Biochemical Pharmacology, Vol. 60, pp. 839–849, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00382-8

Early Stimulation of Acidification Rate by Novel Cytotoxic Pyridyl Cyanoguanidines in Human Tumor Cells: Comparison with *m*-Iodobenzylguanidine

Sara Ekelund,† Gunnar Liminga,* Fredrik Björkling,‡ Erik Ottosen,‡ Charlotte Schou,‡ Lise Binderup‡ and Rolf Larsson**

*Division of Clinical Pharmacology, Akademiska Hospital, S-751 85 Uppsala, Sweden; and ‡Leo Pharmaceutical Products, DK-2750 Ballerup, Denmark

ABSTRACT. CHS 828, a newly recognized pyridyl cyanoguanidine, has shown promising antitumor activity both *in vitro* and *in vivo* and is presently in early phase I clinical trial in collaboration with EORTC. In this study, the effects of CHS 828 and a series of analogues on extracellular acidification and cytotoxicity were compared with those of *m*-iodobenzylguanidine (MIBG) in human tumor cells. The extracellular acidification rate was measured using the Cytosensor microphysiometer, and determination of cytotoxicity and proliferation was [^{14}C] performed by the fluorometric microculture cytotoxicity assay (FMCA) and measurement of [^{14}C]thymidine and leucine uptake. CHS 828 significantly increased the acidification rate during the first 15–24 hr in a concentration-dependent manner. This effect was abolished by removal of glucose from the medium, substituted with 10 mM of pyruvate, indicating stimulated glycolysis as the source of the increased acidification rate. However, CHS 828 induced cytotoxicity at concentrations well below those that affected the rate of acidification; when a series of closely related pyridylguanidine analogues were tested and compared, no apparent relationship between cytotoxicity and acidification could be discerned. Furthermore, comparable increases in the acidification rate were evident in one subline with high-grade resistance to the cytotoxic actions of CHS 828. The results indicate that CHS 828 may share the inhibitory actions of MIBG on mitochondrial respiration with a subsequent increase in glycolysis and acidification rate. However, this mechanism of action appears neither necessary nor sufficient to fully explain the cytotoxic actions of CHS 828 in human tumor cells, actions which remain to be mechanistically clarified. BIOCHEM PHARMACOL **60**;6:839–849, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cyanoguanidine; microphysiometry; extracellular acidification; cytotoxicity

A new cyanoguanidine, *N*-(6-(4-chlorophenoxy)hexyl)-*N′*-cyano-*N″*-4-pyridylguanidine, CHS 828, recently discovered in a routine screening program in rats [1, 2], demonstrated interesting properties as a potential anticancer agent, both *in vitro* and *in vivo* [2, 3]. Notably, potent antitumor activity could be achieved in several *in vivo* xenograft models in the absence of apparent toxicity [3]. The mechanism of action for CHS 828 is not yet known, but the profile of activity does not correlate to any of the standard cytotoxic drugs [3]. At present, CHS 828 is undergoing phase I clinical trials.

Due to their antiproliferative activity, other compounds containing guanidino groups have shown antitumor activity, in particular MIBG§ [4]. Iodinated MIBG is used as a radiopharmaceutical for diagnosis and targeted radiotherapy [5, 6]. The drug itself, a structural and functional analogue of natural norepinephrine, is known to be cytotoxic in micromolar concentrations in human tumor cell lines [4]. MIBG is known to inhibit mitochondrial respiration and ATP production, causing a reactive stimulation of the glycolysis in cells [7, 8].

Recently, CHS 828 was shown to produce an early increase in extracellular acidification in a tumor cell line.‖ The present study was undertaken to further elucidate the potential relationship between the metabolic effects and cytotoxicity. CHS 828 was compared to MIBG in human tumor cell lines and primary cultures of human tumor cells, using the FMCA, kinetic measurements of DNA and protein synthesis, and the Cytosensor microphysiometer.

MATERIALS AND METHODS

Experimental Drugs

CHS-828 (*N*-(6-(4-chlorophenoxy)hexyl)-*N′*-cyano-*N″*-4-pyridylguanidine) and seven structurally related pyridyl cyanoguanidines were supplied by Leo Pharmaceutical Products in stock solutions, dissolved in DMSO of 10 mM,

† Corresponding author. Dr. Sara Ekelund, Dept. of Clinical Pharmacology, Uppsala University, Akademiska Hospital, S-751 85 Uppsala, Sweden. Tel. +46-18-611-52-50; FAX +46-18-51-92-37; E-mail: Sara.Ekelund@medsci.uu.se

§ *Abbreviations:* MIBG, *m*-iodobenzylguanidine; and FMCA, fluorometric microculture cytotoxicity assay.

Received 28 September 1999; accepted 25 February 2000.

‖ Ekelund S and Larsson R, unpublished observation.

and stored frozen at −20°. The drugs were diluted ten times with 33% DMSO and sterile water and further dilutions were made using sterile PBS. MIBG was purchased from Sigma as a powder. The drug was dissolved in 10% DMSO and sterile water to a stock solution of 1 mg/mL, kept in −70°, until further dilution in PBS.

Synthesis of CHS 828 and Its Analogues

CHS 828, compound (**2**), and the new analogues (**4**) to (**7**) (Fig. 1) were prepared by coupling 4-amino pyridine with *S*,*S*′-dimethyl *N*-cyano-dithio-iminocarbonate to give *S*-methyl-*N*-cyano-*N*′-4-pyridylisothiourea as a key intermediate. This was then coupled with a series of primary amines (R-NH_2, cf. Fig. 1) to give the cyanoguanidines in good to excellent yield [1]. The primary amines were prepared by standard methods in good yield from commercially available starting materials. Analogue (**8**) was prepared in similar fashion, but starting from 5-amino-2-methoxy pyridine. Analogue (**3**) was prepared from the corresponding thiourea by reaction with excess cyanamide and *N*,*N*′-dicyclohexylcarbodiimide as previously described [2].

All melting points were uncorrected and recorded using a Büchi 535 apparatus. ^{1}H and ^{13}C NMR data were recorded using a Bruker ARX 300 (300 MHz) spectrometer and chemical shift values were quoted relative to tetramethylsilane. TLC was performed on 0.25-mm Merck silica gel 60-F_{254} plates and flash chromatography on 230–400-mesh Merck silica gel 60. All solvents used were dried over activated 4 Å molecular sieves.

General procedure for synthesis of cyanoguanidines, exemplified by:

N-Cyano-N′-4-pyridyl-N″-(8-tetrahydropyranyloxyoctyl) guanidine (4)

8-Tetrahydropyranyloxy-octylamine (841 mg, 3.66 mmol), *S*-methyl *N*-cyano-*N*′-4-pyridylisothiourea (825 mg, 3.03 mmol) (1), triethylamine (0.8 mL, 5.7 mmol), and 4-(dimethylamino)pyridine (5 mg) were dissolved in pyridine (5.0 mL). The reaction mixture was stirred at 55–60° for 72 hr and then cooled to room temperature. Toluene was added and the reaction mixture was concentrated *in vacuo* to give the crude product which was purified by flash chromatography using a mixture of CH_2Cl_2/MeOH/NH_3(aq.) 98:2:0.2 as eluent and then crystallized from ethyl acetate to afford the title compound as a white amorphous solid. Yield 815 mg, (72%). Mp. 107–108°. ^{1}H NMR (DMSO-d_6) δ: 9.37 (bs, 1H), 8.38 (m, 2H), 7.83 (t, 1H), 7.21 (m, 2H), 4.52 (m, 1H), 3.72 (m, 1H), 3.60 (m, 1H), 3.47–3.19 (m, 4H), 1.80–1.15 (m, 18H). ^{13}C NMR (DMSO-d_6) δ: 157.3, 150.0, 146.1, 116.5, 114.6, 98.0, 66.6, 61.3, 41.8, 30.4, 29.2, 28.8, 28.7, 28.7, 26.2, 25.7, 25.1, 19.3.

