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CHS 828 Inhibits Neuroblastoma Growth in Mice Alone and in Combination with Antiangiogenic Drugs

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ABSTRACT

CHS 828 is a new chemotherapeutic drug, a pyridyl cyanoguanidine. CHS 828 has low toxicity and lacks known patterns of multidrug resistance. Here we report that oral, daily treatment with CHS 828 reduced the growth of SH-SY5Y human neuroblastoma tumors in male NMRI nu/nu mice by 82% without apparent toxicity. CHS 828 induced complete tumor regression for at least 5 weeks in four of nine animals (44%). Combination therapy with CHS 828 and the antiangiogenic drugs TNP-470 or SU5416 decreased neuroblastoma growth by a further 10 and 3%, respectively. Combination therapy induced tumor regression at d 4 with CHS plus TNP and d 6 with CHS plus SU5416, compared with d 14 with CHS 828 alone ($p < 0.05$), and complete tumor regression was seen in nine of 19 animals (47%). Combination treatment of CHS 828 and TNP-470 decreased the total viable tumor volume by 71% compared with treatment with CHS 828 alone. Our findings support CHS 828 as a promising new drug in treatment of childhood cancers. Furthermore, they imply efficiency of daily administration of nontoxic doses of chemotherapy, and a possible additive effect when chemotherapy is combined with angiogenesis inhibitors. **(*Pediatr Res* 51: 607–611, 2002)**

Abbreviations
VEGF, vascular endothelial growth factor
CgA, chromogranin A
TH, tyrosine hydroxylase
Lv, length of vessels per tumor volume
Sv, surface area of vessels per tumor volume
Vv, volume of vessels per tumor volume

Neuroblastoma is one of the most common solid malignant tumors of childhood. The prognosis varies with age at diagnosis, tumor stage, and certain biologic markers. In patients older than 1 y of age with an advanced tumor stage and dismal biologic markers, the outcome remains poor despite aggressive, multimodal therapy; hence, there is a clinical need for new treatment strategies (1).

A new treatment strategy for human solid tumors is antiangiogenic therapy (2). New drugs have been developed that specifically inhibit the formation of new blood vessels in solid tumors, thereby reducing tumor growth and metastasis (3). Two such antiangiogenic drugs in clinical trials are TNP-470, which inhibits endothelial cell proliferation (4), and SU5416, which blocks downstream signaling from VEGF receptor-2, an important pathway in angiogenesis (5). Experimental data suggest that antiangiogenic therapy potentiates chemotherapy (6).

Chemotherapeutic drugs can exert an antiangiogenic effect if administered in a low dose daily [antiangiogenic scheduling, (6, 7)]. Cyclophosphamide and vinblastine have been successfully tested in this respect in animal models, but not a new class of chemotherapeutic drugs, the pyridyl cyanoguanidines, such as CHS 828. CHS 828 has shown promising antitumor activity both *in vitro* and *in vivo* (8). However, the mechanism of its cytotoxicity is still unclear, and the question of whether CHS 828 has antiangiogenic properties has not been addressed. This study was performed to characterize the antitumor efficacy of CHS 828—given in a daily schedule orally—alone and in combination with one of the antiangiogenic agents TNP-470 or SU5416, in a xenograft model for human neuroblastoma.

METHODS

Drugs. All substances were suspended in their solvent immediately before injection. CHS 828 [*N*-(6-chlorophenoxy-

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hexyl)-*N*'-cyano-*N*''-4-pyridylguanidine; Leo Pharmaceutical Products, Ballerup, Denmark] was suspended in peanut oil. TNP-470 (Takeda Chemical Industries Ltd., Osaka, Japan) was suspended in 1% ethanol and 5% gum arabic in 0.9 mg/mL NaCl solution. SU5416 {3-[(2,4-dimethylpyrrol-5-yl) methylidenyl]-indolin-2-one; Sugen Inc., South San Francisco, CA, U.S.A.} was suspended in 0.5% (wt/vol) carboxy-methylcellulose sodium, 0.9% (wt/vol) NaCl, 0.4% (vol/vol) polysorbate 80, and 0.9% (vol/vol) benzyl alcohol in deionized water to the appropriate concentration.

