tumor growth was measured twice weekly for 8 weeks (15). The tumor area was used as expression of the tumor size and was calculated from two perpendicular diameters measured with a digital caliper (16). CHS 828 was given by oral gavage from day 21 when the mean tumor size $\pm$ SE was 29 $\pm$ 3 mm^2. Each treatment group consisted of five to seven mice.

Two treatment schedules with CHS 828 were used:
(a) 20, 50, or 100 mg/kg once daily for 8 weeks; and
(b) 100 or 250 mg/kg once weekly for 8 weeks.

NYH Xenografts. NMRI nu/nu mice were injected with 1×10^7 NYH cells s.c. in both flanks (17, 18). The tumor size was measured as described for the MCF-7 model. CHS 828 was given by oral gavage from day 14, when the tumor size was 30–130 mm^2. The tumor size doubled two to three times in 2 weeks. For this reason, vehicle-treated mice had to be euthanized between week 4 and 6. Each treatment group consisted of 6 to 15 mice.

Two treatment schedules with CHS 828 were used:
(a) 1, 3, 10, 20, or 30 mg/kg once daily for 2 weeks; and
(b) 100 or 250 mg/kg once weekly for 3 weeks.

To ascertain long treatment efficacy, the treated animals were observed for 6 months after the last dose.

CHS 828 at 20 mg/kg was compared with etoposide, paclitaxel, cyclophosphamide, and methotrexate using a once-daily schedule (from day 14 to day 28).

H-460 Xenografts. NMRI nu/nu mice were injected with 5×10^5 H-460 non-SCLC cells in both flanks and treated from the day of inoculation with p.o. doses of CHS 828 ranging from 20 to 100 mg/kg daily for 2 weeks.

Rat Tumors. In the Yoshida ascites hepatosarcoma model, 2×10^7 ascites cells were injected i.p. into inbred female Lewis rats (19, 20), and treatment with CHS 828 was started 3 days after tumor inoculation. CHS 828 was given p.o. at 20–100 mg/kg once daily for a maximum of 21 days. Each treatment group consisted of six rats.

The Walker 256 breast carcinosarcoma tumor was grown by weekly passage in female outbred Sprague Dawley rats (21). Tumor pieces were mechanically disrupted, and a tumor cell suspension of 1×10^7 cells was injected s.c. into the inguinal region. Animals were treated with CHS 828 at a daily oral dose of 20 mg/kg from the day of tumor injection to day 9, when the tumors were dissected and weighed. Each treatment group consisted of 6 animals.

Statistical Methods

In the DNA synthesis and cytotoxicity studies, the IC_{50} values were calculated from the dose-response curves and were expressed as the mean $\pm$ SD of two to three independent experiments.

In the activity profile study, tumor cell survival was presented as the SI, defined as the fluorescence in experimental wells expressed in per cent of that in control wells, with blank values subtracted. The concentration-response data were expressed as the mean $\pm$ SE obtained from three independent experiments. IC_{50} values obtained in the cell line panel were used for comparing the activity of CHS 828 with other compounds using Pearson's correlation coefficient as previously described (22). Curve fitting and parameter estimation in Fig. 1 were performed with the Graphpad Prism software (Graphpad Software, Inc., San Diego, CA), using nonlinear regression and a standard sigmoidal concentration-response model with variable slope. CHS 828 exposure (AUC) was calculated as the CHS 828 concentration multiplied by the exposure time, as the drug was shown to be stable under assay conditions.

In the animal models, tumor sizes were expressed as the mean $\pm$ SE of tumor areas for the animals alive at the time of measurement. Comparison with controls was expressed as T/C % (median tumor area in the treated group $\div$ median tumor area in the vehicle group $\times$ 100). Significance was tested with the Mann-Whitney U test.

RESULTS

DNA Synthesis and Cytotoxicity

NYH SCLC Cells and Lung Fibroblasts. The potency of CHS 828 in NYH SCLC cells measured by the thymidine incorporation assay and by the MTT assay was in the same range as that of paclitaxel and daunorubicin (Table 1). In normal fetal lung fibroblasts, CHS 828 was about 100 times less active than in NYH cells. In contrast, paclitaxel and daunorubicin potently inhibited thymidine incorporation in both NYH cells and lung fibroblasts.