The following compounds were prepared by the above general procedure with minor modifications starting from the respective primary amines. The primary amines were obtained in few steps from commercially available starting materials in good yields by standard reactions.

N-(8-*t*-Butoxycarbonylaminooctyl)-N′-cyano-N″-4-pyridylguanidine (5)

Mp. 132.4–135.3°. ^{1}H NMR (DMSO-d_6) δ: 9.50 (bs, 1H), 8.38 (m, 2H), 7.90 (bs, 1H), 7.21 (m, 2H), 6.75 (t, 1H), 3.26 (bt, 2H), 2.89 (q, 2H), 1.60–1.12 (m, 12H), 1.37 (s, 9H). ^{13}C NMR (DMSO-d_6) δ: 157.3, 155.5, 149.8, 146.1, 116.4, 114.5, 77.2, 41.7, 29.4, 28.6, 28.6, 28.2, 26.1, 26.0.

N-Cyano-N′-(9-diethylphosphinoyloxynonyl)-N″-4-pyridylguanidine (6)

Mp. 79.0–81.8°. ^{1}H NMR (DMSO-d_6) δ: 9.38 (bs, 1H), 8.38 (m, 2H), 7.84 (t, 1H), 7.21 (m, 2H), 4.06–3.87 (m, 6H), 3.26 (q, 2H), 1.59 (m, 2H), 1.52 (m, 2H), 1.37–1.25 (m, 10H), 1.24 (dt, 6H). ^{13}C NMR (DMSO-d_6) δ: 157.2, 149.9, 146.0, 116.4, 114.5, 66.8, 63.0, 41.7, 29.6, 28.7, 28.6, 28.5, 28.3, 26.0, 24.8, 15.9.

N-(6-(4-Chlorobenzoyl)hexyl)-N′-cyano-N″-4-pyridylguanidine (7)

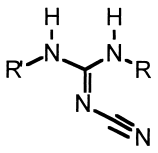
Mp. 138.5–140.0°. ^{1}H NMR (DMSO-d_6) δ: 9.35 (bs, 1H), 8.38 (m, 2H), 7.97 (m, 2H), 7.82 (bt, 1H), 7.58 (m, 2H), 7.22 (bs, 2H), 3.27 (q, 2H), 3.01 (t, 2H), 1.61 (m, 2H), 1.54 (m, 2H), 1.34 (m, 4H). ^{13}C NMR (DMSO-d_6) δ: 199.0, 157.1, 150.0, 145.7, 137.8, 135.3, 129.7, 128.7, 116.3, 114.5, 41.7, 37.7, 28.5, 28.1, 25.9, 23.5.

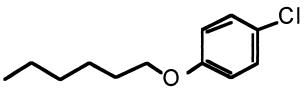
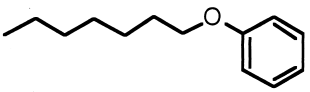
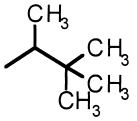
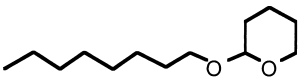
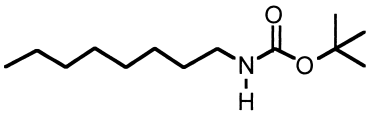
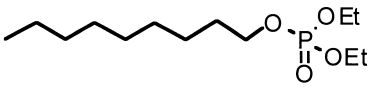
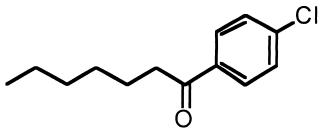
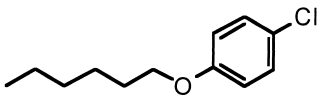
N-(6-(4-Chlorophenoxy)hexyl)-N′-cyano-N″-(2-methoxy-5-pyridyl)guanidine (8)

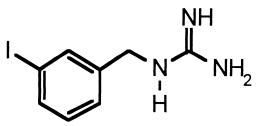
S-Methyl *N*-cyano-*N*′-(2-methoxy-5-pyridyl)-isothiourea was used in place of s-methyl *N*-cyano-*N*′-4-pyridylisothiourea. Mp. 127.5–128.3°. ^{1}H NMR (DMSO-d_6) δ: 8.82 (bs, 1H), 8.00 (d, 1H), 7.55 (dd, 1H), 7.30 (m, 2H), 7.08 (bt, 1H), 6.94 (m, 2H), 6.81 (d, 1H), 3.95 (t, 2H), 3.84 (s, 3H), 3.16 (q, 2H), 1.70 (m, 2H), 1.50 (m, 2H), 1.41 (m, 2H), 1.31 (m, 2H). ^{13}C NMR (DMSO-d_6) δ: 161.2, 158.6, 157.4, 143.4, 137.0, 129.1, 127.9, 123.9, 117.3, 116.1, 110.3, 67.7, 53.2, 41.2, 28.7, 28.4, 25.8, 25.1.

S-Methyl N-cyano-N′-(2-methoxy-5-pyridyl)-isothiourea

To 5-amino-2-methoxypyridine (5.0 g, 40 mmol) and *S*,*S*′-dimethyl *N*-cyano-dithio-iminocarbonate (6.7 g, 46 mmol) in dimethylformamide (40 mL), NaH (1.9 g, 60% dispersion in mineral oil, 0°) was slowly added. The mixture was stirred at 0° for 6 hr, then at room temperature for 72 hr after which ether (165 mL) and petroleum ether (33 mL) were added. After decanting of the supernatant phase, the oily residue was stirred twice with ether:petroleum ether (5:1) (165 mL). After decanting, the resulting semisolid was triturated with ice water (100 mL) and filtered. The stirred ice-cooled filtrate was treated with glacial acetic acid (3.7 mL), and the precipitate was collected by filtration and washed with water and small portions of ether. The crude product was crystallized from methanol by addition of a small amount of water. Yield (4.9 g, 55%). Mp. 167.2–168.0°. ^{1}H NMR (DMSO-d_6) δ: 10.13 (bs, 1H), 8.17 (d, 1H), 7.75 (dd, 1H), 6.87 (d, 1H), 3.86 (s, 3H), 2.67 (bs, 3H). ^{13}C NMR (DMSO-d_6) δ: 171.2, 161.7, 143.1, 136.6, 127.9, 114.6, 110.2, 53.4, 14.6.



Compound	R	R'
CHS 828		4-pyridyl
2		4-pyridyl
3		4-pyridyl
4		4-pyridyl
5		4-pyridyl
6		4-pyridyl
7		4-pyridyl
8		(2-methoxy)-5-pyridyl



MIBG

FIG. 1. The chemical structure of *N*-(6-chlorophenoxy-hexyl)-*N'*-cyano-*N''*-4-pyridylguanidine, CHS 828, a series of structurally related CHS 828 analogues, and the guanidine MIBG.