Cells. SH-SY5Y cells, derived from a poorly differentiated, non-MYCN–amplified human neuroblastoma tumor (9), were used. These cells express VEGF and several other angiogenesis stimulators (10, 11). The cells were maintained in Eagle's minimal essential medium (SVA, Uppsala, Sweden) supplemented with 10% FCS, 1 μM L-glutamine, penicillin (100 U/mL), and streptomycin (50 μg/mL; Sigma Chemical Co., St. Louis, MO, U.S.A.). The cells were grown in humidified air (95%) and 5% CO_2 at 37°C.

Animals. Seventy-five male NMRI nu/nu mice (B & M, Ry, Denmark) were used for xenografting at an age of 6 wk (body weight, 25–30 g). The animals were housed at a temperature of 24°C, with a 12-h light, 12-h dark cycle. They were fed *ad libitum* with water and food pellets. All handling of the animals was carried out under aseptic conditions. The animal weight and general appearance were recorded every other day throughout the experiment. The experiment was approved by the regional ethics committee for animal research.

Xenografting. Subconfluent cells in triple-chamber culture flasks (Nunc, Roskilde, Denmark) were harvested by adding trypsin and 0.02% EDTA for 10 min. The cells were spun down and resuspended in medium to 150×10^6 cells/mL. The recipient mice were anesthetized with 2% Fluothane (Zeneca Ltd., Macclesfield, U.K.) supplemented with 50% N_2O in oxygen, and 0.2 mL of the cell suspension was injected s.c. in the right hind leg. Care was taken to avoid leakage from the puncture site and not to inject intramuscularly.

Measurement of tumor volume and administration of drugs. Tumor volume measurement began when the tumor became palpable (approximately 0.1 mL) and was then repeated every second day. The animal was anesthetized, and the longest diameter of the tumor and the width perpendicular to it were measured with a caliper. Tumor volume was then calculated as length $\times$ width^2 $\times$ 0.44. When a tumor reached a volume of 0.3 mL, the treatment began. Animals with a tumor volume less than 0.3 mL, 4 wk after injection of tumor cells, were excluded from the study. At the time when the animals reached this volume, they were randomized into treatment groups in the following order: control (n = 10), CHS 828 only (n = 9), TNP-470 only (n = 10), SU5416 only (n = 9), CHS 828 plus TNP-470 (n = 10), and CHS 828 plus SU5416 (n = 9). All animals received treatment within 2 to 4 wk after injection of tumor cells. Control animals were given vehicle without active drug. CHS 828 at a dose of 20 mg/kg was given daily by oral gavage with a 1.2-mm-diameter umbilical vessel catheter (Argyle, Sherwood Medical, St. Louis, MO, U.S.A.). SU5416 and TNP-470 at doses of 50 mg/kg and 15–30 mg/kg, respectively (see below for doses of TNP-470), were injected every second day s.c. in the neck.

Perfusion fixation and autopsy. Animals were anesthetized by an i.p. injection of 25 mg/kg of 2,2,2-tribromoethanol (Sigma Chemical Co.) in 2.5% 2-methyl-2-butanol (Sigma Chemical Co.) in 0.9 mg/mL NaCl solution. A cannula was inserted in the thoracic aorta, and the animal was perfusion-fixed with 4% paraformaldehyde in 1.47 mg/mL $NaH_2PO4 \cdot H_2O$, 12.62 mg/mL $Na_2HPO4 \cdot 2\ H_2O$, and 4.09 mg/mL NaCl in distilled H_2O (Millonig's buffer, pH 7.4, 37°C). The internal organs were examined for macroscopic metastases.

Blood analyses. Venous blood was drawn from the right atrium with a heparinized syringe before the perfusion fixation. The heparinized blood was put on ice and spun within 30 min at 2000 $\times$ g for 20 min. The plasma was then removed and stored at −20°C.

Determination of CHS 828 concentrations was performed by Leo Pharmaceuticals, by an HPLC method with UV detection at 277 nm (12).

