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European Journal of Pharmacology 418 (2001) 39–45

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Cellular pharmacodynamics of the cytotoxic guanidino-containing drug CHS 828. Comparison with methylglyoxal-bis(guanylhydrazone)

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Received 15 January 2001; received in revised form 19 March 2001; accepted 28 March 2001

Abstract

N-(6-(4-chlorophenoxy)hexyl)-*N′*-cyano-*N″*-4-pyridylguanidine (CHS 828) is a new guanidino-containing compound with antitumoral activity both in vitro and in vivo. Its activity profile differs from those of standard cytotoxic drugs but the mechanism of action is not yet fully understood. CHS 828 is presently in early phase I and II clinical trials. In the present study, the pharmacodynamic effects at the cellular level of CHS 828 was compared to another compound containing two guanidino groups, methylglyoxal-bis(guanylhydrazone) (MGBG). MGBG is known to inhibit the synthesis of polyamines, which are important in, e.g., proliferation and macromolecular synthesis. The concentration–response relationship of CHS 828 closely resembled that of MGBG and the drugs were similar with respect to inhibition of DNA and protein synthesis. On the other hand, CHS 828 induced a significant increase in cellular metabolism while MGBG did not. The cytotoxic effect of MGBG was reversed by the addition of exogenous polyamines, while that of CHS 828 was unaffected. Unlike MGBG, there was also no effect of CHS 828 on the levels of decarboxylating enzymes in the polyamine biosynthesis. In conclusion, CHS 828 does not appear to share any major mechanisms of action with the polyamine synthesis inhibitor MGBG. Further studies will be required to define the exact mechanism of action of CHS 828.

Keywords: Cytosensor; Microphysiometer; CHS 828; MGBG (methylglyoxal-bis(guanylhydrazone)); Cytotoxicity; Polyamine

1. Introduction

CHS 828, *N*-(6-(4-chlorophenoxy)hexyl)-*N′*-cyano-*N″*-4-pyridylguanidine, is a new drug with antitumor activity and with a profile of activity different from those of standard cytotoxic drugs (Vig Hjarnaa et al., 1999). The new drug candidate has been thoroughly investigated both in vitro and in vivo (Ekelund et al., 2000, 2001; Mork Hansen et al., 2000; Vig Hjarnaa et al., 1999) and is now in early clinical trials. The mechanism of action is not yet known, but it was recently shown that CHS 828 may share the inhibitory actions of MIBG (*meta*-iodobenzylguanidine) on mitochondrial respiration with subsequent increase in glycolytic flux (Ekelund et al., 2000). However, this does not fully explain the cytotoxic action of CHS 828 and additional mechanisms of action remain to be defined.

Many guanidino-containing compounds have gained interest due to their antiproliferative activity. One of these compounds is methylglyoxal-bis(guanylhydrazone), MGBG (Porter et al., 1981; Seiler et al., 1998). MGBG shares some structural features with natural polyamines, especially with spermidine (Porter et al., 1981). The polyamines are small cations that are critically involved in many biological processes, e.g., regulation of cell proliferation and macromolecular synthesis (Cohen, 1978; Jänne et al., 1978; Pegg, 1988). Elevated polyamine contents have been noted in tumors (Jänne et al., 1978). Therefore, the polyamine metabolism might be a target for chemotherapy.

MGBG has shown antitumor activity in several phase II clinical trials in a variety of different diagnoses. Still, the exact mechanism whereby MGBG exerts its cytotoxic effects is not fully understood (Ekelund et al., 2001; Jänne et al., 1991; Marsoni et al., 1987; Von Hoff, 1994). However, the drug is a potent inhibitor of the biosynthesis of the polyamines spermidine and spermine originating

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00944-X

from ornithine and putrescine (Jänne et al., 1991; Seiler et al., 1998). It has also been suggested that the cytotoxic effect of MGBG is exerted by the prevention of mitochondrial spermine flux (Toninello et al., 1999). Yet, other reports have demonstrated that MGBG is involved in inhibition of mitochondrial respiration and ATP production, similar to that of MIBG (Chaffee et al., 1979; Mikles-Robertson et al., 1979; Toninello et al., 1988). Most probably, there are several mechanisms for the antiproliferative activity.