Cell Lines and Patient Tumor Samples

The human histiocytic lymphoma cell line U-937 GTB [9] was used for most experiments, but the CHS 828-resistant subline U-937 GTB/CHS, the myeloma cell line RPMI 8226/S, the small cell lung cancer cell line NCI-H69, and the leukemic cell line CCRF-CEM were also used. The cell lines were grown in cell culture flasks kept in an atmosphere containing 5% CO_2 and at a temperature of 37°.

Cell culture medium RPMI-1640 (Sigma-Aldrich) was supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 2 mM glutamine, 50 μg/mL of streptomycin, and 60 μg/mL of penicillin (HyClone). Growth and morphology of the cell lines were monitored two or three times per week. The resistance pattern of U-937 GTB/CHS was routinely confirmed in control experiments every second month. Screening for mycoplasma was done on a yearly basis.

Experiments were also performed using tumor samples from patients with chronic lymphatic leukemia (CLL) and ovarian carcinoma. Cell preparations of both hematological and solid tumors have been described in detail earlier [10, 11].

The Cytosensor Method

To investigate the metabolic activity of cells, a silicon microphysiometer, the Cytosensor® (Molecular Devices), was used to detect the excretion rate of metabolic products. The acidification of the extracellular environment is due to lactic acid and carbon dioxide, metabolic byproducts of cells [12, 13]. Cells are retained by the Cytosensor in a flow chamber in aqueous diffusive contact with the surface of a pH-sensitive silicon chip. The chip together with a reference electrode and other components form a light-addressable potentiometric sensor (LAPS) that is used to detect small changes in the extracellular acidification rate [12, 14, 15]. Once per second, the LAPS makes a voltage measurement that is linearly related to pH.

The Cytosensor consists of two units with altogether eight parallel measurement channels. Each channel contains a microvolume flow chamber in which the cells were immobilized during experiments using a mixture of 25% agarose (Molecular Devices) and 75% cell suspension. Non-adherent cells need to be trapped in a matrix inside the cell capsule to ensure that they are not washed away to one side by the flow of medium through the sensor chamber. The cell suspension–agarose mixture is retained in a disk-shaped region 50 μm high and 6 mm in diameter between two track-etched microporous polycarbonate membranes. The sensing surface of the LAPS contacts the lower membrane and the culture medium flows tangentially across the surface of the upper membrane [13]. Culture medium is pumped from a reservoir by a peristaltic pump and passes through a debubbler–degasser, a selection valve, and finally through the flow sensor chamber. The cell concentration used in each chamber was 1.5×10^5 in a volume of 10 μL cell suspension–agarose mixture.

The experimental medium was a low-buffering capacity medium (National Veterinary Institute) with no HEPES or bicarbonate, but with 6 mL/L of 4 M NaCl to preserve the osmotic balance. The medium also contained 10 ml/L of 200 mM L-glutamine and 10 ml/L of penicillin and streptomycin. Medium, with or without drug, was constantly pumped through the chamber at a flow rate of 100 μL/min. In order to measure the extracellular acidification rate,

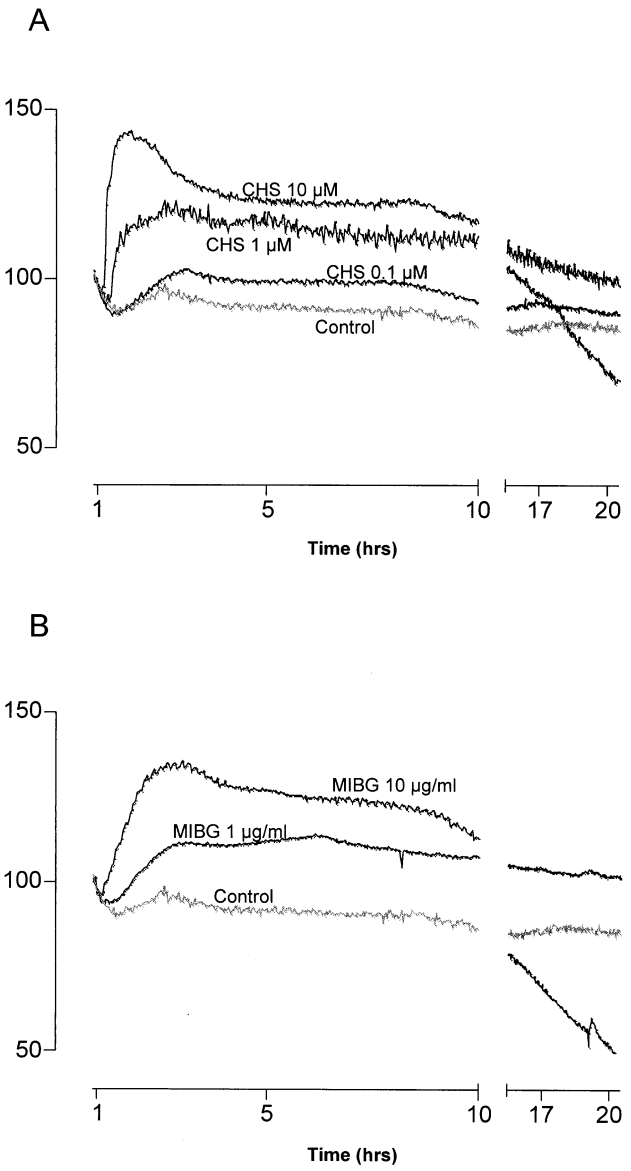


FIG. 2. Concentration–response curves for the guanidine CHS 828 (A) and MIBG (B) using the Cytosensor microphysiometer for continuous drug exposure for approximately 20 hr.

there were brief halts (30 sec) in the medium flow. During this period, acidic metabolites were built up with a consequent decrease in extracellular pH. These acidifications are typically <0.1 pH units and cause no significant perturbations of cell physiology [13]. The Cytosensor measures the rate of proton excretion from 10^4 to 10^6 cells and the noise of the instrument is low, between 0.0005 and 0.001 pH units. A decrease of 61 mV is approximately equal to an acidification of 1 pH unit at 37°. Numerically, 1 μvolt/sec is close to 1×10^{-3} pH units per min [13]. The halt was followed by 90 sec of perfusion, and this 120-sec pump cycle was used throughout the 20-hr long experiments. During fluid flow, the sensor output is stable and reflects a pH near that of the culture medium entering the flow chamber.

Drugs were diluted in the medium and perfused through a second channel after 1 hr of baseline establishment. The