Tissue analyses. The paraformaldehyde-fixed tumors were dehydrated and embedded in paraffin. Tissue sections, 3 μm thick, were cut and put on diaminoalkyl-silane treated glass slides, dehydrated, and stained immunohistochemically.

Cell proliferation was detected by Ki-67 nuclear antigen staining, which recognizes all cell cycle phases except G_0. The tissue sections were blocked in 0.3% hydrogen peroxide for 20 min, microwave treated (750 W) for 2 $\times$ 5 min in citrate buffer (21 mg/mL $C_6H_8O_7 \cdot H_2O$ in distilled H_2O, pH 6.0), and blocked for 10 min in 1% BSA in PBS consisting of 2 mg/mL KH_2PO_4, 2 mg/mL KCl, 80 mg/mL NaCl, and 28.8 mg/mL $Na_2HPO_4 \cdot 2\ H_2O$ in distilled H_2O. The primary antibody (MIB 1, monoclonal mouse anti-human Ki-67 nuclear antigen, Dianova, Hamburg, Germany) was applied at 1:100 for 1 h at room temperature. The secondary antibody (polyclonal, biotinylated rabbit anti-mouse immunoglobulins, E 354, Dako A/S, Glostrup, Denmark), 1:200, was applied for 30 min. For detection, ABComplex/HRP (K 355, Dako A/S), 1:200, was applied for 30 min. This was followed by development with diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) and counterstaining with Harris's hematoxylin. Sections of human breast carcinoma served as a positive control, and omission of the primary antibody as a negative control. All antibodies were diluted in 1% BSA in PBS.

Staining specific for neuroendocrine and adrenergic cells, *i.e.* neuroblastoma cells, was performed by CgA and TH immunohistochemistry (13). Tissue sections were blocked in 0.3% hydrogen peroxide for 20 min and microwave treated (750 W) for 2 $\times$ 5 min in citrate buffer for TH staining only. This was followed by blocking in 1% BSA and 10% normal rabbit serum for 30 min. The primary antibody (monoclonal mouse anti-human CgA, No. 1199 021, or monoclonal rabbit anti-TH, No. 1017381; Boehringer Mannheim GmbH, Mannheim, Germany) was applied at 1:500 for 30 min or 1:80 overnight, respectively. The secondary antibody (polyclonal, biotinylated rabbit anti-mouse immunoglobulins, E 354, Dako A/S) was applied at 1:80 and 1:200, respectively, for 30 min. For detection, ABComplex/HRP (K 355, Dako A/S), 1:200 for CgA and 1:100 for TH, was applied for 30 min, followed by

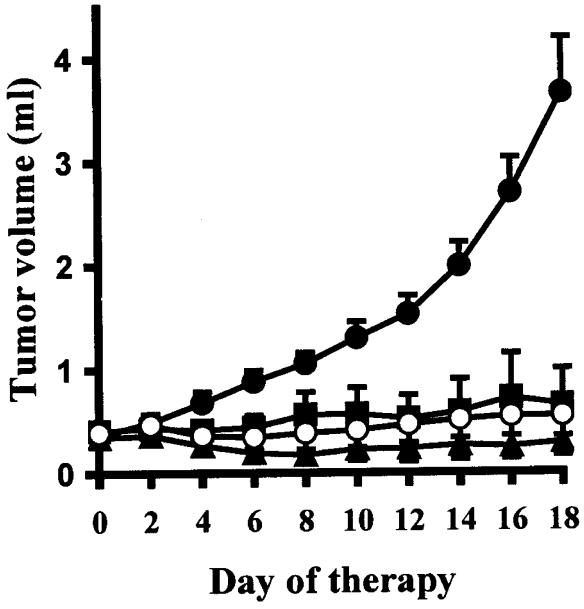

Figure 1. Neuroblastoma growth in nude mice: control ($n = 10$; ●), CHS 828 ($n = 9$; ■), CHS 828 + TNP-470 ($n = 10$; ▲), and CHS 828 + SU5416 ($n = 9$; ○). Results are expressed as mean ± SEM.

development with diaminobenzidine tetrahydrochloride and counterstaining with hematoxylin. Sections from human jejunum served as a positive control for CgA, and human adrenal medulla for TH. Omission of the primary antibody served as a negative control. All antibodies were diluted in 1% BSA in PBS.

Apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay (14). After deparaffinization, the sections were digested with proteinase K (Sigma Chemical Co., 20 μg/mL) for 50 min. After four washes in distilled water and blocking in 2.0% hydrogen peroxide in PBS, the Apoptag kit (Oncor, Gaithersburg, MD, U.S.A.) was applied according to the manufacturer's instructions. As a positive control, DNase I was added (20 min at 37°C) after blocking in hydrogen peroxidase, thus producing DNA breaks in virtually all cells. Replacement of TdT with water served as a negative control. The slides were counterstained with hematoxylin.

Vascular counts were performed on hematoxylin and eosin–stained tissue sections. The blood vessels are visible as punched out holes in stained sections after perfusion fixation, and hence no specific endothelial cell marker was necessary. Lv, Sv, and Vv were calculated as previously described (13).

Stereological quantifications. All sections were quantified by one observer in a blinded fashion. Structures were counted at ×400 with an eyepiece grid (506800, Leica, Singapore, Singapore) of 10 × 10 squares (0.25 × 0.25 mm). The grid was placed at random at the upper left corner of a section, and then systematically advanced every 1 to 3 mm (depending on the tumor size) in both directions with use of the microscope goniometer stage. Morphologic variables from at least 15 grids were quantified for each tumor. Areas with hemorrhage and apoptotic or necrotic cells were considered as nonviable and were excluded from analysis of other variables (13), but were used for calculation of the viable tissue fraction. The estimate of viable tissue fraction was used to calculate the total viable tumor volume (viable tissue fraction × tumor volume). The fractions of Ki-67–positive and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling–positive tumor cells were determined among 2,000 cells in each section.

Statistical methods. Data were processed in GraphPad Prism for Windows (GraphPad Software Inc, San Diego, CA, U.S.A.). Differences among groups in tumor growth and tissue variables were analyzed with the Kruskal-Wallis test and Dunn's multiple comparison test. Differences for which $p < 0.05$ were considered statistically significant.

RESULTS

Neuroblastoma growth. The tumor growth rate was significantly lower in all CHS 828 treatment groups than in the control animals (Fig. 1). This difference was statistically evident as early as d 4 in the combination treatment group CHS 828 + TNP-470 and d 6 in the CHS 828 + SU5416 group, but not until d 14 in the group treated with CHS 828 alone. Tumor growth was not significantly decreased by treatment with any of the antiangiogenic drugs when given alone (data not shown).

The experiment was terminated when the control animals' tumors reached a volume of 4.0 mL (approximately 10% of body weight). The mean tumor volume of treated animals divided by the mean tumor volume of control animals (the T/C ratio) on d 18 was 0.18 for CHS 828 alone, 0.15 for CHS 828 + SU5416, and 0.08 for CHS 828 + TNP-470. In each treatment group, some animals exhibited complete tumor regression at d 18: four of nine (44%) in the group given CHS 828 alone, four of nine (44%) in the CHS 828 + SU5416

Table 1. *Overview of experiments*

Variable	Controls	CHS 828	CHS 828 + SU5416	CHS 828 + TNP-470
n	10	9	9	10
Dosage (mg/kg)	0	20 daily	20 daily + 50 qod	20 daily + 15–30 qod
Tumor volume d 18 (mL)	3.7 ± 1.7	0.65 ± 1.0†	0.55 ± 0.59‡	0.29 ± 0.40†
Total viable tumor volume (mL)§	2.60 ± 1.36	1.43 ± 0.32	0.87 ± 0.32†	0.41 ± 0.30‡
T/C	1.0	0.18	0.15	0.080
Complete tumor regression	0/10	4/9	4/9	5/10

Mean ± SD. * $p \leq 0.05$, † $p \leq 0.01$, and ‡ $p \leq 0.001$, Kruskal-Wallis test.