MCF-7 Breast Cancer Cells and Endothelial Cells. Measured by the thymidine incorporation assay, the potency of CHS 828 in breast

Table 1 *DNA synthesis and cytotoxicity in NYH SCLC cells and normal lung fibroblasts: effects of CHS 828, paclitaxel, and daunorubicin*

Results are expressed as the mean IC_{50} concentration $\pm$ SD of two to three experiments.

| | NYH SCLC (IC_{50} nM) | | Lung fibroblasts (IC_{50} nM) |
	^{3}H-TdR inc.[a]	MTT	^{3}H-TdR inc.
CHS 828	2.7 $\pm$ 2.0	0.5 $\pm$ 0.0	188 $\pm$ 130
Paclitaxel	4.3 $\pm$ 3.3	7.0 $\pm$ 1.7	6.2 $\pm$ 1.5
Daunorubicin	3.9 $\pm$ 0.9	8.3 $\pm$ 1.7	5.8 $\pm$ 0.9

[a] Incorporation of tritiated thymidine.

cancer cells was in the same range as that of daunorubicin, whereas paclitaxel was 6 times more potent (Table 2). Measured by the cytotoxicity MTT assay, paclitaxel and daunorubicin showed the same potency as in the thymidine incorporation studies, whereas CHS 828 was about 4 times more potent. In HUVEC cells, CHS 828 was markedly less active than in tumor cells. Paclitaxel and daunorubicin had IC_{50} values more similar to those obtained with tumor cells.

Activity Profile

To further study the antiproliferative activity profile of CHS 828, a panel of 10 cell lines with different resistance patterns was chosen. Clear differences in the potency of CHS 828 were observed among the 10 cell lines with respect to both IC_{50} and maximal effect (E_{max}), and the concentration-response curves were typically plateau-shaped at higher concentrations (Fig. 1*A*). At 100 μM, the drug was highly cytotoxic against all of the cell lines with SI values <10% (data not shown). The U937 cell lines were the most sensitive, whereas the ACHN cell line was the most resistant cell line in terms of IC_{50} (Fig. 1*B*). When the overall activity pattern was compared with the corresponding activity data for 10 standard drugs and 2 clinically used guanidine compounds (MIBG and MGBG), low-to-moderate correlation coefficients were observed (Table 3). When this comparison was extended to 100 other investigational cytotoxic or antiproliferative compounds, all of the correlation coefficients obtained were less than 0.65 (range, -0.45 to 0.65), except for eosinophilic cationic protein (ECP), for which the correlation was 0.79 (data not shown).
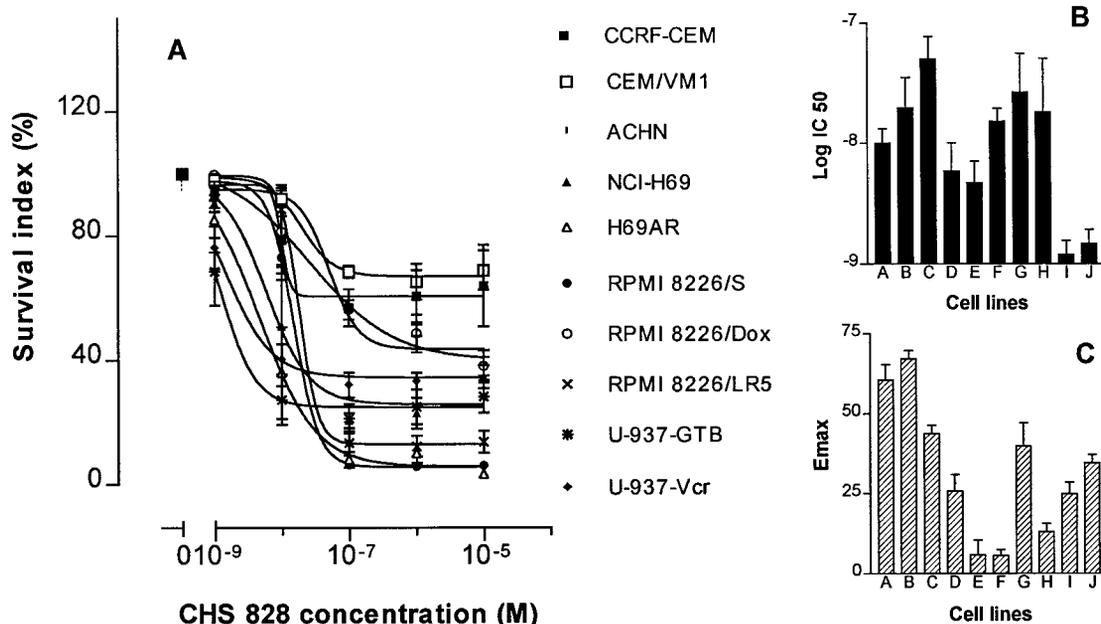
Fig. 1. Cytotoxic activity of CHS 828 in a panel of 10 human tumor cell lines measured by the FMCA after a 72-h incubation with continuous exposure (*A*). Calculated log IC_{50}s are shown in *B* and the maximum effect (E_{max}) in *C*. Curve fitting and parameter estimation were performed with a standard sigmoidal concentration-response model with variable slope. The results are presented as mean values $\pm$ SE obtained from three independent experiments.