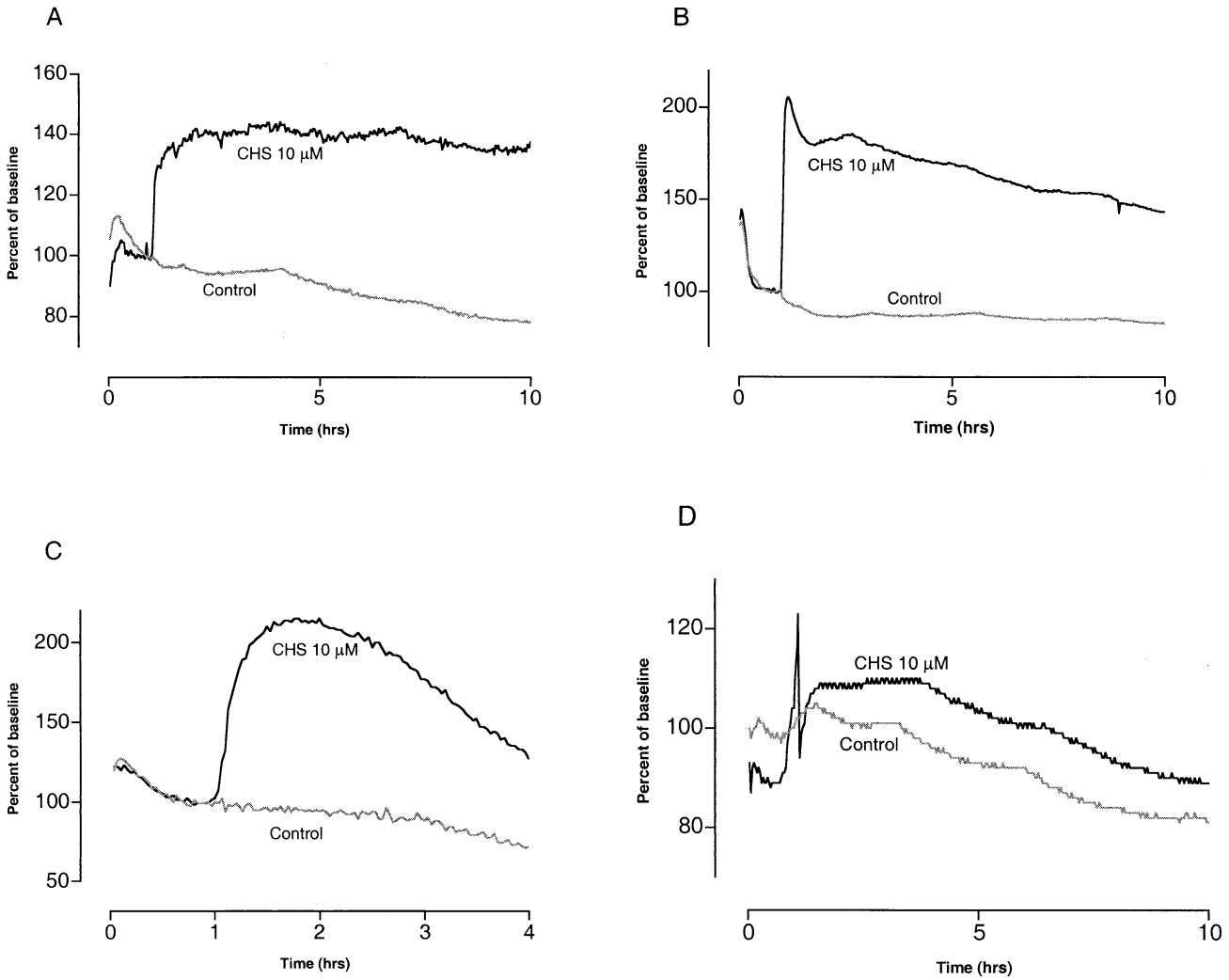


FIG. 3. Effect of CHS 828 on the acidification rate in different tumor cell systems: the small cell lung cancer cell line NCI-H69 (A), the leukemic cell line CCRF-CEM (B), a CLL patient sample (C), and an ovarian carcinoma patient sample (D).

acidification rate was calculated by the Cytosoft program as $-\mu V$/sec and was later normalized to a percent value.

FMCA: Reagents and Procedure

Viability of cells was determined by the FMCA. The assay is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes. This method has been described in detail previously [10]. Experimental V-shaped, 96-well microtiter plates are prepared in advance, using a pipetting robot (Pro/Pette; Perkin Elmer) that dispenses 20 μL of drug solution at ten times the final experimental concentration into the plates. Each drug is dispensed in triplicate. The plates are then kept frozen at −70° until further use.

Cell suspension, 20×10^3 cells/well and 180 μL/well, was added to the plates before incubation for 72 hr at 37° and 5% carbon dioxide. Blank wells received only culture medium, while control wells contained cell suspension and PBS but no drug. Medium was not changed during this incubation period and drugs and medium were subsequently removed and the cells washed once with PBS. One hundred microliters of fluorescein diacetate (FDA), 10 μg/mL, was added to each well and the plates were incubated for an additional 40 min. The generated fluorescence in each well was then measured in a Fluoroscan II (Labsystems OY). FMCA cytotoxicity results have previously been shown to correlate well with those of other well-established non-clonogenic cytotoxicity assays [16, 17].

Measurement of pH

Measurement of intracellular pH was performed using a fluorescence spectrophotometer F 2000 (Hitachi) and the tetraacetoxymethyl (AM) ester of BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein). The AM ester penetrates into cells where it is hydrolyzed to the highly fluorescent BCECF by non-specific esterases. BCECF is a poor permeate with a pK_a value close to 7. Within the

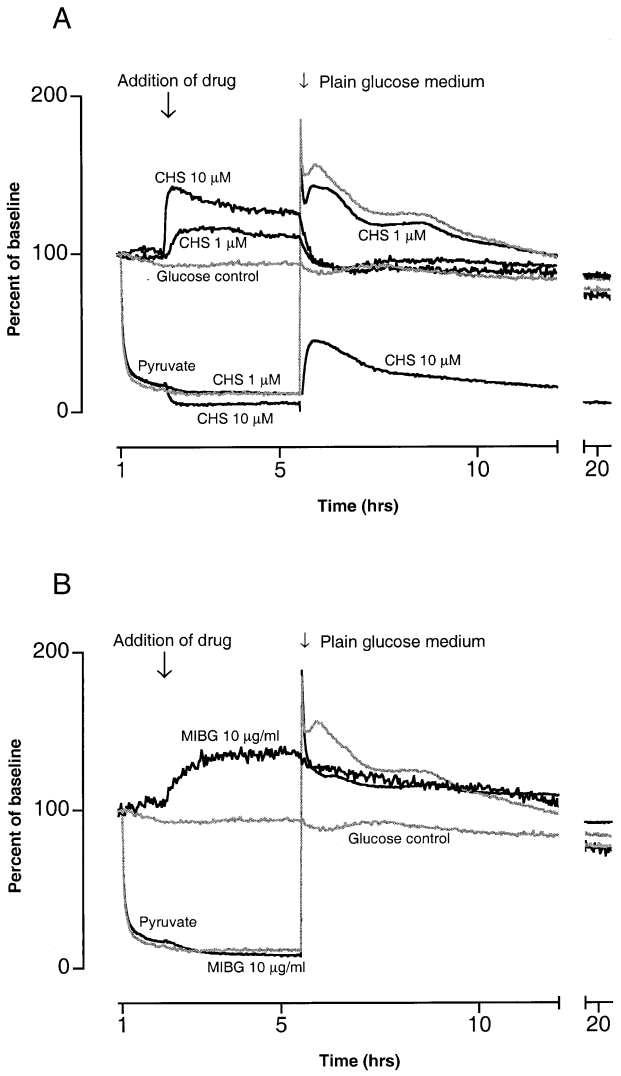


FIG. 4. Effect of CHS 828 (A) and MIBG (B) on the acidification rate during exposure of glucose-containing medium or medium without glucose but supplemented with 10 mM pyruvate. Results are presented as one experiment out of three.

range of pH 6.5–7.5, there is a linear relationship between its fluorescence intensity and pH, and BCECF can therefore serve as a cytoplasmic fluorescent pH-sensitive indicator.