The purpose of this study was to compare cellular effects of the new guanidino-containing compound, CHS 828, with the more well known MGBG, in order to increase the understanding of the mechanism of the cytotoxic action of CHS 828.

2. Materials and methods

2.1. Experimental drugs

CHS-828, *N*-(6-(4-chlorophenoxy)hexyl)-*N′*-cyano-*N″*-4-pyridylguanidine was supplied by Leo Pharmaceutical Products in stock solutions, dissolved in dimethylsulphoxide (DMSO) at 10 mM and were stored frozen at −20°C. The drug was diluted 10 times with 33% DMSO in sterile water and further dilution was made using sterile phosphate buffered saline (Sigma). The final concentration of DMSO was 0.33% in the highest concentration tested (CHS 828, 10 μM), and did not affect cell survival (not shown). MGBG was purchased from Sigma as a powder, and was dissolved and diluted in sterile water to a stock solution of 1 mg/ml. The drug was stored at −70°C until use. Spermidine was also obtained from Sigma. Dilution to a stock solution of 10 mM using sterile water was made prior to each experiment.

2.2. Cell line

The experiments were performed using the human histiocytic lymphoma cell line, U-937-GTB (Sundström and Nilsson, 1976). The cells were cultured under standard incubator conditions in 75 cm^2 flasks (Nunc, Roskilde, Denmark). The culture medium RPMI 1640 (Hyclone, Cramlington, UK), supplemented with 10% heat-inactivated fetal calf serum (HyClone), 2 mM glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin (HyClone), was used. Cell growth and morphology were monitored on a weekly basis.

2.3. Extracellular pH measurements

The Cytosensor microphysiometer (Molecular Devices, Sunnyvale, CA) was used to study the effect of CHS 828 and MGBG on cellular metabolism measured by monitoring the rate at which cells excrete acidic metabolic by-products, e.g., lactic acid and carbon dioxide. There are several different biochemical pathways in cells that contribute to the generation of acidic by-products, for example glycolysis and respiration (McConnell et al., 1992; Owicki and Parce, 1992). A pH sensitive silicon chip together with a reference electrode and other components form a light-addressable potentiometric sensor (LAPS) is used to measure small changes of extracellular acidification rate (Hafeman et al., 1988; Owicki et al., 1994). Once each second, the LAPS makes a voltage measurement that is linearly related to pH.

The Cytosensor consists of two units with eight parallel flow chambers. In the chambers, the cell capsule cups are loaded. 1.5×10^5 U-937-GTB cells were immobilized in the capsule cup in 10 μl of agarose mixture (Molecular Devices). The cup was perfused with low buffered RPMI 1640 medium (National Veterinary Institute, Uppsala, Sweden) with no HEPES or bicarbonate, but with 6 ml/l 4 M NaCl to preserve the osmotic balance. The medium also contained 10 ml/l 200 mM L-glutamine, 60 mg/l peni-