From the concentration-effect graphs in Fig. 1, *A* and *C*, it is apparent that CHS 828 showed a diminished activity (E_{max}) against the Pgp-expressing subline compared with the parental one. Addition of the Pgp-blocker SDZ PSC 833 at 1–3 μg/ml to RPMI 8226/Dox40 cells only marginally increased the sensitivity to CHS 828, and the effect was of similar magnitude in the parental line. At these concentrations, SDZ PSC 833 produced a near complete reversal of doxorubicin resistance (data not shown). In addition, CHS 828 was shown not to interfere with the function of the Pgp in Pgp-overexpressing MCF-7$_{adr}$ cells, in the standard chemosensitizer assay performed by Panlabs, Inc. (Bothell, WA; data not shown). No significant sensitivity to MRP, GSH, or tubulin-associated MDR, was apparent when comparing the concentration-response curves of the parental cell lines and their resistant sublines (Fig. 1, *A-C*).

To investigate the time dependency of the induction of the cytotoxic effects of CHS 828, RPMI 8226/S cells were incubated with the drug for 2 and 72 h, respectively. Continuous exposure for 72 h, compared with 2 h, produced a left-shifted dose-response curve (Fig. 2*A*). However, when the SI was plotted against the AUC (concentration $\times$ h), the difference between the two incubation schedules disappeared (Fig. 2*B*).

Animal Models

MCF-7 Xenografts in Nude Mice. CHS 828 was administered daily at 20, 50, or 100 mg/kg p.o. to mice with established tumors from day 21 to 70 after inoculation (Fig. 3*A*). The dose of 20 mg/kg tended to delay tumor growth for the first 3 weeks of treatment, but, thereafter, tumor growth resumed. The dose of 50 mg/kg arrested tumor growth, but two mice died during the experiment. The remaining mice showed no sign of toxicity (no weight loss). The highest dose of CHS 828 (100 mg/kg/day) was toxic to all of the mice. CHS 828 was next administered once weekly at 100 or 250 mg/kg/week (Fig. 3*B*). At 100 mg/kg, no significant effect on tumor regression was observed. Mice that were given 250 mg/kg had tumor regression

Table 2 *DNA synthesis and cytotoxicity in MCF-7 breast cancer cells and endothelial cells: effects of CHS 828, paclitaxel, and daunorubicin*

Results are expressed as the mean IC_{50} concentration $\pm$ SD of two to four experiments.

	MCF-7 breast cancer cells (IC_{50} nM)		Endothelial cells (IC_{50} nM) ^{3}H-TdR inc.
	^{3}H-TdR inc.[a]	MTT	
CHS 828	31.0 ± 24.0	7.3 ± 3.3	5650 ± 212
Paclitaxel	4.9 ± 0.8	8.6 ± 0.3	5.3 ± 0.2
Daunorubicin	39.0 ± 4.9	38.5 ± 36.0	45.0 ± 1.0

[a] Incorporation of tritiated thymidine.

Table 3 *Activity pattern of CHS 828: correlation with chemotherapeutic agents with various mechanisms of action and with two structurally related guanidines, MIBG and MGBG*

Drugs	Mechanistic class	R[a]
Etoposide	Topo II inhibitor	0.57
MIBG	Other	0.30
Daunorubicin	Topo II inhibitor	0.20
MGBG	Other	0.16
Cladribine	Antimetabolite	−0.01
Paclitaxel	Tubulin-active	−0.03
Vincristine	Tubulin-active	−0.06
Topotecan	Topo I inhibitor	−0.19
5-Fluorouracil	Antimetabolite	−0.28
4-Hc[b]	Alkylating agent	−0.29
Cisplatin	Alkylating agent	−0.32
Cytarabine	Antimetabolite	−0.34

[a] R, Pearson's correlation coefficient.
[b] 4-Hc, hydroperoxy-cyclophosphamide (active metabolite of cyclophosphamide); Topo II, topoisomerase II.
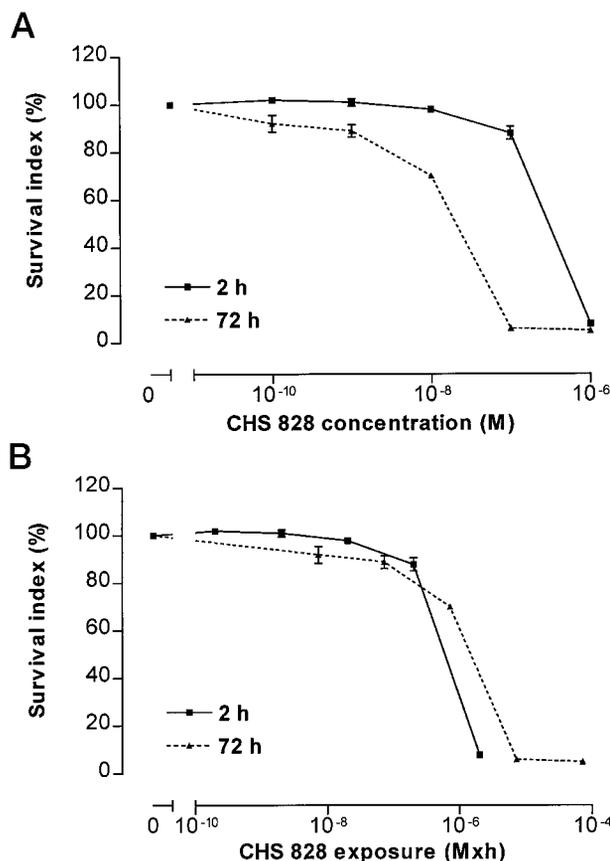
Fig. 2. Dependency of exposure time in CHS 828-induced cytotoxicity. RPMI 8226/S cells were cultured for 72 h, CHS 828 was present for 2 h (■) or for 72 h (▲). In *A*, the drug concentration is shown on the *X*-axis, whereas in *B*, the total drug exposure (AUC = drug concentration $\times$ exposure time) is used. The results are presented as mean values $\pm$ SE obtained from three independent experiments.