In each experiment, 10×10^6 cells were suspended in 5 mL of culture medium and loaded with BCECF-AM during 30 min of incubation at 37° with an equal amount of medium containing 2 μM dye. The cells were centrifuged and washed once with medium before dilution in a 10-mL standard HEPES buffer (Sigma-Aldrich) supplemented with 100 mM glucose and 7.5 mM calcium chloride, pH adjusted to 7.4. Cell suspension (2 mL) was incubated with constant stirring at 37° in a 1-cm cuvette in the fluorescence spectrophotometer with excitation and emission wavelengths set at 490 and 520 nm, respectively. All additions to the cell suspensions were made in a volume of 20 μL. The fluorescence after each addition was monitored until stability was attained.

Measurements of DNA and Protein Synthesis

Protein and DNA synthesis were measured with a Cytostar-T plate, available in Amersham's "*In Situ* mRNA Cytostar-T assay" kit, (Amersham International plc), a pre-made scintillating 96-well microtiter plate with scint fluid molded into the bottom of the wells [18, 19]. Cells were suspended in fresh media containing 111 nCi/mL [^{14}C]thymidine (Amersham CFA.532: 56 mCi/mmol, 50 μCi/mL) for DNA synthesis experiments or 222 nCi/mL [^{14}C]leucine (Amersham CFB.183: 56 mCi/mmol, 50 μCi/mL) for protein synthesis experiments, yielding a final radioactivity in the wells of approximately 20 and 40 nCi, respectively. Aliquots of 180-μL cell suspension containing 50,000 U-937 GTB cells were added to each well, except for blank wells where only medium containing isotope was added. Radioactivity was measured with a Wallac 1450 MicroBeta trilux liquid scintillation counter, (Wallac OY) using MicroBeta Windows workstation software (Wallac OY). Data were collected at 0 through 48 hr as indicated (Fig. 7).

TABLE 1. A summary of the comparison of CHS 828, the seven related analogues (described in Fig. 1), and the cyanoguanidine MIBG with respect to Cytosensor data and cytotoxicity

Drug	Concentration (μM)	% of control at peak	% of control at 3 hr of drug exposure	IC_{50}	E_{max}	IC_{50} (RPMI 8226/S)	E_{max}
CHS 828	10	121.9	118.2	0.0022	3.9	0.039	13.6
2	10	111.6	108.7	2.4×10^{-5}	5.5	0.0023	10.9
3	10	—	100.9	>10	77.2	>10	92.5
4	10	110.9	115.0	2.9×10^{-5}	6.3	0.0032	14.5
5	10	108.7	110.6	1.9×10^{-5}	5.4	$<1 \times 10^{-3}$	14.7
6	10	114.2	114.8	$<1 \times 10^{-3}$	4.9	$<1 \times 10^{-3}$	10.7
7	10	110.2	108.4	2.1×10^{-5}	5.7	0.0041	13.9
8	10	108.8	112.7	>10	62.4	>10	95.1
MIBG	10 μg/mL	123.7	120.6	12.53	0.43	ND	ND

The increase in the acidification rate induced by each compound is presented as percent of control at the peak and after 3 hr of drug exposure. The cell line U-937 GTB was used along with RPMI 8226/S, as indicated in the table. The results are presented as the mean values of three to four experiments for each analogue and six experiments for MIBG. IC_{50} = inhibitory concentration reducing the survival index (SI) to 50%, E_{max} = maximal effect of drug, lowest SI value, ND = not done.

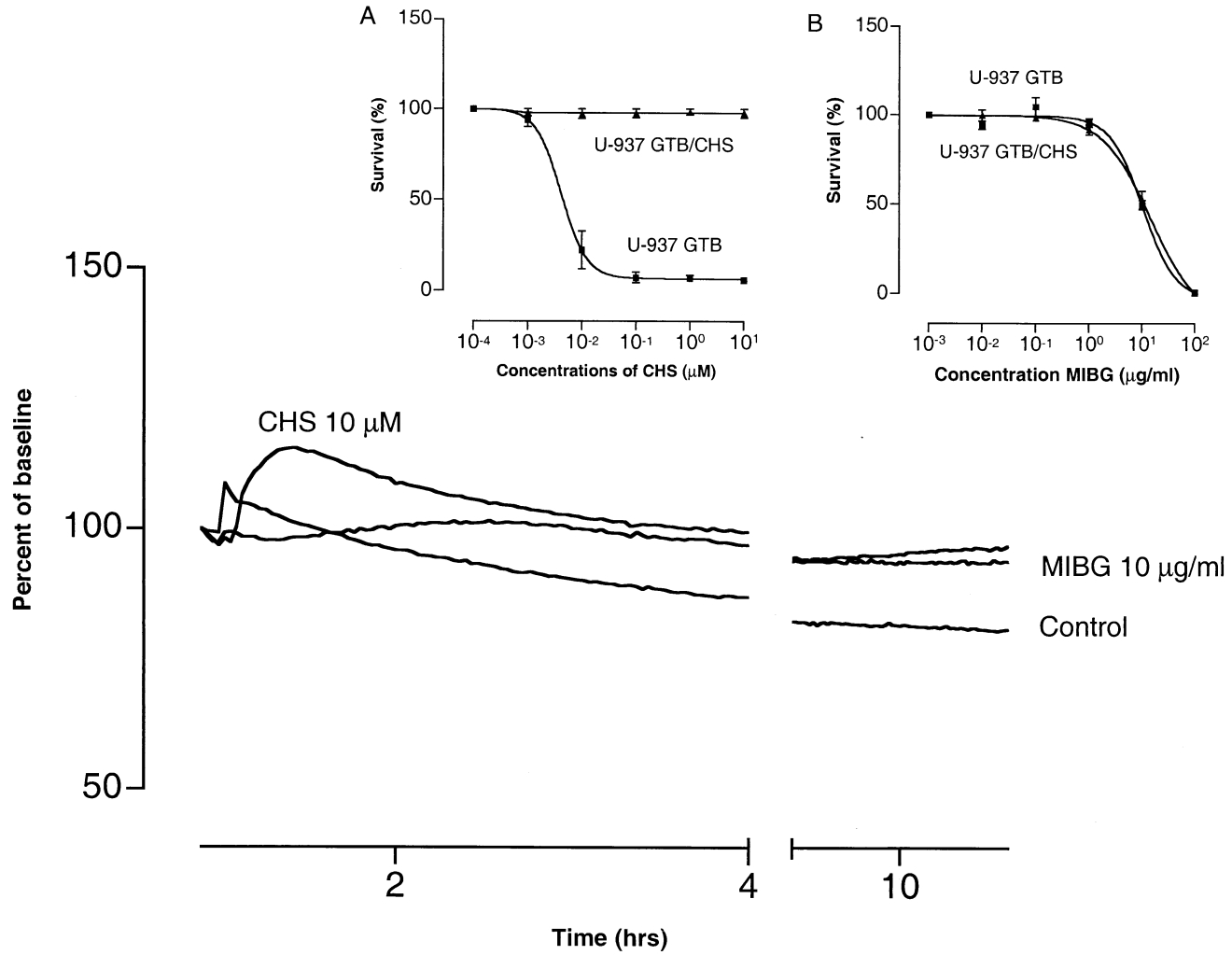


FIG. 5. Effect of CHS 828 and MIBG on the acidification rate in the high-grade CHS 828-resistant subline of U-937 GTB. The insets show the cytotoxicity of CHS 828 (A) and MIBG (B) on both the parental and resistant cell line.