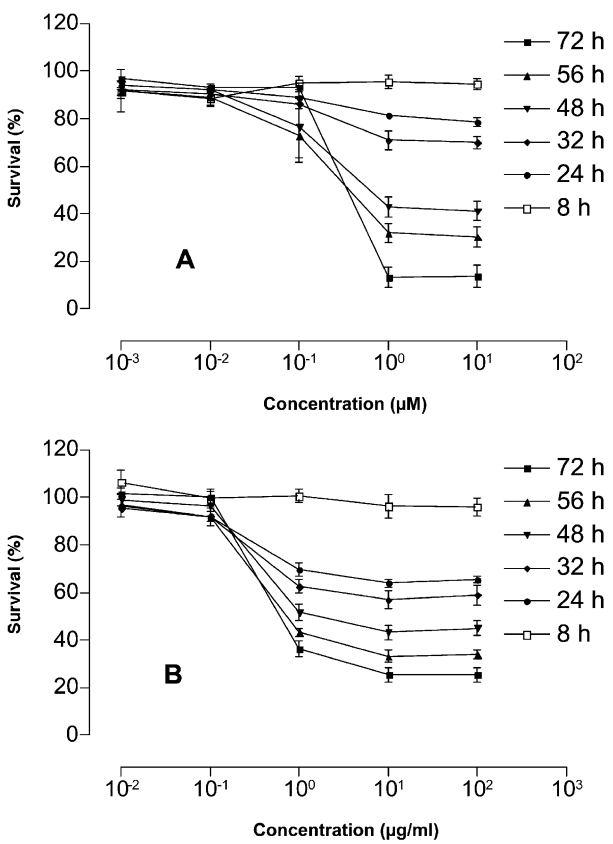

Fig. 1. Concentration–response curves, demonstrating the cytotoxic effect of CHS 828 (A) and MGBG (B). Cells were exposed to drugs during the indicated exposure times, then plates were washed once with PBS and incubated further until 72 h. Cell survival was measured using the FMCA method. The concentrations indicated are in μM for CHS 828 and in μg/ml for MGBG, where 1 μM CHS 828 equals 0.26 μg/ml of MGBG.

cillin G and 50 mg/l streptomycin. The flow rate through the cup was 100 μl/min and at this flow rate, cells are not washed out of the cups (Parce et al., 1989). Culture medium was pumped from a reservoir by a peristaltic pump and passes through a debubbler–degasser, a selection valve and finally through the flow sensor chamber. Every 90 s, the flow was stopped for 30 s and the extracellular acidification rate was measured. During fluid flow, the sensor output is stable and reflects a pH near that of the culture medium. Since the medium is in a closed system, no drift in pH occurs because of equilibration with atmospheric CO_2 (Parce et al., 1989).

2.4. Measurement of cellular cytotoxicity

The Fluorometric Microculture Cytotoxicity Assay (FMCA) is a semi-automated, non-clonogenic assay that was employed for drug cytotoxicity testing. The method is based on the measurement of fluorescence generated from cellular hydrolysis of fluorescein diacetate (Sigma) to fluorescent fluorescein by cells with intact plasma membranes (Larsson et al., 1992; Nygren et al., 1994).

Fluorescein diacetate was dissolved in DMSO to 10 mg/ml (Sigma) and kept frozen as a stock solution, protected from light. V-shaped 96-well microtiter plates were prepared in advance with triplicates of drugs and stored at −70°C until further use. Wells were filled with 20 μl of drug solution at 10 times the final concentration and control wells with 20 μl PBS. Drugs could be stored in this way for at least 6 months without decline in drug activity as judged by repeated testing (not shown).

On day 1, 180 μl per well of cell suspension (20 000 cells/well) were seeded into the thawed microtiter plates and the plates were then incubated for 72 h. Six wells with cells but without drugs served as controls and six wells with only culture medium served as blanks. After incubation, the plates were centrifuged at 200 × *g* during 5 min, followed by medium aspiration using a microtitre plate washer (Dynatec Laboratories, Chantilly, VA) and washing once with 200 μl PBS. Thereafter, 100 μl HEPES buffer containing fluorescein diacetate at 10 μg/ml was added and the plates were incubated another 40 min for the hydrolysis of fluorescein diacetate to take place. The fluorescence generated was measured in the microtiter plate

