PERGAMON

European Journal of Cancer 37 (2001) 260–267

European Journal of Cancer

www.ejconline.com

Temporal effects of the novel antitumour pyridyl cyanoguanidine (CHS 828) on human lymphoma cells

P. Martinsson [a,*], G. Liminga [a], S. Dhar [a], M. de la Torre [b], A. Lukinius [b], E. Jonsson [a], S. Bashir Hassan [a], L. Binderup [c], J. Kristensen [a], R. Larsson [a]

[a] *Division of Clinical Pharmacology, University Hospital, Uppsala University, S-751 85 Uppsala, Sweden*
[b] *Division of Genetics and Pathology, University Hospital, Uppsala University, S-751 85 Uppsala, Sweden*
[c] *Leo Pharmaceutical Products, Industriparken 55, DK-2750 Ballerup, Denmark*

Received 10 May 2000; received in revised form 30 August 2000; accepted 31 August 2000

Abstract

CHS 828, a novel pyridyl cyanoguanidine, has shown potent antitumour activity both *in vitro* and *in vivo* and is currently undergoing phase I evaluation in humans in collaboration with the European Organization for Research and Treatment of Cancer (EORTC). Here we study the temporal effects of CHS 828 on cytotoxicity, protein and DNA synthesis, cellular morphology and ultra structure using the lymphoma cell line U-937 GTB as the primary tumour model. *In vitro* analysis of tumour cell survival in response to CHS 828 revealed a cytotoxic effect progressively increased as a function of exposure time with maximum efficacy observed after 72 h. Activity of CHS 828 on U-937 GTB cells grown *in vivo* was also found. CHS 828 induced-cell death was dependent on intact protein synthesis and most cells appeared to lose their membrane integrity in the presence of a relatively well preserved nuclear structure. The results indicate that CHS 828 induced active and delayed cell death with a non-apoptotic morphology.

Keywords: Antineoplastic agent; Human lymphoma; Cell line; Morphology; Cytotoxicity

1. Introduction

In the systematic search for new agents for cancer therapy, it was discovered that a number of pyridyl cyanoguanidines showed antitumour activity after oral administration in a routine screening programme in a rat model [1]. Subsequent studies in a panel of human tumour cell lines showed potent antiproliferative activity of CHS 828 in the nanomolar to micromolar range [1]. CHS 828 also showed significant antitumour activity in several *in vivo* tumour models at doses causing little or no toxicity [1,2]. This was especially pronounced in a nude mouse model of small cell lung cancer (NYH) and in the xenograft model of MCF-7 breast cancer cells, two tumour model systems highly and generally resistant to standard drugs [2]. *In vivo* activity was also observed in Yoshida hepato-sarcoma and Walker 256 breast carcinoma, as well as in chemically induced breast tumours in the rat [1,2].

Further studies using a panel of cell lines representing defined mechanisms of resistance, revealed a differential pattern of antitumour activity with some cell lines sensitive in the subnanomolar range. In addition, there was a low correlation with clinically used standard agents and no significant influence of permeability glycoprotein (Pgp), multidrug resistance-associated protein (MRP), glutathione S-transferase (GSH), topoisomerase II or tubulin-associated multidrug resistance (MDR) on CHS 828 sensitivity was apparent [2]. CHS 828 is currently undergoing phase I evaluation in patients with various solid tumours, and a phase II study in patients with chronic lymphatic leukaemia has been initiated.

In the present study, we attempt to characterise the mode and kinetics of CHS 828-induced cytotoxicity using the histiocytic lymphoma cell line U-937 GTB as a model. This cell line is readily inducible to apoptosis by various stimuli [3–9] and is CHS 828-sensitive [2].

* Corresponding author. Tel.: +46 (18) 6111010; fax: +46 (18) 519237.
E-mail address: petra.martinsson@medsci.uu.se (P. Martinsson).

0959-8049/01/$ - see front matter
PII: S0959-8049(00)00375-0

2. Materials and methods

2.1. Drugs and reagents

N-(6-(4-chlorophenoxy)hexyl)-N′-cyano-N″-4-pyridylguanidine (CHS 828) was obtained from Leo Pharmaceuticals, Denmark. The chemical structure is depicted in Fig. 1. CHS 828 was dissolved in 100% dimethyl sulphoxide (DMSO) and kept at −20°C as a stock solution of 10 mM, further dilutions were made in 30% DMSO (1 mM CHS 828) and phosphate-buffered saline (PBS). Etoposide (Vepesid, Bristol-Myers Squibb, Bromma, Sweden) at 15 μg/ml (25 μM), was used as positive control for apoptotic features [9,10] in several of the experiments. Cycloheximide was used as a positive control during the protein synthesis experiments and aphidicolin as a positive control during the DNA synthesis experiments (both from Sigma Chemical Co., St Louis, MO, USA). The probe for fluorometric microculture cytotoxicity assay (FMCA) (FDA; Sigma Chemical Co.) was dissolved in 100% DMSO and kept frozen (−20°C) as a stock solution (10 mg/ml) protected from light. All other solvents and chemicals were of analytical grade, and obtained through commercial sources. Drugs were freshly prepared from stock solutions for each experiment.