already in the week after the first dose. One mouse died during this experiment, whereas the remaining mice showed no loss of body weight after 7 weeks of dosing.

NYH Xenografts in Nude Mice. CHS 828 was administered daily at 3, 10, 20, and 30 mg/kg p.o. to mice with established tumors from day 14 to 28 after inoculation (Fig. 4*A*). The dose of 3 mg/kg was without effect, whereas tumor growth arrest was observed with 10 mg/kg. Twenty and 30 mg/kg caused regression of tumors. No weight loss was observed in any of the treatment groups.

Once-a-week administration of 100 mg/kg or 250 mg/kg of CHS 828 on day 14, 21, and 28 caused rapid regression of tumors (Fig. 4*B*). Regression of tumors continued throughout the observation period, which lasted for more than 6 months after cessation of treatment (Fig. 4*B*). In the group treated with 250 mg/week, regression was observed after the first dose, and at the end of the experiment, no tumors persisted. All of the mice survived, and no loss of body weight was observed in any of the mice.

The effects of CHS 828 were compared with those of reference chemotherapeutic agents in the NYH xenograft model (Table 4). CHS 828, etoposide, methotrexate, and cyclophosphamide were administered p.o., whereas paclitaxel was given s.c. All of the reference compounds were used at the maximally tolerated dosages. Of these, only paclitaxel showed significant activity with a *T/C* value of 48%. CHS 828 was extremely efficient, having a *T/C* value of 0%, at doses below the maximum tolerated dose.

H-460 Xenografts in Nude Mice. CHS 828 was administered daily at doses ranging from 20 to 100 mg/kg p.o. for 2 weeks from the day of the inoculation of tumor cells. No effect of CHS 828 was
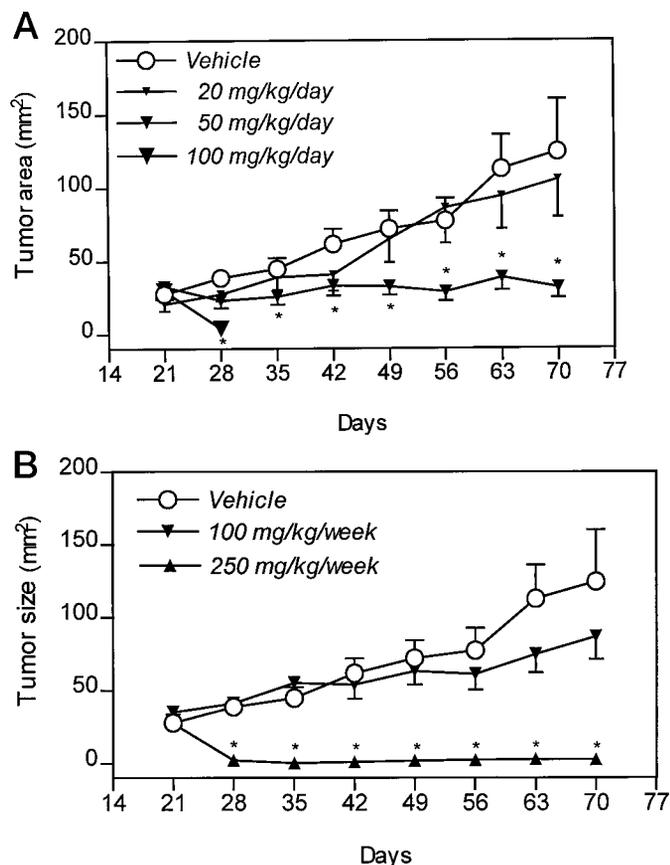
Fig. 3. Effects of CHS 828 in nude mice with MCF-7 breast cancer tumors. Ovariectomized and estrogen-substituted female mice were inoculated with 5×10^6 MCF-7 cells in both flanks. CHS 828 was administered p.o. from day 21 to day 70. *A*, effects on tumor size after treatment once daily; *B*, effects after treatment once weekly.