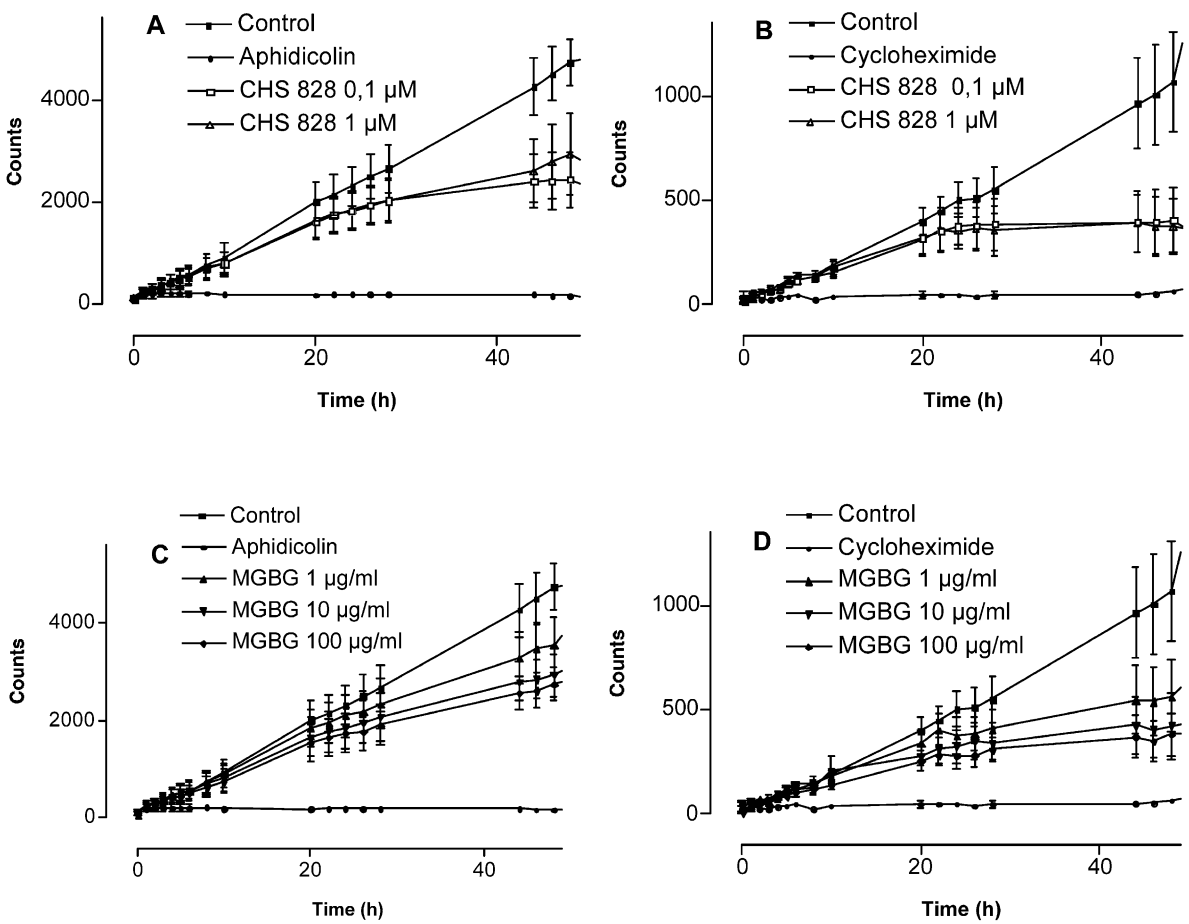

Fig. 2. Effect of CHS 828 (A, C) and MGBG (B, D) on DNA and protein synthesis over time, using tymidine and leucine incorporation, respectively. One typical experiment out of three is shown. The concentrations indicated are in μM for CHS 828 and in μg/ml for MGBG.

fluorescence spectrophotometer Fluoroscan II (Labsystems OY, Helsinki, Finland). The fluorescence is proportional to the number of living cells in a well and cell survival was presented as survival index (SI), defined as the fluorescence in experimental wells in percent of that in control wells, with blank values subtracted.