2.2. Cell lines

The histiocytic lymphoma cell line U-937 GTB [11] was used for all experiments, and the myeloma cell line Roswell Park Memorial Institute (RPMI) 8226/S, the leukaemia cell line CEM/S and the lung cancer cell line NCI H69 where indicated. All cells were maintained in RPMI 1640 complete medium (without phenol red for microculture kinetic (MiCK) experiments, Sigma-Aldrich Co. Ltd, Irvine, UK), supplemented with 10% heat inactivated fetal bovine serum (FBS; Hy Clone, Cramlington, UK), 2 mM L-glutamine, 50 μg/ml streptomycin and 60 μg/ml penicillin (Hy Clone) in a controlled humidified atmosphere of 37°C, 5% CO_2, and were harvested in log phase. Cultures were monitored and passaged twice weekly, receiving fresh growth medium the day before subcultivation.

2.3. Fluorometric Microculture Cytotoxicity Assay (FMCA)

This total cell kill assay was performed as previously described [12]. In brief, 96-well microtitre plates (MTP, Nunc, Roskilde, Denmark) were prepared with 20 μl CHS 828 at 10 times the desired final concentration, or 10 μl of CHS 828 and 10 μl of either aphidicolin or cycloheximide at 20 times the desired final concentrations, kept at −70°C and thawed upon use. Aliquots of 180 μl of cell suspension containing 2×10^4 cells per well were used. At the desired time-points, the plate was washed with PBS, FDA was added and fluorescence measured after 40 min in a 96-well scanning fluorometre (Fluoroskan II, Labsystems Oy, Helsinki, Finland). Six wells containing only culture medium served as blanks and six wells containing cell suspension, but no drug, served as untreated controls in each experiment.

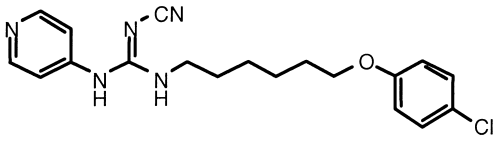

Fig. 1. Structural formula for CHS 828.

2.4. Light and electron microscopy

Cytospin preparations of cells cultured in MTPs in the presence or absence of drug were air-dried and stained with May–Grünwald/Giemsa dye (MGG). The slides were microscopically examined for viability and the presence of apoptosis, based on morphological criteria previously established [13]. 10 fields of vision (40×) were examined in each sample.

Cells for electron microscopy were kept and treated in culture flasks at 2.5×10^5 cells/ml. Drug (or PBS for control culture) was added at ten times the desired final concentration at one-tenth of the final volume. After rinsing in PBS at desired time-points, cells were processed for ultra-structural analysis and embedded in epoxy resin using a conventional protocol [14].

2.5. Microculture kinetic (MiCK) assay

The method measures changes in optical density (OD) in a cell culture due to early apoptotic changes in cell morphology, such as membrane blebbing and has previously been described by Kravtsov and co-workers [15]. The correlation of results obtained by the MiCK assay to time-lapse video microscopy, flow cytometry of light scattering properties, Annexin V binding and DNA gel electrophoresis has been elegantly shown [16]. Flat-bottomed MTPs were prepared with 20 μl drug solution in triplicates, at 10 times the desired final concentration, and kept at −70°C. Cells were washed and resuspended in complete medium without phenol red and cell density was adjusted to 1.1×10^6 cells/ml. Aliquots of 180 μl of cell suspension were seeded into the wells and the MTP placed in a standard incubator for 30 min of gas and temperature equilibration. Six blank wells received only growth medium and control wells received cell suspension without drug. Subsequently each well was layered with 50 μl of sterile mineral oil (Sigma-Aldrich, Stockholm, Sweden) to prevent evaporation and contamination, and MTPs placed in a Spectramax Plus spectrophotometer (Göteborgs Termometerfabrik,

Sweden). OD at 405 nm was measured automatically every 14 min for 48 h. During this monitoring, MTPs were kept at 37°C protected from light. Data from the Spectramax Plus were analysed by the SOFTmax® PRO (Molecular Devises, Sunnyvale, CA, USA).

2.6. Measurements of DNA and protein synthesis

Protein and DNA synthesis were measured with a Cytostar-T® plate, available in Amershams 'In Situ mRNA Cytostar-T® assay' kit, (Amersham International plc, Buckinghamshire, UK), a pre-made scintillating MTP, with scint fluid moulded into the bottom of the wells [17,18]. 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 222nCi/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 5×10^4 U-937 GTB cells were added to each well, except blank wells where only medium containing isotope was added. Drugs (test wells) and PBS (blank and untreated control wells) were added in duplicates (20 μl/well) 2 h after cell seeding when the measured radioactivity in cell-containing wells was at least twice the baseline value. Radioactivity was measured with a Wallac 1450 MicroBeta® trilux liquid scintillation counter (Wallac OY, Turku, Finland) using MicroBeta® Windows workstation software (Wallac OY).