CHS 828 were studied in MCF-7 human breast cancer cells and in NYH human SCLC cells—the two cell types that were also used for the *in vivo* tumor studies in nude mice. The MCF-7 cells are broadly used as a representative of a classic estrogen-dependent tumor, with an intact p53-dependent repair mechanism (25). The NYH cells are aggressive SCLC cells that have lost several tumor suppressor proteins such as pRB and p130 (26). The NYH cells are resistant to the alkylating agent 1,3-bis-(2-chloroethyl)-1-nitroso-urea (18).

In vitro, CHS 828 displayed a potency similar to that of the reference cytotoxic agents daunorubicin and paclitaxel. Comparative studies with normal cells were performed using fetal lung fibroblasts as normal counterparts for the SCLC cells. The choice of a normal cell type for comparative studies with the MCF-7 breast cancer cells was more difficult. We decided to use human endothelial cells stimulated by vascular endothelial growth factor, which, in addition, allowed us to gain information on the possible effects of CHS 828 on angiogenesis. In contrast to daunorubicin and paclitaxel, CHS 828 was found to have considerably less antiproliferative activity against the normal cell types than against the tumor cells. The results suggest a decreased risk of toxicity against normal tissues but also indicate that CHS 828 does not exert antiangiogenic effects at the level of endothelial cell proliferation.

The mechanism for induction of cell death by CHS 828 remains to be clarified. The assays used for the determination of the antiproliferative and cytotoxic effects of CHS 828 do not distinguish between
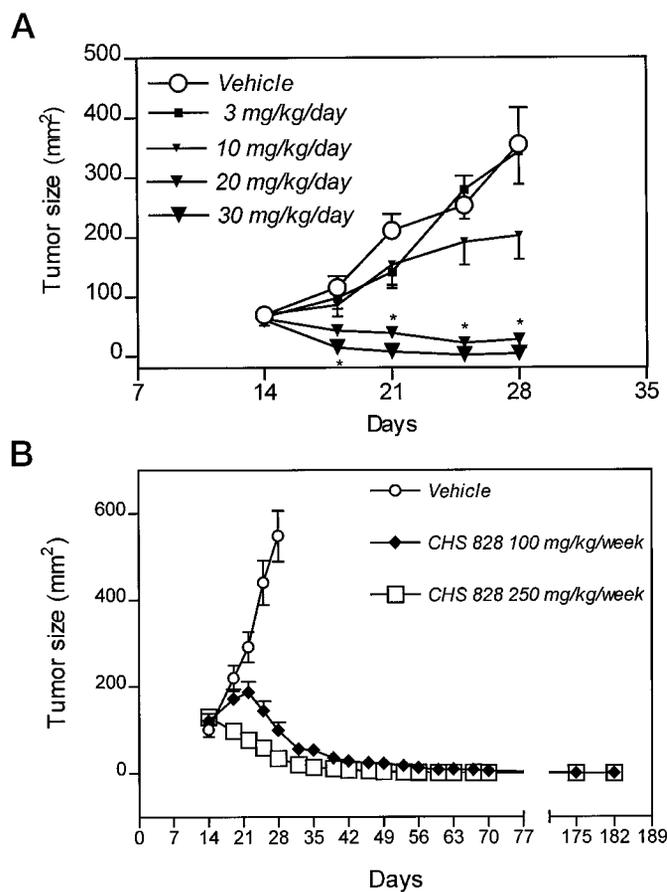
observed on tumor growth in this model of non-SCLC (data not shown).

Rat Tumors. In rats with i.p. implanted Yoshida hepatosarcoma cells, CHS 828 was found to prolong survival time by more than 100%, when administered at doses ranging from 20 to 50 mg/kg p.o. (given once daily or once every second day). In rats with s.c. Walker 256 breast carcinosarcomas, CHS 828 at 20 mg/kg p.o. once daily reduced tumor weight by 80%, compared with control tumors (data not shown).

DISCUSSION

CHS 828 is a recently discovered antitumor drug candidate, belonging to a group of pyridyl cyanoguanidines that hitherto have attracted pharmacological interest as hypotensive agents because of their activity as potassium channel openers. This activity is, however, not associated with the antitumor activity, and CHS 828 has no potassium channel-opening activity (1). Pyridyl cyanoguanidines have not previously been shown to exert antitumor effects, but two structurally related benzylguanidines (MIBG and MGBG) have shown cytotoxic effects in cultured cancer cells and antitumor responses in animals (23, 24). MIBG is a structural and functional analogue of epinephrine, and its radio-iodinated form has been used for scintigraphic detection and radiotherapy of tumors derived from adrenergic tissues (24). MIBG and MGBG were included as reference compounds in our cell line activity pattern studies, but no significant correlation with the profile of CHS 828 was observed.