2.5. Measurement of decarboxylase activity

One million cells in 10 ml of growth medium were exposed to drug (CHS 1 μM and MGBG 10 μg/ml) in cell culture flasks during 1 or 20 h. The cell suspension was then centrifuged and resuspended in 1 ml cold PBS and centrifuged once more before being sonicated in 50 μl of a lysis buffer. The lysis buffer contained 50 mM Tris–HCl (pH 7.4), 0.1% Triton-X 100, 4 mM EDTA, 5 mM dithiothreitol, 200 000 U/ml Trasylol, 10 mM benzamidine and 0,1 mg/ml albumin. The homogenates were centrifuged at $100\,000 \times g$ for 10 min at +4°C. For ornithine decarboxylase measurements, 30 μl of supernatant was mixed with 30 μl incubation buffer containing 2 mM pyridoxal phosphate, 0,4 mM L-ornithine and 10 μCi/ml L-[1-^{14}C]ornithine (Nycomed Amersham, Buckinghamshire, England). The assay was performed in small glass vials inserted into an outer flask tightly sealed with a rubber membrane. After 60 min incubation at 37°C in a slowly shaking water bath, reactions were terminated by addition of 100 μl 5 M H_2SO_4 and 250 μl Hyamine were injected into the outer flask to trap liberated $^{14}CO_2$. Following the addition of 0.4 M Na_2HPO_4 to liberate CO_2, Unisolve scintillation fluid was added and the radioactivity quantified by scintillation counting. The *S*-adenosyl methionine decarboxylase assay was carried out in the same way except that the incubation buffer consisted of 5 mM $NaPO_4$, 0.4 mM *S*-adenosyl-L-methionine and 4 μCi/ml *S*-adenosyl-L-[carboxyl-^{14}C]methionine (Nycomed Amersham) (Sjöholm et al., 1993, 1994).

2.6. Measurement of DNA and protein synthesis

Protein and DNA synthesis were measured with a Cytostar-T plate (available in the "In Situ mRNA Cytostar-T assay" kit, Amersham International), a pre-made scintillating 96-well microtiter plate, with scintillation fluid molded into the bottom of the wells (Graves et al., 1997; Harris et al., 1996). 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 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 was collected at 0 through 72 h.

3. Results

When comparing the cytotoxic effects of CHS 828 and MGBG using concentration–response curves from FMCA experiments, there was an apparent resemblance in the shape between the two curves (Fig. 1). The longer the time of drug exposure, the higher the degree of cell death was observed in a dose-dependent manner, reaching a plateau at each exposure time. However, in correlation analysis of

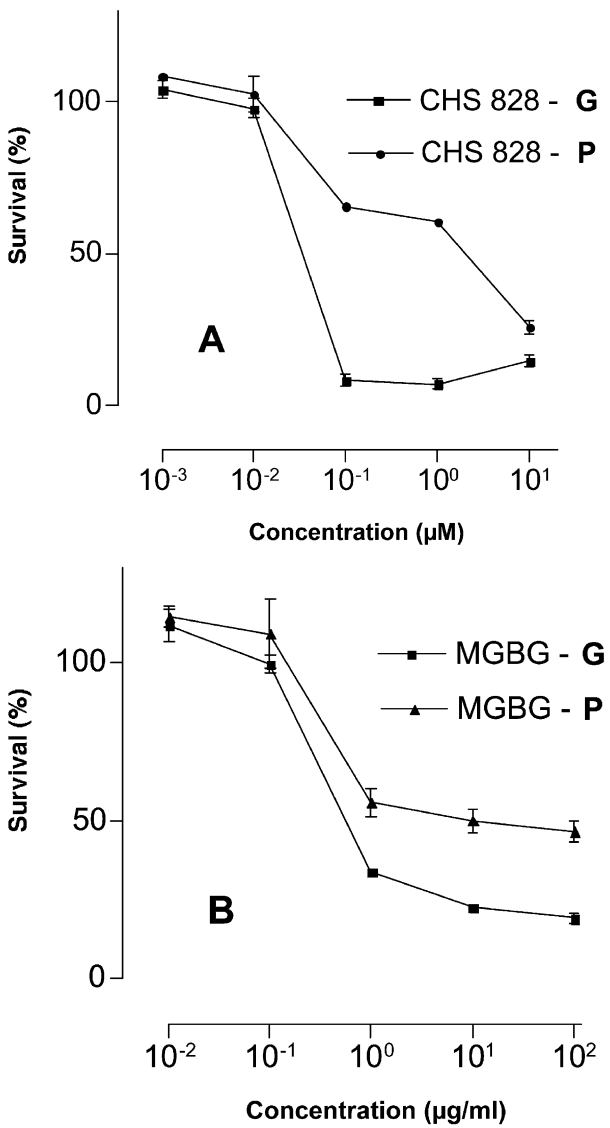

Fig. 3. The effect of CHS 828 (A) and MGBG (B) on U-937 GTB cell survival using medium containing either glucose (G) or pyruvate (P) as the energy source. The results are presented as mean values ± S.E.M. of three to five experiments.