2.7. *Hollow fibre* in vivo *assay*

In the *in vivo* experiment, polyvinylidene fluoride hollow fibres (inner diameter 1 mm, molecular cut-off 500 kDa; Spectrum Medical Industries, Los Angeles, CA, USA) were filled with cells at a density of 2×10^6 cells/ml and heat sealed in 20 mm parts, method modified from Hollingshead and colleagues [19]. The fibres were incubated *in vitro* for 48 h before implantation subcutaneously (s.c.) in male Sprague–Dawley rats (Charles River, Uppsala, Sweden). On the day after implantation (day 0) and four subsequent days the rats received 75 mg/kg of CHS 828 in a 10 mg/ml suspension with 2% methylcellulose or saline by oral gavage. On day 5, fibres were retrieved and cell density determined by staining with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) for 4 h. MTT is converted to formazan by metabolically active cells, the amount of formazan being proportional to the number of living cells. After rinsing the fibres and drying them, the formed formazan was extracted with DMSO and the OD read at 570 nm. Rats were monitored for 2 weeks with respect to weight and haematological parameters.

The study included two control rats and two CHS 828-treated rats, each one carrying three fibres with U-937 GTB and one empty fibre. Results are expressed as net growth (%), calculated for each fibre as: $((OD_{retrieval\ day}) - (\text{mean } OD_{implantation\ day}))/(\text{mean } OD_{implantation\ day})$. Hence, a net growth of −100% represents total cell kill, while a value greater than 0% represents net growth in the fibre.

3. Results

3.1. Cytotoxic activity

The cytotoxic *in vitro* activity of CHS 828 was investigated in the U-937 GTB human lymphoma cell line with the total cell kill assay FMCA. Detailed temporal analysis of viability revealed an exposure time-dependent pattern of cytotoxic effect (Fig. 2). No cytotoxic effect was observed at 24 h, whereas maximum efficacy was reached at very low concentrations (nanomolar) after 72 h continuous incubation with the drug. Utilising the hollow fibre assay, the *in vivo* cytotoxicity was studied. Cells retrieved from treated animals showed a total cell kill (−75% compared with the implantation day), while the cells had been proliferating in the fibres in the control animals, showing a mean cell number 170% greater than on the implantation day (Fig. 3). Cell survival in fibres retrieved only 24 h after the first dose of CHS 828 showed full viability. At the dose intensity used, toxicity was low with no lowering of white blood cells and almost no effect on platelets, haemoglobin, weight gain or gross appearance.

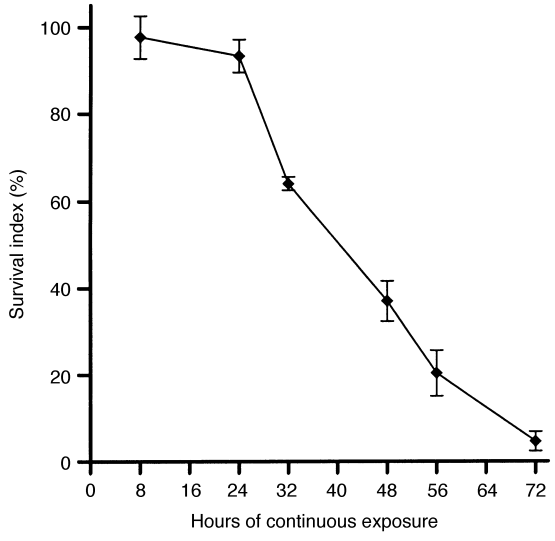

Fig. 2. Exposure time-dependent cytotoxic effect for 0.1 μM CHS 828. Total cell kill was measured by FMCA. Survival index = (fluorescence of treated wells-blank/fluorescence of untreated control-blank)×100. Error bars indicate the standard error of the mean (SEM) of three separate experiments.

3.2. Effects on DNA and protein synthesis

Next, the effect of CHS 828 on DNA and protein synthesis was investigated by measurements of cellular ^{14}C-labelled thymidine and leucine incorporation sequentially over time using Cytostar-T® microplate technology. No inhibitory effects were observed during the first 24 h of exposure. However, within a few hours from this time-point leucine and thymidine uptakes were almost completely inhibited (Fig. 4a, b). The effect of CHS 828 on protein synthesis was detected slightly earlier and was relatively more pronounced compared with the effect on DNA synthesis. These temporal effects on macromolecular synthesis were in sharp contrast to the pattern of inhibition observed for the positive control agents, cycloheximide and aphidicolin, as well as for etoposide at equitoxic concentrations (Fig. 4a, b).

In order to determine the dependence on macromolecular synthesis of the cytotoxic effect of CHS 828, cytotoxicity was studied in the presence of cycloheximide or aphidicolin, relatively specific inhibitors of protein and DNA synthesis, respectively (Fig. 5a, b). Continuous exposure to 1 μg/ml cycloheximide, which completely inhibits protein synthesis and proliferation without a significant effect on viability for 72 h, protected U-937 GTB cells from undergoing CHS 828-induced cell death. At 0.1 μg/ml cycloheximide, proliferation and protein synthesis was inhibited to approximately 50% and also under these conditions protection from CHS 828-induced toxicity was observed, although less pronounced. In contrast, blocking preferentially DNA synthesis with aphidicolin at 0.1–1.0 μg/ml did not prevent the effects of CHS 828. However, protection was observed at lower CHS 828 concentrations (0.001–0.01 μg/ml).