In the present study, the antiproliferative and cytotoxic effects of

Fig. 4. Effects of CHS 828 in nude mice with NYH SCLC tumors. Female mice were inoculated with 1×10^7 NYH cells in both flanks. CHS 828 was administered p.o. from day 14 to day 28. *A*, effects on tumor size after treatment once daily; *B*, effects after treatment once weekly. Tumor growth was monitored for 6 months after the cessation of treatment (*B*).

Table 4 *Effects of CHS 828 in nude mice with NYH SCLC tumors: comparison with reference antineoplastic compounds*

Compound	CHS 828	Etoposide	Paclitaxel	Methotrexate	Cyclophosphamide
Dose (once daily)	20 mg/kg	20 mg/kg	10 mg/kg	5 mg/kg	10 mg/kg
Route	p.o.	p.o.	s.c.	p.o.	p.o.
T/C %[a]	0%	64%	48%	67%	86%
	$P < 0.05$	ns[b]	$P < 0.05$	ns	ns

[a] T/C %: Median tumor area in the treated group/median tumor area in the vehicle group after 14 days of treatment (from day 14 to day 28). (Statistics by Mann Whitney *U*-test).
[b] ns, not significant.

necrotic and apoptotic cell death. Additional studies, including DNA fragmentation and caspase 3 activity studies, have been undertaken.[3]

When the activity pattern of CHS 828 in a human cell panel (consisting of four sensitive parental cells lines, five drug-resistant sublines with different mechanisms of resistance, and one cell line with primary resistance) was correlated with the activity data from standard chemotherapeutic agents, correlation coefficients were low to medium. Previous studies using this system have shown that anticancer agents with closely related mechanisms of action generally show high correlation coefficients (>0.85; Refs. 3, 27, 28, 29). These results suggest a potentially new mechanism of action for CHS 828.

The studies in the drug-resistant cell lines indicated no sensitivity of CHS 828 to drug resistance mediated by MRP, GSH, topoisomerase II, or tubulin-associated MDR. CHS 828 was less cytotoxic against the Pgp-overexpressing RPMI 8226/Dox40 cell line than against the parental RPMI 8226/S line. This finding suggested a role for Pgp in protecting the cells from the effects of CHS 828. However, the lack of reversibility of CHS 828 resistance by Pgp-blockers indicates that this is not the case. Measurements of the rate of proliferation during the incubation time showed a slightly reduced rate of proliferation in the doxorubicin-resistant subline compared with the parental cell line, which might, at least to some extent, contribute to the observed difference in sensitivity to CHS 828.

Studies in the RPMI 8226/S cell line showed that CHS 828 induced less cytotoxicity when present in the incubation for only 2 h compared with continuous presence for 72 h. This difference disappeared after taking AUC (concentration × time) into account; the AUC indicated that the effect of CHS 828 was dependent on the total exposure rather than on exposure time alone. The fact that even a 2-h incubation with CHS 828 resulted in the induction of cytotoxicity after 72 h of incubation suggests that the activation of cell death pathways by CHS 828 is an early event that does not require prolonged exposure. These results together with the *in vivo* data also suggest that single or intermittent dosing schedules may be sufficient to obtain tumor responses in the clinical setting, at least from a strictly pharmacodynamic point of view.

CHS 828 was tested *in vivo* in a number of animal models, including nude mice xenografted with human cancer cells and rats with transplanted rodent tumors. CHS 828 was active after oral administration in most of the models. In the MCF-7 breast cancer model, a weekly dose of 250 mg/kg had a significant antitumor effect after the first dose, leading to regression of established tumors. The lower weekly dose of 100 mg/kg was less efficient. The daily dosing schedule was associated with significant toxicity at doses that were able to induce tumor regression. Long-term observation of treated animals was not performed because of mortality in both the vehicle- and the drug-treated groups after the depletion of the estrogen content of the implanted pellets, which occurred after day 60.

MCF-7 cells seem to be generally refractory to standard chemotherapy when grown as xenografts in nude mice. In a study of drug

activity in xenograft models from the National Cancer Institute (30), a panel of 12 standard drugs encompassing alkylating agents, DNA binders, antimetabolites, and mitotic inhibitors were tested. None of the 12 drugs was found to induce tumor regression in the MCF-7 model.

In accordance with the differences in sensitivity observed *in vitro* between the MCF-7 and NYH cells, NYH tumors in mice were more sensitive to CHS 828 than the MCF-7 tumors were. The weekly dosing schedule was again the most efficient one. A weekly dose of 250 mg/kg induced immediate tumor regression and, in contrast to the MCF-7 tumors, NYH tumors were also very sensitive to 100 mg/kg. Continued regression and no tumor regrowth were seen during a 6-month observation period after the third (and last) dose of CHS 828. No toxic effects of CHS 828 were noted in these experiments. These results are particularly interesting because the NYH xenograft model does not seem to be very sensitive to standard drugs. As shown in the present study, all of the reference chemotherapeutic drugs, including paclitaxel, induce little or no growth inhibition in this model despite being tested at their maximum tolerated doses. The significant tumor regression induced by CHS 828 in these xenograft models is, therefore, encouraging.