§ Calculated on controls ($n = 9$), CHS 828 ($n = 3$), CHS 828 + SU5416 ($n = 3$), and CHS 828 + TNP-470 ($n = 4$). Comparison with controls or combination treatment groups compared with CHS 828 only indicated significance for CHS 828 + TNP-470 ($p < 0.05$).

T/C, treatment to control ratio for tumor volume on d 18.

group, and five of 10 (50%) in the CHS 828 + TNP-470 group (Table 1).

All animals gained weight and appeared healthy during the experiment, apart from the CHS 828 + TNP-470 group, which exhibited moderate weight loss. In this group, therefore, half the dose of TNP-470 (*i.e.* 15 mg/kg every other day) was given. The tumor volume at d 18 in the combination groups did not differ significantly from that in the group treated with CHS 828 alone. In animals from each CHS 828 treatment group, eight animals in all, that exhibited complete tumor regression, treatment was withheld for more than 40 d without regrowth of tumors.

Plasma CHS 828 concentrations. Plasma CHS 828 concentrations were 0.94 ± 0.68 μg/mL in 12 of 20 analyzed samples; eight samples had concentrations below the detection limit of 0.1 μg/mL.

Angiogenesis. Owing to the unforeseen tumor regression, the tissue variables could be quantified only in three to four tumors from each CHS 828 treatment group. Hence, lack of statistical significance may be owing to the small sample size. However, a consistent reduction of angiogenesis, as reflected in a lower Lv, Vv, and Sv than in controls, was observed in most treatment groups (Table 2). The mean tumor blood vessel diameter (46 ± 17 μm) was similar in all groups, indicating that the methodology was reproducible.

Tumor cell proliferation. The fraction of proliferating neuroblastoma cells was not significantly reduced in any of the treatment groups (Table 3).

Tumor cell apoptosis. The fraction of apoptotic neuroblastoma cells was increased (by 1.05- to 2.2-fold) in all treatment groups except the group given CHS 828 alone, in which it was paradoxically decreased. These differences did not reach significance (Table 3).

All tumors exhibited cells staining positively for CgA and TH, confirming that the tumors were of neuroblastoma origin. There was no difference in the amount of labeled cells among the treatment groups.

Viable tumor tissue fraction and total viable tumor volume. Antiangiogenic treatment alone decreased the viable tumor tissue fraction, although the decrease was not statistically significant. The total viable tumor volume was significantly decreased by 71% ($p < 0.05$) in animals treated with CHS 828 plus TNP-470 compared with treatment with CHS 828 alone (Table 1).

Table 2. *Quantification of tumor angiogenesis on d 18 of therapy*

Variable	Controls	CHS 828	CHS 828 + SU5416	CHS 828 + TNP-470
n	9	3	3	3
Lv (mm^{-2})	115 ± 45	68 ± 44	103 ± 45	40 ± 31
Difference (%)		−41	−10	−65
Vv (10^{-3})	0.17 ± 0.09	0.09 ± 0.06	0.12 ± 0.03	0.061 ± 0.02
Difference (%)		−48	−29	−64
Sv (mm^{-1})	4.2 ± 1.4	2.8 ± 1.2	4.2 ± 0.78	2.4 ± 0.86
Difference (%)		−33	0	−43

Mean ± SD. For definitions of Lv, Vv, and Sv, see Wassberg *et al.* (13). Differences were not significant ($p < 0.05$, Kruskal-Wallis test).

Table 3. *Quantification of tumor dynamics on d 18 of therapy*

Variable	Control	CHS 828	CHS 828 + SU5416	CHS 828 + TNP-470
n	9	3	3	3
Viable tissue (%)	66 ± 24	86 ± 24	75 ± 34	67 ± 47
Difference		30	14	1.5
Proliferating cells (%)	4.4 ± 3.2	4.0 ± 3.9	2.4 ± 0.88	3.6 ± 1.1
Difference		−9.1	−52	−18
Apoptotic cells (%)	6.3 ± 5.7	3.1 ± 1.6	6.6 ± 5.4	14 ± 12
Difference		−51	4.8	110

Mean ± SD. Differences were not significant ($p < 0.05$, Kruskal-Wallis test).