RESULTS

The effects of the guanidines MIBG (1–10 μg/mL) and CHS 828 (0.01–10 μM) on extracellular acidification and cytotoxicity were compared in the histiocytic lymphoma cell line U-937 GTB, a cell line sensitive to CHS 828 (data not shown). Using the Cytosensor, MIBG and CHS 828 significantly increased the rate of acidification during the first 15–24 hr of drug exposure in a concentration-dependent manner (Fig. 2, A and B). In the case of CHS 828, the increase was rather rapid, reaching a maximum value within the first hour of drug exposure, while for MIBG the increase was marginally slower. A higher concentration both increased the magnitude of the acidification rate and shortened the time period of the stimulated metabolic activity for both compounds. The CHS 828-induced stimulation of the acidification rate could also be observed in other human tumor cell systems (Fig. 3, A–D) and appears to be a general effect of the drug. There was no apparent decrease in intracellular pH following CHS or MIBG addition, indicating efficient transport of protons across the plasma membrane (not shown).

The increase in the acidification rate was abolished by removal of glucose from the medium, substituted with 10 mM pyruvate, indicating stimulated glycolysis as the source of the increased metabolic activity (Fig. 4, A and B). These observations are consistent with the previously described inhibition of mitochondrial function with compensatory activation of glycolysis by MIBG [7, 8, 20]. With pyruvate as energy source, the level of the control curve was considerably lowered and even more so with the addition of drug. This decrease in the acidification rate was reversible in all cases, except for the highest concentration of CHS 828 (10 μM) where the pyruvate medium seemed to potentiate the cytotoxic effect of the drug. When exchanging the pyruvate and drug-containing medium back to plain glucose medium, a rebound increase in the acidification rate was observed before the curves returned to the level of untreated control. It was also evident that the increase in the acidification rate could be reversed by the removal of drug, allowing the cells to recover in plain medium (Fig. 4A).

A series of closely related CHS 828 analogues (Fig. 1)

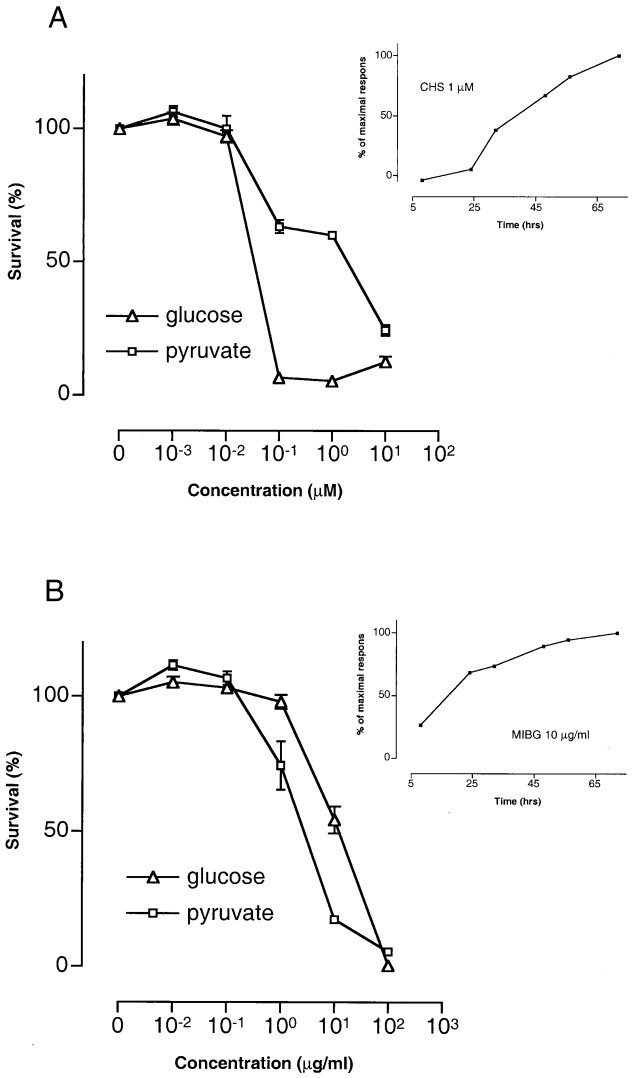


FIG. 6. The effect of CHS 828 (A) and MIBG (B) on U-937 GTB cell survival using medium containing either glucose or pyruvate as the energy source. The results are presented as mean values of three to five experiments. The time dependence of the cytotoxic effect on cells exposed to CHS 828 and MIBG, in a single experiment out of three, is also shown (insets A and B).

were tested in the U-937 GTB cell line and compared regarding both their influence on the rate of acidification and cytotoxicity measured as survival index (SI) by the FMCA (Table 1). Cytotoxicity was also measured in the myeloma cell line RPMI 8226/S. In the latter cell line, the most potent analogue was (**6**) followed by (**5**), while (**3**) and (**8**) were inactive (Table 1). Most of the investigated analogues had a similar effect and affected the acidification rate positively, but not to the same extent as CHS 828. No apparent relationship between cytotoxicity, measured as the IC_{50} value for cytotoxicity, and acidification, measured after 3 hr of drug exposure, could be discerned (R = 0.47). Best effect, measured as peak height and E_{max} value respectively, was observed for (**6**) (Table 1).

Furthermore, comparable increases in the acidification rate were also evident in one subline with high-grade resistance (>1000-fold) to the cytotoxic actions of CHS 828 (Fig. 5). This cell line also showed a low degree of cross-resistance to MIBG (Fig. 5, inset). The results indicate that although CHS 828 may share the inhibitory actions of MIBG on mitochondrial respiration with a subsequent increase in glycolysis and acidification rate, this is not sufficient to induce cytotoxicity.

In another series of experiments using the FMCA, the effects of glucose and pyruvate medium on cell survival of CHS 828- and MIBG-treated cells were compared (Fig. 6, A and B). A difference in cytotoxic effect between the two guanidines was distinguished, as the best effect of CHS 828 was seen using glucose medium, while the opposite was true regarding MIBG. In addition, the effects of CHS 828 and MIBG at different time points were compared by interrupting drug exposure after 8, 24, 32, 48, 56, and 72 hr (Fig. 6, inset A and B). The cell survival appeared time-dependent in the case of CHS 828, but this tendency was less pronounced for MIBG.

Yet another difference between CHS 828 and MIBG activity was evident from experiments using thymidine and leucine incorporation for the study of actions on DNA and protein synthesis over time (Fig. 7). In the case of the CHS 828-treated cells, the curves diverged from the control curve after approximately 25 hr for all concentrations tested (0.1, 1, and 10 μM), while the effect of MIBG was more rapid and concentration-dependent. The kinetics for inhibition of macromolecular synthesis thus further suggest different mechanisms by which the two compounds induce cytotoxicity.

The stability of CHS 828 and MIBG was investigated after 72 hr of preincubation prior to experiments, and these drugs were found stable under assay conditions (not shown).