CHS 828 was also tested in nude mice bearing H-460 non-small cell lung tumors. These cells had previously shown sensitivity to CHS 828 *in vitro*, comparable with that seen with the MCF-7 cells (results not shown). Surprisingly, no effect of CHS 828 was seen on H-460 tumor growth in the nude mice. The reason for this treatment failure is not clear, but it may be associated with the aggressive growth rate of the tumors, which severely limits the life span of the animals and reduces the treatment time to a few weeks (starting at the time of inoculation of the tumor cells).

CHS 828 showed a substantial antitumor activity against rodent tumors, both in the form of i.p.-implanted Yoshida hepatosarcoma cells (ascitic tumors) and s.c.-growing Walker 256 breast carcinosarcomas.

The toxicological profile of CHS 828 is currently under investigation in rodents and dogs. The predominant toxicity seems to be gastrointestinal irritation with mucositis, diarrhea, and vomiting. In genotoxicity tests, CHS 828 has not shown any mutagenic or clastogenic effects.

In conclusion, CHS 828 is a new antineoplastic agent with a broad spectrum of activity against a variety of human cancer cells, including multidrug resistant cells. The mechanism of action of CHS 828 is still unknown, but it seems to be different from that of currently used chemotherapeutic agents. CHS 828 is presently in Phase I clinical studies.

REFERENCES

1. Schou, C., Ottosen, E. R., Björkling, F., Latini, S., Hjarnaa, P. V., Bramm, E., and Binderup, L. Novel cyanoguanidines with potent oral antitumour activity. Bioorg. & Med. Chem. Lett., *7:* 3095–3100, 1997.
2. Petersen, H. J., Kærgaard Nielsen, C., and Arrigoni-Martelli, E. Synthesis and hypotensive activity of *N*-alkyl-*N''*-cyano-*N'*-pyridylguanidines. J. Med. Chem., *21:* 773–781, 1978.
3. Dhar, S., Nygren, P., Csoka, K., Botling, J., Nilsson, K., and Larsson, R. Anti-cancer drug characterisation using a human cell line panel representing defined types of drug resistance. Br. J. Cancer, *74:* 888–896, 1996.

4. Sundström, C., and Nilsson, K. Establishment and characterisation of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer, *17:* 565–577, 1976.

5. Botling, J., Liminga, G., Larsson, R., Nygren, P., and Nilsson, K. Development of vincristine resistance and increased sensitivity to cyclosporin A and verapamil in the human U-937 lymphoma cell line without overexpression of the 170kDa P-glyco-protein. Int. J. Cancer, *58:* 269–274, 1994.

6. Dalton, W. S., Durie, B. G, Alberts, D. S., Gerlach, J. H., and Cress, A. E. Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. Cancer Res., *46:* 5125–5130, 1986.

7. Bellamy, W. T, Dalton, W. S, Gleason, M. C., Grogan, T. M, and Trent, J. M. Development and characterization of a melphalan-resistant human multiple myeloma cell line. Cancer Res., *51:* 995–1002, 1991.

8. Mulcahy, R. T., Bailey, H. H., and Gipp, J. J. Up-regulation of γ-glutamylcysteine synthetase activity in melphalan-resistant human multiple myeloma cells expressing increased glutathione levels. Cancer Chemother. Pharmacol., *34:* 67–71, 1994.

9. Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., and Deeley, R. G. Overexpression of a transporter gene in a multidrug-resistance human lung cancer cell line. Science (Washington DC), *258:* 1650–1654, 1992.

10. Mirski, S. E., Gerlach, J. H., and Cole, S. P. Multidrug resistance in a human small cell lung cancer cell line selected in Adriamycin. Cancer Res., *47:* 2594–2598, 1987.

11. Danks, M. K., Schmidt, C. A, Cirtain, M. C., Suttle, D. P., and Beck, W. T. Altered catalytic activity of and DNA cleavage by DNA topoisomerase II from human leukemic cells selected for resistance to VM-26. Biochemistry, *27:* 8861–8869, 1988.

12. Danks, M. K., Yalowich, J. C., and Beck, W. T. Atypical multidrug resistance in a human leukemic cell line selected for resistance to teniposide (VM-26). Cancer Res., *47:* 1297–1301, 1987.

13. Nygren, P., and Larsson, R. Verapamil and cyclosporin A sensitize human kidney tumor cells to vincristine in absence of membrane P-glycoprotein and without apparent changes in the cytoplasmic free Ca^{2+} concentration. Biosci. Rep., *10:* 231–237, 1990.