DISCUSSION

There is now accumulating clinical and experimental evidence that solid tumor growth is angiogenesis dependent, in that an increase in the tumor cell population has to be preceded by an increase in nutritive blood vessels (2). This hypothesis is supported by the findings that rapidly growing and metastasizing human tumors exhibit more blood vessels than do less aggressive tumors (15, 16) and that circulating levels of angiogenic peptides are elevated in patients with disseminated disease (17–19). Neuroblastoma has a high expression of angiogenesis stimulators (10). Furthermore, correlation of angiogenesis with poor outcome in neuroblastoma patients has been shown (20). Thus, antiangiogenic therapy may improve the long-term outcome in patients with this tumor. The tumor vasculature may be targeted therapeutically, either by specific inhibitors of endothelial proliferation and migration, or by cytotoxic drugs given at a low dose continuously (antiangiogenic scheduling), or by a combination of these approaches (6, 7, 21, 22). SH-SY5Y neuroblastoma cells xenografted to nude mice will inevitably kill the animals within 3 wk after tumor take. We have shown here that a new chemotherapeutic drug, CHS 828, administered daily, efficiently inhibits neuroblastoma growth in our xenograft model. When CHS 828 is combined with two specific antiangiogenic drugs, tumor regression occurs more rapidly and decreases the total viable tumor volume of the tumors.

CHS 828 is a pyridyl cyanoguanidine with potent antitumor activity both *in vitro* and *in vivo*. It shows low correlation with the activity patterns of known anticancer drugs, and of known patterns of multidrug resistance (8). Interestingly, CHS 828 has structural similarities to the catecholamine precursor metaiodobenzylguanidine, which is known to accumulate in neuroblastomas and is used for imaging and radiotherapy (23, 24). We are currently investigating whether it is CHS 828 as such or an antiangiogenic schedule that reduces neuroblastoma growth. Also, we are addressing the issue of cyclic therapy, *i.e.* the question of whether regressed tumors regrow, and if so, whether they are still sensitive to CHS 828 therapy. Yet another question at issue is whether the antiangiogenic scheduling can be more effective if the drug is given continuously by an implantable osmotic minipump.

TNP-470 is a synthetic analog to the fungal antibiotic fumagillin, and inhibits endothelial cell migration and proliferation (4, 25–27). It has been shown to inhibit tumor growth and

metastasis in both murine tumors and human xenografts *in vivo*, but not to induce tumor regression. TNP-470 has been shown to reduce angiogenesis and to increase tumor necrosis by increased differentiation and apoptosis in experimental neuroblastoma [agonal differentiation (13)]. TNP-470 alone in mice reduces angiogenesis consistent with our previous results in rats (22) but with a lower treatment to control ratio of 0.69 (unpublished data). TNP-470 in combination with CHS 828 induced complete tumor regression in five of 10 animals compared with four of nine animals with CHS 828 treatment alone. TNP-470 in combination with CHS 828 gave an earlier tumor regression and decreased the total viable tumor tissue volume. Thus TNP-470 increases the efficacy of CHS 828 treatment.

SU5416 is an angiogenesis inhibitor currently in phase 3 clinical trials. SU5416 inhibits VEGF signaling by acting as a VEGF receptor-2 tyrosine kinase inhibitor. It is an ATP competitive inhibitor of the kinase (5). A variety of human tumors express VEGF and its receptors. VEGF is considered to be one of the key mediators of angiogenesis (28). Our experimental neuroblastomas express VEGF on both the mRNA and protein levels (unpublished data). When SU5416 was combined with CHS 828, complete tumor regression was seen in four of nine animals, but did not decrease the total viable tumor volume, although an earlier tumor regression than for CHS 828 treatment alone was seen when compared with controls.

We conclude that the new therapeutic drug CHS 828 alone, as well as in combination with antiangiogenic drugs, is a potent inhibitor of experimental neuroblastoma growth *in vivo* in doses not causing systemic toxicity. The additional facts that our study and previous investigations of CHS 828 have shown no patterns of multidrug resistance and low toxicity make it a promising new drug in treatment of childhood cancers.

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