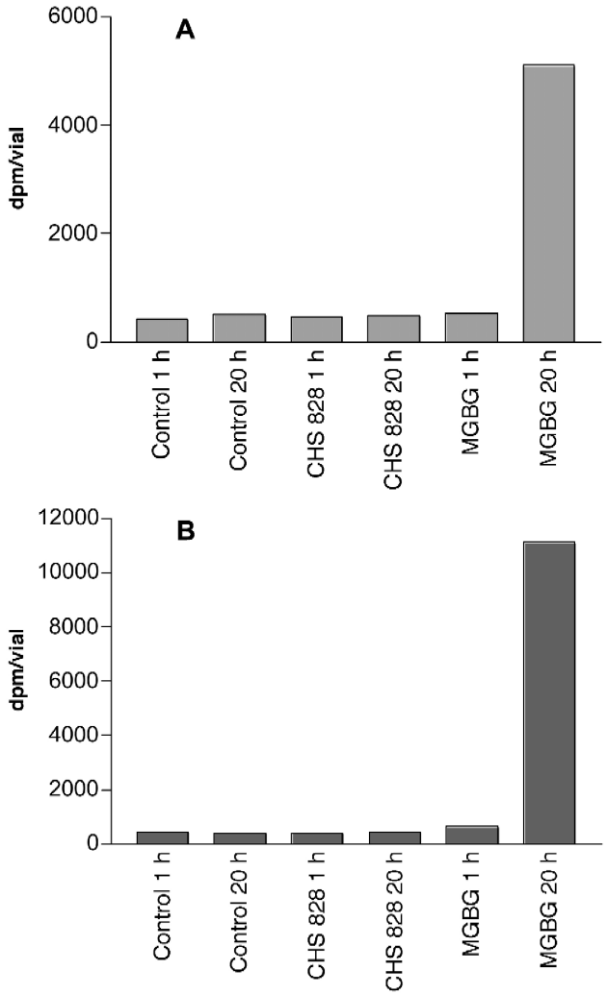

Fig. 4. Effects of CHS 828 and MGBG on decarboxylase activities in U-937-GTB cells. Cells were exposed to the drugs for 1 or 20 h. Decarboxylase activities were assayed by measuring the generation of $^{14}CO_2$ from cell homogenates incubated with L-[^{14}Cl]ornithine for ornithine decarboxylase (A) or *S*-adenosyl-L-[carboxyl-^{14}C]methionine for *S*-adenosyl methionine decarboxylase (B). Data are presented with blank values subtracted.

log IC_{50} values of CHS 828 and MGBG, Pearson's coefficient of correlation was 0.67, which only corresponds to a moderate correlation. For agents sharing the same mechanism of action, Pearson's coefficient of correlation is usually > 0.85.

In experiments measuring DNA and protein synthesis, there were also similarities between CHS 828 and MGBG showing similar kinetics (Fig. 2). The synthesis of both DNA and protein for both drugs began to decline after approximately 20–25 h.

Fig. 3 shows the cytotoxic effects measured as cell survival in cells exposed to CHS 828 (Fig. 3A) and MIBG (Fig. 3B) using medium containing glucose or pyruvate. Pyruvate as the only energy source blocks the glycolysis (Ekelund et al., 2000) and protected cells from the cytotoxic actions of CHS 828 and MGBG. This phenomenon was more pronounced for CHS 828.

In experiments measuring the effects of CHS 828 (1 μM) and MGBG (10 μg/ml) on polyamines synthesis, the two drugs differed (Fig. 4). After 1 h of drug incubation, there was no effect on enzyme activity, neither for ornithine decarboxylase or *S*-adenosyl methionine decarboxylase. After 20 h of drug exposure, there was still no effect of CHS 828, while MGBG significantly enhanced the activity of both enzymes with an increase of 10 and 15 times, respectively.