14. Larsson, R., Kristensen, J., Sandberg, C., and Nygren, P. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia, using a fluorometric microculture cytotoxicity assay (FMCA). Int. J. Cancer, *50:* 177–185, 1992.

15. Brünner, N., Bronzert, D., Vindeloev, L. L., Spang-Thomsen, M., and Lippmann, M. E. Effect on growth and cell cycle kinetics of estradiol and tamoxifen on MCF-7 human breast cancer cells grown *in vitro* and in nude mice. Cancer Res., *49:* 1515–1520, 1989.

16. Tomayko, M. M., and Reynolds, C. P. Determination of subcutaneous tumor size in athymic (nude) mice. Cancer Chemother. Pharmacol., *24:* 148–154, 1989.

17. Aabo, K., Vindeloev, L. L., and Spang-Thomsen, M. Clonal dominance between subpopulations of mixed small cell lung cancer xenografts implanted ectopically in nude mice. Eur. J. Cancer, *31A:* 222–229, 1995.

18. Aabo, K., Roed, H., Vindeloev, L. L., and Spang-Thomsen, M. A dominated and resistant subpopulation causes regrowth after response to 1,3-bis(2-chloroethyl)-1-nitrosurea treatment of a heterogeneous small cell lung cancer xenograft in nude mice. Cancer Res., *54:* 3295–3299, 1994.

19. Marusic, M., Allegretti, N., and Culo, F. The timing of cyclophosphamide therapy in tumor-bearing rats affects the resistance to tumor challenge in survivors. Experientia, *34:* 1355–1356, 1978.

20. Fujita, H., Sakurai, T., and Toyoshima, S. New antitumor amino acid derivative, A-748, evaluated in rat ascites hepatomas. Cancer Treat. Rep., *63:* 223–224, 1979.

21. Minne, H. W., Raue, F., Bellwinkel, S., and Ziegler, R. The hypercalcaemic syndrome in rats bearing the Walker carcinosarcoma 256. Acta Endocrinol., *78:* 613–624, 1975.

22. Boyd, M. R., and Paull, K. D. Some practical considerations and applications of the National Cancer Institute *in vitro* anticancer drug discovery screen. Drug Dev. Res., *34:* 91–109, 1995.

23. Smets, L. A., Bout, B., and Wisse, J. Cytotoxic and antitumour effects of the norepinephrine analogue *meta*-iodo-benzylguanidine (MIBG). Cancer Chemother. Pharmacol., *21:* 9–13, 1988.

24. Loesberg, C., Van Rooij, H., Romijn, J. C., and Smets, L. A. Mitochondrial effects of the guanidino group-containing cytostatic drugs, *m*-iodobenzylguanidine and meth-ylglyoxal bis (guanylhydrazone). Biochem. Pharmacol., *42:* 793–798, 1991.

25. Thompson, A. M., Steel, C. M., Chetty, U., Hawkins, R. A., Miller, W. R., Carter, D. C., Forrest, A. P., and Evans, H. J. *p53* gene mRNA expression and chromosome *17p* allele loss in breast cancer. Br. J. Cancer, *61:* 74–78, 1990.

26. Helin, K., Holm, K., Niebuhr, A., Eiberg, H., Tommerup, N., Hougaard, S., Poulsen, H. S., Spang-Thomsen, M., and Nørgaard, P. Loss of the retinoblastoma protein-related p130 protein in small cell lung carcinoma. Proc. Natl. Acad. Sci. USA, *94:* 6933–6938, 1997.

27. Fridborg, H., Nygren, P., Dhar, S., Csoka, K., Kristensen, J., and Larsson, R. *In vitro* evaluation of new anticancer drugs, exemplified by vinorelbine, using the fluoromet-ric microculture cytotoxicity assay on human tumour cell lines and patient biopsy cells. J. Exp. Ther. Oncol., *1:* 286–295, 1996.

28. Jonsson, E., Fridborg, H., Csoka, K., Dhar, S., Sundström, C., Nygren, P., and Larsson, R. Cytotoxic activity of topotecan in human tumour cell lines and primary cultures of human tumour cells from patients. Br. J. Cancer, *76:* 211–219, 1997.

29. Dhar, S., Nygren, P., Liminga, G., Sundström, C., de la Torre, M., Nilsson, K., and Larsson, R. Relationship between cytotoxic drug response patterns and activity of drug efflux transporters mediating multidrug resistance. Eur. J. Pharmacol., *346:* 315–322, 1998.

30. Plowman, J., Dykes, D. J., Hollingshead, M., Simpson-Herren, L., and Alley, M. C. Human Tumor Xenograft Models in NCI Drug Development. *In:* B. A. Teicher (ed.), Anticancer Drug Development Guide, pp. 101–125. New Jersey: Humana Press, 1997.