DISCUSSION

CHS 828 dose dependently induced an increase in the acidification rate of long duration, which is unusual when comparing with standard cytotoxic drugs used for cancer treatment [21]. In this earlier study, standard cytotoxic drugs representing different mechanisms of action generally produced an early inhibition of the acidification rate (within 10 hr) with or without preceding transient stimulation [21]. The CHS 828-induced increase was similar to that observed in response to another guanidine, MIBG, which is used clinically as a radioiodinated diagnostic and therapeutic agent for adrenergic tumors [5, 6]. MIBG is known to affect mitochondrial respiration, resulting in ATP depletion followed by a compensatory increased glycolytic activity and lactate production [7, 8, 20]. This chain of events may also mechanistically explain the increase in the acidification rate observed with the two guanidines. Indeed, replacing glucose with pyruvate to block glycolytic activity from the medium completely abolished the CHS 828 and MIBG stimulation of the acidification rate, thus supporting this notion. MIBG inter-

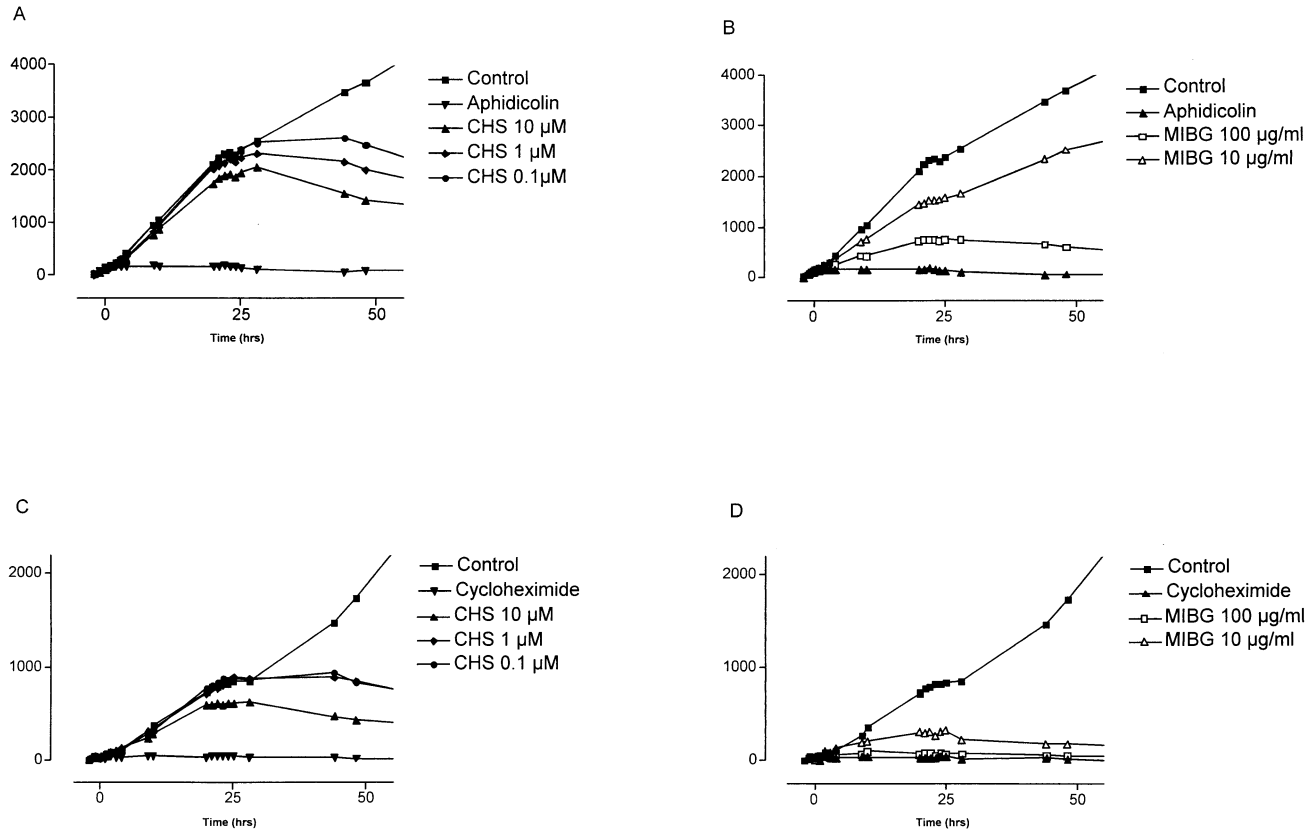


FIG. 7. Effect of CHS 828 (A, C) and MIBG (B, D) on DNA and protein synthesis over time, using thymidine and leucine incorporation, respectively. One experiment out of three is shown.

acts with complex I of the respiratory chain without affecting F1 ATP-ase, but the site of interaction of the pyridyl cyanoguanidines is not yet known [7].

Stimulation of the acidification rate could be observed in all cell systems tested, including primary cultures of human tumor cells from patients, indicating that this effect of CHS 828 is not cell type-specific. The ability to increase the acidification rate was, to a variable degree, also shared by other cytotoxic pyridyl cyanoguanidines, suggesting a class-specific effect.

What is then the relationship between an increased acidification rate and CHS 828-induced cytotoxicity? Several lines of evidence indicate that these effects are not directly coupled. First, maximal cytotoxicity of CHS 828 was already observed at concentrations producing no or only a marginal increase in the rate of acidification. Second, comparing the pyridyl cyanoguanidine analogues, there was no apparent relationship between acidification rate and cytotoxicity. Third, comparable increases in the acidification rate were evident in one U-937 subline with high-grade resistance (>1000-fold) to the cytotoxic actions of CHS 828, and this cell line also showed a low degree of cross-resistance to MIBG. Finally, the cytotoxic response pattern and the kinetics of cell kill induced by CHS 828 were clearly different from those observed in response to MIBG [3]. In addition to this, the present study also demonstrated a dissimilar profile with respect to inhibition of DNA and protein synthesis, further underlining the mechanistic difference between the two drugs in inducing antitumor activity. The overall results thus indicate that although CHS 828 may share the inhibitory actions of MIBG on mitochondrial respiration [7, 8, 20] with a subsequent increase in glycolysis and acidification rate, this mechanism of action appears neither necessary nor sufficient to fully explain the cytotoxic actions of CHS 828 in the U-937 GTB model.

However, it cannot be excluded that CHS 828-induced stimulation of the acidification rate may contribute to the cytotoxic response observed to CHS 828 in some cell systems. For example, the pronounced increase in chronic lymphatic leukemia (CLL) compared to the lower effect in ovarian carcinoma corresponds to the extent of cytotoxicity observed in these cell types [22]. Furthermore, blocking glycolysis by the removal of glucose paradoxically reduced CHS 828-induced cytotoxicity while potentiating that of MIBG. This might indicate that the extent of glycolysis may be an important determinant for optimal CHS 828 activity. However, if and to what extent the acidification rate and glycolysis are contributing to the efficacy of CHS 828 remains to be elucidated.

It should also be noted that the CHS 828-induced stimulation of the acidification rate may hypothetically be utilized therapeutically. Lowering of intracellular pH has been shown to induce a specific mode of cell death [23, 24]

and could theoretically contribute positively to the actions of CHS 828. This may be accomplished by pharmacological maneuvers affecting intracellular pH regulation, for example by blocking Na^+/H^+ transport with amiloride [25–28]. This may result in intracellular acidification and augmented cell kill. Another way to utilize the CHS 828-induced stimulation of the acidification rate therapeutically would be in combination with bioreductive drugs such as mitomycin C, which shows improved efficacy in acid environments found in the central parts of solid tumors [29–31]. These potential approaches to improve CHS 828 efficacy will be subjects for future studies.

This study was supported by grants from the Swedish Cancer Society and the Lions Cancer Research Foundation. The skillful technical assistance of Lena Lenhammar and Christina Leek are gratefully acknowledged. Also, our thanks to Sumeer Dhar for the work done with the CHS-resistant cell line.