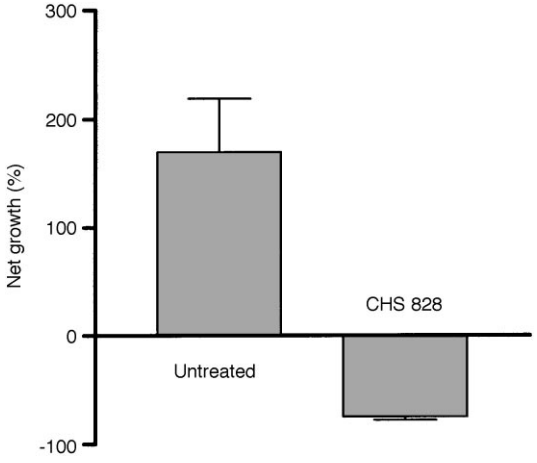

Fig. 3. Effect of CHS 828 in the *in vivo* hollow fibre experiment. The bars show mean net growth (%) of the cells in fibres from untreated and CHS 828-treated rats. Error bars indicate standard error of the mean (SEM) of five–six fibres/group.

3.3. Mode of cell death

In order to further characterise the kinetics and mode of CHS 828-induced cell death, we employed the MiCK assay [15,16]. The MiCK assay is based on the continuous monitoring of changes in OD in undisturbed microcultures of tumour cells and has been used to monitor the onset, extent and duration of the apoptotic process [16]. In the MiCK assay, apoptosis is reflected by a steep increase in OD of the cell cultures caused by early morphological changes, i.e. membrane blebbing. This contrasts to cells undergoing necrosis for which a decline in OD is typically observed. Typical apoptotic and necrotic control curves are shown in response to etoposide and Triton X-100 0.1%, respectively (Fig. 6). In response to CHS 828, however, the OD curve for cells exposed to concentrations 0.1–10 μM increased at

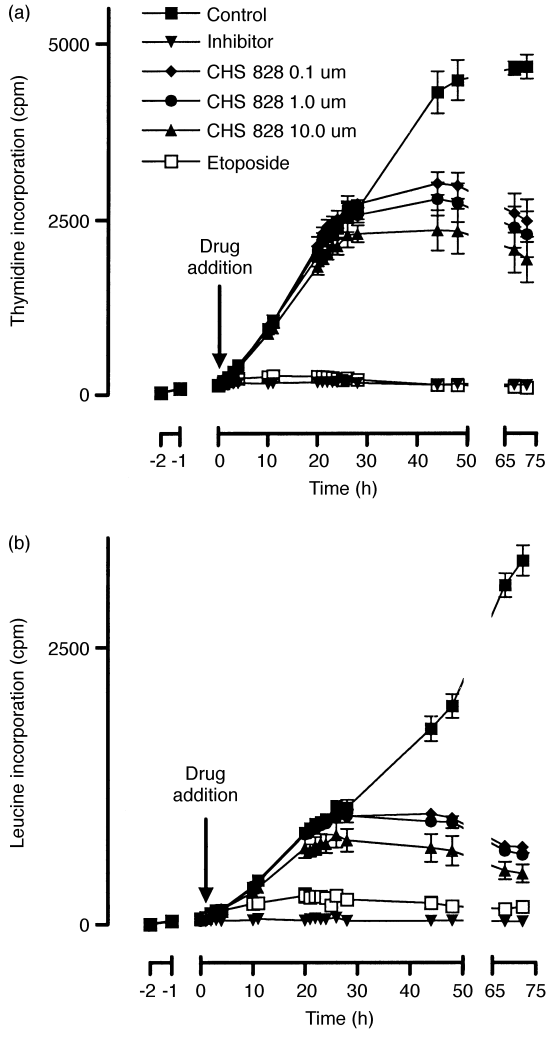

Fig. 4. U-937 GTB cells monitored in a Cytostar-T® plate for 72 h showing: (a) DNA synthesis measured by the incorporation of ^{14}C-labelled thymidine. Inhibitor: aphidicolin. (b) Protein synthesis measured by the incorporation of ^{14}C-labelled leucine. Inhibitor: cycloheximide 10 μg/ml. Error bars indicate standard error of the mean (SEM) of three to four separate experiments. cpm, counts per minute.

approximately the same slope as that for untreated control cultures (the increase representing proliferation) for approximately 24 h, and then decreased steeply. The same patterns of OD changes were evident for CHS 828-treated CEM/S and RPMI 8226/S cell line cultures (data not shown).

Based on this kinetic information, a series of cytospin slides were prepared at various times (8, 24, 32, 44, 56 and 72 h) of exposure to CHS 828 0.1 μM and subsequently stained with MGG. As a positive control, etoposide was employed and slides were prepared after 1, 2, 4, 6 and 8 h exposure. CHS 828 exposure for 44–72 h induced extensive and increasing cytoplasmic fragmentation, vacuolisation of nuclei and cytoplasm and eventually total cellular collapse, but little or no apparent karyorrhexis or formation of apoptotic bodies in cells with intact plasma membranes. Cells that lost membrane integrity showed relatively intact nuclear structures. At 44 h viability (defined as microscopic integrity of the nucleus and cytoplasm) was estimated to 70%, at 56 h 50%, decreasing rapidly towards zero at 72 h (Fig. 7). These morphological changes in response to CHS 828 appear to be a general phenomenon, being reproducible in other types of cell lines (CEM/S, NCI H69 and RPMI 8226/S), as well as in primary cultures of patient leukaemia cells (data not shown).