The influence of the natural polyamine, spermidine on the cytotoxic effects of CHS 828 and MGBG was tested using the FMCA. The polyamine was added to the culture medium at the concentrations 0.125 and 0.0625 mM. These concentrations protected the cells from the cytotoxic action of MGBG (Fig. 5), but in the case of CHS 828, there was no change in cell survival with either of the two concentrations of spermidine.

In Cytosensor experiments measuring the extracellular acidification rate, there was no effect of MGBG compared

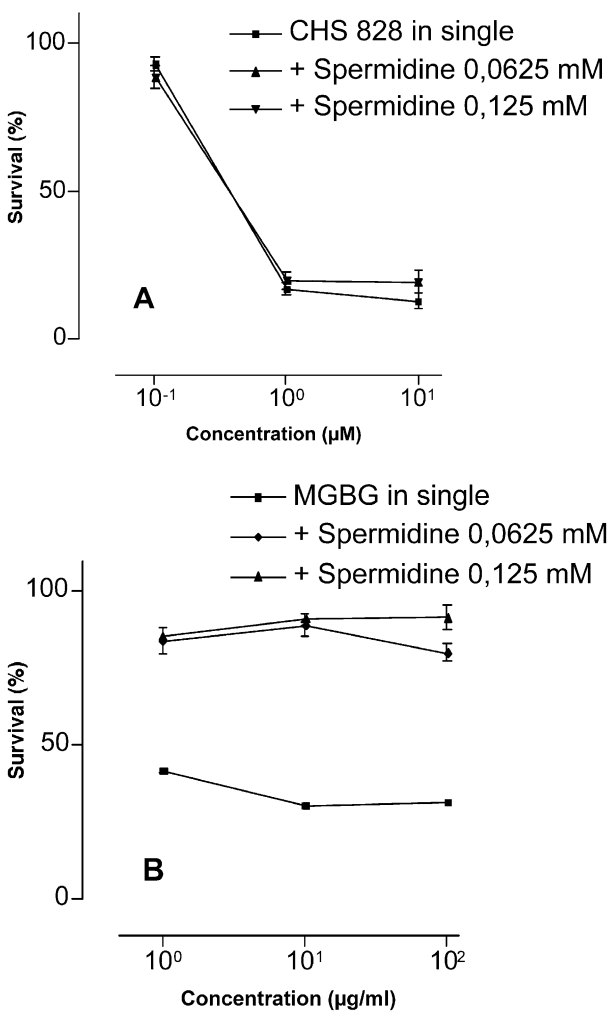

Fig. 5. Concentration–response curves from FMCA experiments with CHS 828 (A) and MGBG (B), alone and in combination with the indicated concentrations of the polyamine spermidine. The results are presented as mean values ± S.E.M. of four experiments.

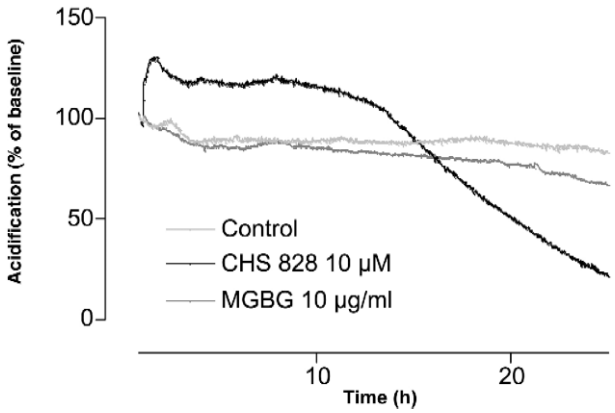

Fig. 6. Concentration–response curves for indicated concentrations of the guanidines CHS 828 and MGBG using the Cytosensor microphysiometer for continuous drug exposure during 24 h. One typical experiment out of four is shown.

to the control. On the other hand, CHS 828 induced an early increase in metabolic activation with a duration of approximately 12–15 h (Fig. 6) similar to what has been shown previously (Ekelund et al., 2000).