References

1. Schou C, Ottosen ER, Petersen HJ, Björkling F, Latini S, Hjarnaa PV, Bramm E and Binderup L, Novel cyanoguanidines with potent oral antitumour activity. *Bioorg Med Chem Lett* **7:** 3095–3100, 1997.
2. Petersen HJ, Kaegaard 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. Vig Hjarnaa P-J, Dhar S, Latini S, Jonsson E, Larsson R, Bramm E, Skov T and Binderup L, CHS 828, a novel pyridyl cyanoguanidine with potent antitumor activity *in vitro* and *in vivo*. *Cancer Res* **59:** 5751–5757, 1999.
4. Smets LA, Bout B and Wisse J, Cytotoxic and antitumor effects of the norepinephrine analogue meta-iodo-benzylguanidine (MIBG). *Cancer Chemother Pharmacol* **21:** 9–13, 1988.
5. Sisson JC, Frager MS, Valk TW, Gross MD, Swanson DP, Wieland DM, Tobes MC, Beierwaltes WH and Thompson NW, Scintigraphic localization of pheochromocytoma. *N Engl J Med* **305:** 12–17, 1981.
6. Sisson JC, Shapiro B, Beierwaltes WH, Glowniak JV, Nakajo M, Mangner TJ, Carey JE, Swanson DP, Copp JE, Satterlee WG and Wieland DM, Radiopharmaceutical treatment of malignant pheochromocytoma. *J Nucl Med* **24:** 197–206, 1984.
7. Loesberg C, Van Rooij H, Nooijen WJ, Meijer AJ and Smets LA, Impaired mitochondrial respiration and stimulated glycolysis by *m*-iodobenzylguanidine (MIBG). *Int J Cancer* **46:** 276–281, 1990.
8. Cornelissen J, Wanders RJ, Van den Bogert C, Van Kuilenburg AB, Elzinga L, Voute PA and Van Gennip AH, Meta-iodobenzylguanidine (MIBG) inhibits malate and succinate driven mitochondrial ATP synthesis in the human neuroblastoma cell line SK-N-BE(2c). *Eur J Cancer* **31A:** 582–586, 1995.
9. Sundström C and Nilsson K, Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* **17:** 565–577, 1976.
10. 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.
11. Nygren P, Fridborg H, Csoka K, Sundström C, De La Torre M, Kristensen J, Bergh J, Hagberg H, Glimelius B, Rastad J, Tholander B and Larsson R, Detection of tumor-specific cytotoxic drug activity *in vitro* using the fluorometric microculture cytotoxicity assay and primary cultures of tumor cells from patients. *Int J Cancer* **56:** 715–720, 1994.
12. Owicki JC and Parce JW, Biosensors based on the energy metabolism of living cells: The physical chemistry and cell biology of extracellular acidification. *Biosens Bioelectron* **7:** 255–272, 1992.
13. McConnell HM, Owicki JC, Parce JW, Miller DL, Baxter GT, Wada HG and Pitchford S, The Cytosensor microphysiometer: Biological applications of silicon technology. *Science* **257:** 1906–1912, 1992.
14. Owicki JC, Bousse LJ, Hafeman DG, Kirk GL, Olson JD, Wada HG and Parce JW, The light-addressable potentiometric sensor: Principles and biological applications. *Annu Rev Biophys Biomol Struct* **23:** 87–113, 1994.
15. Hafeman DG, Parce JW and McConnell HM, Light-addressable potentiometric sensor for biochemical systems. *Science* **240:** 1182–1185, 1988.
16. Carson R, Nigerian P, Ekberg M and Slater L, Chemotherapeutic drug sensitivity testing of leukaemic cells *in vitro* using a semiautomated fluorometric assay. *Leukemia* **4:** 567–571, 1990.
17. Nygren P, Kristensen J, Jonsson B, Lönnerholm G, Kreuger A and Larsson R, Feasibility of the fluorometric microculture cytotoxicity assay (FMCA) for cytotoxic drug sensitivity testing of tumor cells from patients with acute lymphocytic leukemia. *Leukemia* **6:** 1121–1128, 1992.
18. Harris DW, Kenrick MK, Pither RJ, Anson JG and Jones DA, Development of a high-volume *in situ* mRNA hybridization assay for the quantification of gene expression utilizing scintillating microplates. *Anal Biochem* **243:** 249–256, 1996.
19. Graves R, Davies R, Brophy G, O'Beirne G and Cook N, Noninvasive, real-time method for the examination of thymidine uptake events: Application of the method to V-79 cell synchrony studies. *Anal Biochem* **248:** 251–257, 1997.
20. Loesberg C, Van Rooij H, Romijn JC and Smets LA, Mitochondrial effects of the guanidino group-containing cytostatic drugs, *m*-iodobenzylguanidine and methylglyoxal bis (guanylhydrazone). *Biochem Pharmacol* **42:** 793–798, 1991.
21. Ekelund S, Nygren P and Larsson R, Microphysiometry: New technology for evaluation of anticancer drug activity in human tumor cells *in vitro*. *Anti-Cancer Drugs* **9:** 531–538, 1998.
22. Åleskog A, Bashir-Hassan S, Jonsson E, Alvfors C, Kristensen J and Larsson R, *In vitro* activity of CHS 828 in primary cultures of human hematological and solid tumors. *90th Annual Meeting of the American Association for Cancer Research* 40, p. 299. Cadmus Journal Services, Philadelphia, 1999.
23. Tannock IF and Rotin D, Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res* **49:** 4373–4384, 1989.
24. Horvat B, Taheri S and Salihagic A, Tumour cell proliferation is abolished by inhibition of Na^+/H^+ and CHO_3^-/Cl^- exchange. *Eur J Cancer* **29A:** 132–137, 1992.
25. Rotin D, Wan P, Grinstein S and Tannock I, Cytotoxicity of compounds that interfere with the regulation of intracellular pH: A potentially new class of anticancer drugs. *Cancer Res* **47:** 1497–1504, 1987.
26. Maidorn RP Jr, Cragoe EJ and Tannock IF, Therapeutic potential of analogues of amiloride: Inhibition of the regulation of intracellular pH as a possible mechanism of tumour selective therapy. *Br J Cancer* **67:** 297–303, 1993.
27. Kuin A, Aalders M, Lamfers M, van Zuidam DJ, Essers M, Beijnen JH and Smets LA, Potentiation of anti-cancer drug activity at low intratumoral pH induced by the mitochondrial

inhibitor *m*-iodobenzylguanidine (MIBG) and its analogue benzylguanidine (BG). *Br J Cancer* **79:** 793–801, 1999.
28. Garcia-Canero R, Trilla C, Perez de Diego J, Diaz-Gil J and Cobo J, Na^+/H^+ exchange inhibition induces intracellular acidosis and differentially impairs cell growth and viability of human and rat hepatocarcinoma cells. *Toxicol Lett* **106:** 215–228, 1999.
29. Sakamoto N, Toge T and Nishiyama M, Tumor-specific synergistic therapy of mitomycin C: Modulation of bioreductive activation. *Hiroshima J Med Sci* **46:** 67–73, 1997.
30. Cummings J, Spanswick V, Tomasz M and Smyth JF, Enzymology of mitomycin C metabolic activation in tumour tissue. *Biochem Pharmacol* **56:** 405–414, 1998.
31. Wike-Hooley JL, Haveman J and Reinhold HS, The relevance of tumour pH to the treatment of malignant disease. *Radiother Oncol* **2:** 343–366, 1984.