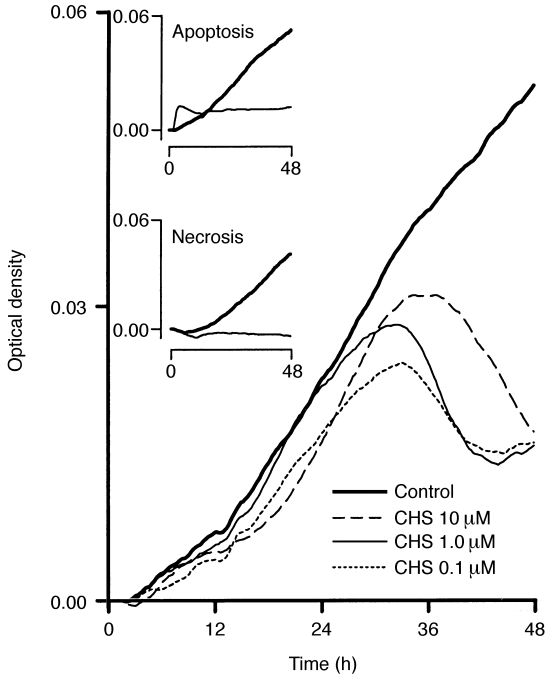

Fig. 6. MiCK assay curves; optical density (OD) kinetics of cell cultures treated with CHS 828 at three different concentrations. The inset shows the effects of etoposide as an example of apoptosis, and Triton X-100 0.1% as an example of immediate necrosis (bold lines represent untreated control culture). One representative experiment out of three.

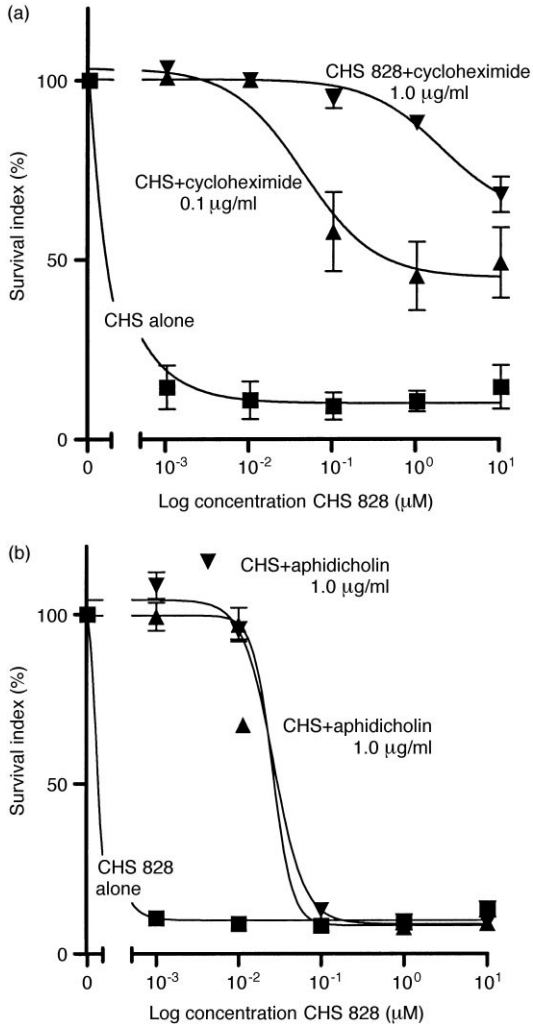

Fig. 5. Dependence of CHS 828-induced cytotoxicity on (a) protein synthesis; and (b) DNA synthesis during 72 h of exposure to CHS 828 0.1 μM as measured by the fluorometric microculture cytotoxicity assay (FMCA). Error bars indicate standard error of the mean (SEM) of three separate experiments.

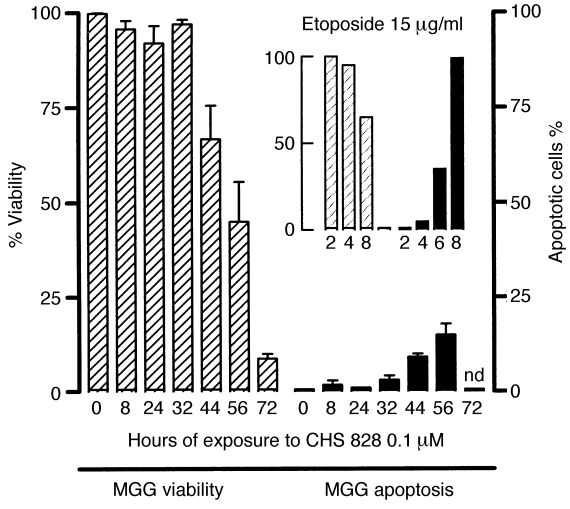

Fig. 7. Effect of 0.1 μM CHS 828 on cell viability and frequency of May–Grünwald/Giemsa (MGG) stained apoptotic cells. The experiments were repeated three times with similar results. nd, not determined. Error bars indicate SEM of three different experiments.