4. Discussion

The purpose of the present study was to compare the cellular pharmacodynamics of the two guanidino-containing compounds, CHS 828 and MGBG. The latter is known since long to be a potent inhibitor of the biosynthesis of polyamines as it has structural similarities with the natural polyamine spermidine with the same number of atoms in the main chain (Williams-Ashman and Schenone, 1972). The polyamines are important in many biological processes like cell proliferation and macromolecular synthesis. Furthermore, it has been suggested in literature that MGBG may exert metabolic effects unrelated to polyamine metabolism, e.g., antimitochondrial action (Ekelund et al., 2001).

CHS 828 is a newly recognized compound in the guanidino family (Petersen et al., 1978). It was discovered when screening for cyototoxic activity among compounds with antihypertensive activity (Schou et al., 1997). The drug has shown interesting results both in vitro and in vivo and is presently in phase I and II trials.

The mechanism of action of CHS 828 is not known and the drug is presently under intense investigation to reveal more information about the mechanisms behind its cytotoxicity. In a recent study, we compared CHS 828 to MIBG, a known mitochondrial inhibitor which is also a guanidino-containing compound (Ekelund et al., 2000). The two drugs both induced a prolonged and sustained increase in the extracellular acidification, more pronounced than have been shown previously for standard cytotoxic drugs (Ekelund et al., 1998). This lowering of pH is due to a compensatory increase in glycolytic flux when mitochondrial respiration is inhibited resulting in an overproduction of acidic byproducts (Cornelissen et al., 1995; Loesberg et al., 1990).

In a previous study, it was demonstrated that MGBG did not affect glycolysis during the first 24 h of drug exposure in Ehrlich ascites cells (Alhonen-Hongisto et al., 1980). Also, in the present study, extracellular pH was not influenced by MGBG, suggesting that MGBG does not share the inhibitory effect of CHS 828 on mitochondrial function.

Cells exposed to CHS 828 and with pyruvate as the only energy source were partly protected against the cytotoxic actions of the drug. With pyruvate, glycolysis is blocked (Ekelund et al., 2000) and we speculate that without this pathway, CHS 828 cannot fully exert its cytotoxic action. Protection in the same way was also afforded by MGBG, but not to the same extent as for CHS 828, indicating less dependency of this drug on intact glycolysis.

In the literature, the inhibitory effect of MGBG on DNA and protein synthesis has been described to appear already after 4–8 h (Alhonen-Hongisto et al., 1980). In our study, the first clear signs of inhibition exerted by both CHS 828 and MGBG appeared after approximately 20 h of drug exposure, more clearly in the case of CHS 828.

The resemblance between CHS 828 and MGBG, in cytotoxicity described by dose–response curves and different exposure times, was not reflected in the way the drugs affected the activity of the enzymes *S*-adenosyl methionine decarboxylase and ornithine decarboxylase, enzymes important in the synthesis of the polyamines. The present observation with an increased activity of the enzymes in cells exposed to MGBG may seem paradoxical. However, this phenomenon has been encountered in other cell systems and is likely to reflect a large increase in the amount of enzyme protein due to stabilization against breakdown. Such stabilization occurs with competitive inhibitors like MGBG (Pegg, 1988; Pegg and McCann, 1982). Initially, there is an inhibition of enzyme activity resulting in a decreased polyamine content and the subsequent increase is believed to be a late event that represents a compensatory mechanism for the decreased spermidine and spermine contents (Sjöholm et al., 1993, 1994).

MGBG effectively reduces the intracelullar concentrations of spermidine and spermine at micromolar levels and its antiproliferative action is fully reversible upon addition of exogenous polyamines (Jänne et al., 1978; Porter et al., 1981). Most of the effects of MGBG on cultured cells can be prevented by the concurrent administration of spermidine at equimolar or higher concentrations (Porter et al., 1981). This is in line with the present study where all concentrations of spermidine fully reversed the cytotoxic effects of MGBG. On the contrary, there was no effect of spermidine on survival of cells exposed to CHS 828.

In conclusion, CHS 828 does not appear to share the same mechanism of action as MGBG with respect to

inhibition of the polyamine synthesis. Further studies with CHS 828 are ongoing and the exact mechanisms for its cytotoxic effects remain to be determined.

Acknowledgements

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 Emica Dizdarevic are gratefully acknowledged.

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