Morphological evaluation of cells from the hollow fibres of the *in vivo* experiments revealed the same appearance of cells as those treated *in vitro*.

Electron microscopy performed at 24, 48 and 64 h largely confirmed the findings from light microscopy. All control cells were spherical with short clumsy protrusions. The nuclei showed deep invaginations and one or two nucleoli, and the cytoplasm was filled with mitochondria, ribosomes, endoplasmic reticulum, Golgi apparatus and transport vesicles. After 6 h exposure to etoposide, cells showed rounding of nuclei, condensed chromatin in a spotty or crescent fashion, apoptotic bodies, extensive vacuolisation and intact plasma membranes (Fig. 8a, b). After 24 h exposure to CHS 828, the ultrastructure was indistinguishable from that of untreated control. However, CHS 828 treatment for 48 and 64 h showed cells with swollen mitochondria with the inner membrane structure under decomposition and dilated endoplasmic reticulum and apparently normal plasma membrane (Fig. 8c, d). Some cells showed nuclei with condensed chromatin in a spotty pattern (Fig. 8c), other cells exhibited largely unaffected nuclei with deep invaginations (Fig. 8d). Occasionally a cell displayed typical (early) apoptotic ultrastructure with the chromatin condensed in apoptotic bodies or in a crescent fashion.

4. Discussion

There is a constant need for new cytotoxic drugs with novel mechanisms of action and resistance patterns, and CHS 828 may have the potential to meet this need [2]. The present study was undertaken to further elucidate the effects of this drug, and the results provide us with a temporal map of the events leading to the death of cells treated with CHS 828.

CHS 828 was found to induce a delayed inhibition of macromolecular synthesis with unusual kinetics. No difference in thymidine or leucine incorporation compared with control cultures was observed during the first 24 h. Furthermore, no decrease in viability during this period of time was observed. In fact a concentration-dependent stimulation of metabolic activity in response to CHS 828 during this period has been reported [20]. At the 24 h time-point, there were no changes in the cell

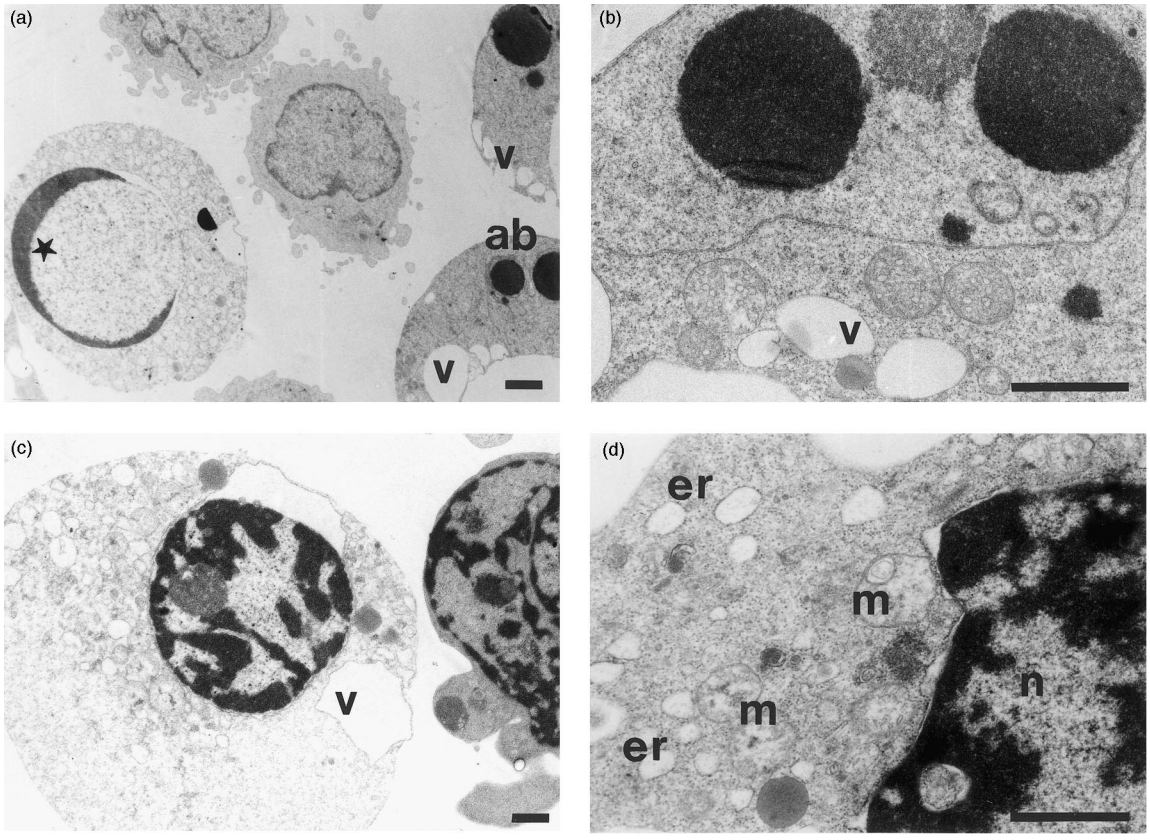

Fig. 8. Electron microscopy study of U-937 GTB cells. (a, b) Classical apoptosis induced by 6 h exposure to etoposide (15 μg/ml). The condensed chromatin is arranged as a crescent, ⋆, along an intact nuclear membrane or as apoptotic bodies, **ab**. The cytoplasm contains destroyed mitochondria, **m**, and numerous vacuoles, **v**. The plasma membrane is intact. (c, d) Cells exposed to CHS 828 for 64 h show (c) two cells with condensed chromatin gathered along the nuclear membranes, and cytoplasms under decomposition enveloped by intact plasma membranes. (d) An apparently normal nucleus, **n**, dilated endoplasmic reticulum, **er**, swollen and affected mitochondria, **m**, and an intact plasma membrane are demonstrated. Magnifications: (a and c)×5400; (b and d)×18000. Scale bars = 1 μm.

cycle distribution measured by fluorescence activated cell separator (FACS) nor any signs of differentiation, as judged by measurements of cell determinant (CD) 11c, CD 14 and CD 49f expression, as well as routine cytochemical testing of naphthol esterase activity (data not shown). However, shortly after this time-point, both DNA and protein syntheses were affected and the first morphological signs of cell death were observed after 44 h. The kinetics of inhibition of DNA and protein synthesis was clearly different from that observed in response to standard cytotoxic agents at equitoxic concentrations [21]. These drugs, representing several mechanistic classes, were shown to induce inhibition within 1–10 h of exposure. The described effects on viability and macromolecular synthesis were observed at low concentrations of CHS 828 and increasing this concentration 100-fold did not increase the maximum efficacy. A similar pattern of events was observed for other cell lines as well, including those of solid tumour origins (data not shown). The same cell death kinetics were observed also in the *in vivo* setting, supporting potential clinical relevance of the *in vitro* results. These data suggest the inhibition/activation of a highly specific cellular process apparently saturated at CHS 828 concentrations well below those achievable and tolerable *in vivo* in rats and dogs (>1 μM, data not shown), indicating a potentially favourable therapeutic index.

What then is the mode of cell death in the CHS 828-treated cultures? Apoptosis is proposed to be the main mechanism whereby chemotherapy and radiation induce cell killing of tumour cells [22–27]. In the present study, the delayed CHS 828-induced cytotoxicity did not conform completely to the typical morphology of classical apoptosis.

Using the MiCK assay to monitor early events associated with the apoptotic process did not show an OD curve indicative of apoptosis, but a curve rather compatible with stimuli producing delayed necrosis. In fact, as evident from light microscopy of MGG-stained cells, many cells appear to die subsequent to extensive cytoplasmic alterations with relatively intact nuclei with only early changes of nuclear chromatin consistent with classical apoptosis. The described morphology of CHS 828-induced cell death does not readily conform to the patterns of nuclear fragmentation described for U-937 GTB cells in response to a variety of apoptogenic stimuli, including oxidative stress [28]. Active cell death with non-apoptotic features has previously been described in different cell systems, including neuronal cells and fibroblasts [29–32]. These cell systems can all undergo cell death in an organised manner involving signalling and execution that results in cell morphology traditionally associated with necrosis. However, the precise mechanisms underlying CHS 828-induced cell death remain to be investigated, using more specific markers of the apoptotic pathway.

Acknowledgements

We are indebted to Carina Alvfors, Maria Hellström and Christina Leek for technical assistance. We also thank Drs Kenneth Nilsson, Christer Sundström and Fredrik Öberg, Department of Genetics and Pathology, Uppsala University, for their help with measurements of cellular differentiation and cell cycle distribution. This study was supported in part by Leo Pharmaceutical Products, Denmark.

References

1. Schou C, Ottosen E, Björkling F, *et al*. Novel cyanoguanidines with potent oral antitumour activity. *Bioorg Med Chem Lett* 1997, **7**, 3095–3100.
2. Vig Hjarnaa PJ, Jonsson E, Latini S, *et al*. CHS 828, a novel pyridyl cyanoguanidine with potent antitumor activity in vitro and in vivo. *Cancer Res* 1999, **59**, 5751–5757.
3. Lennon S, Martin S, Cotter T. Dose-dependent induction of apoptosis in human tumor cell lines by widely diverging stimuli. *Cell Prolif* 1991, **24**, 203–214.
4. Lai Y, Chen Y, Wu T, *et al*. Induction of apoptosis in human leukemic U937 cells by tetrandine. *Anticancer Drugs* 1998, **9**, 77–81.
5. Larochette N, Decaudin D, Jacotot E, *et al*. Arsenite induces apoptosis via a direct effect on the mitochondrial permeability transition pore. *Exp Cell Res* 1999, **249**, 413–421.
6. Ashush H, Rozenszajn LA, Blass M, *et al*. Apoptosis induction of human myeloid leukemic cells by ultrasound exposure. *Cancer Res* 2000, **60**, 1014–1020.
7. Yang GH, Jarvis BB, Chung YJ, Pestka JJ. Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. *Toxicol Appl Pharm* 2000, **164**, 149–160.
8. Okuma E, Saeki K, Shimura M, Ishizaka Y, Yasugi E, You A. Induction of apoptosis in human hematopoietic U937 cells by granulocyte-macrophage colony-stimulating factor: possible existence of caspase 3-like pathway. *Leukemia* 2000, **14**, 612–619.
9. Shrivastava P, Sodhi A, Ranjan P. Anticancer drug-induced apoptosis in human monocytic leukemic cell line U937 requires activation of endonuclease(s). *Anticancer Drugs* 2000, **11**, 39–48.
10. Dubrez L, Savoy I, Hamman A, Solary E. Pivotal role of a DEVD-sensitive step in etoposide-induced and Fas-mediated apoptotic pathways. *EMBO J* 1996, **15**, 5504–5512.
11. Sundström C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* 1976, **17**, 565–577.
12. Dhar S, Nygren P, Liminga G, *et al*. Anti-cancer drug characterisation using a human cell line panel representing defined types of drug resistance. *Br J Cancer* 1996, **74**, 888–896.
13. Levin S. Apoptosis, necrosis, or oncosis: what is your diagnosis? A report from the Cell Death Nomenclature Committee of the Society of Toxicologic Pathologists. *Toxicol Sci* 1998, **41**, 155–156.
14. Lukinius A, Jansson L, Korsgren O. Ultrastructural evidence for blood microvessels devoid of an endothelial cell lining in transplanted pancreatic islets. *Am J Pathol* 1995, **146**, 429–435.
15. Kravtsov VD, Fabian I. Automated monitoring of apoptosis in suspension cell cultures. *Lab Invest* 1996, **74**, 557–570.
16. Kravtsov V, Daniel T, Koury M. Comparative analysis of different methodological approaches to the in vitro study of drug-induced apoptosis. *Am J Pathol* 1999, **155**, 1327–1339.

17. Harris DW, Kenrick MK, Pither RJ, Anson JG, Jones DA. Development of a high-volume *in situ* mRNA hybridization assay for the qualification of gene expression utilizing scintillating microplates. *Analyt Biochem* 1996, **243**, 249–256.
18. Graves R, Davies R, Brophy G, O'Beirne G, Cook N. Non-invasive, real-time method for the examination of thymidine uptake events — application of the method to V-79 cell synchrony studies. *Analyt Biochem* 1997, **248**, 251–257.
19. Hollingshead MG, Alley MC, Camalier RF, *et al*. In vivo cultivation of tumor cells in hollow fibers. *Life Sci* 1995, **57**, 131–141.
20. Ekelund S, Liminga G, Björkling F, *et al*. Early stimulation of acidification rate by novel cytotoxic pyridyl cyanoguanidines in human tumor cells: comparison with *m*-iodobenzylguanidine. *Biochem Pharmacol* 2000, **60**, 839–849.
21. Liminga G, Martinsson P, Jonsson B, Nygren P, Larsson R. Calcein acetoxymethyl ester induces apoptosis in the human histiocytic lymphoma cell line U-937 GTB. *Biochem Pharmacol* 2000, in press.
22. Hickman J. Apoptosis induced by anticancer drugs. *Cancer Metast Rev* 1992, **2**, 121–139.
23. Huschtscha L, Bartier W, Malmström A, Tattersall M. Cell death by apoptosis following anticancer drug treatment in vitro. *Int J Oncol* 1995, **6**, 585–593.
24. Sachs L, Lotem J. Control of programmed cell death in normal and leukemic cells: new implications for therapy. *Blood* 1993, **82**, 15–21.
25. Hannun Y. Apoptosis and the dilemma of cancer chemotherapy. *Blood* 1997, **89**, 1845–1853.
26. Hickman J. Apoptosis and chemotherapy resistance. *Eur J Cancer* 1996, **32A**, 921–926.
27. Dive C, Evans C, Whetton A. Induction of apoptosis — new targets for cancer chemotherapy. *Semin Cancer Biol* 1992, **3**, 417–427.
28. Dini L, Coppola S, Ruzittu M, Ghibelli L. Multiple pathways for apoptotic nuclear fragmentation. *Exp Cell Res* 1996, **223**, 340–347.
29. Nicotera P, Leist M, Manzo L. Neuronal cell death: a demise with different shapes. *Trends Pharmacol Sci* 1999, **20**, 46–51.
30. Schwartz LM, Smith SW, Jones ME, Osborne BA. Do all programmed cell deaths occur via apoptosis? *Proc Natl Acad Sci USA* 1993, **90**, 980–984.
31. Zakeri ZF, Quaglino D, Latham T, Lockshin RA. Delayed internucleosomal DNA fragmentation in programmed cell death. *FASEB J* 1993, **7**, 470–478.
32. Umemura S, Osamura RY, Tsutsumi Y. Cell renewal and functional morphology of human lactating breast: presentation of a new mode of cell death ('magentosis') characterized by nuclear periodic acid-Schiff reactivity. *Pathol Int* 1996, **46**, 105–121.