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Cell death with atypical features induced by the novel antitumoral drug CHS 828, in human U-937 GTB cells

Petra Martinsson [a,*], Manuel de la Torre [b], Lise Binderup [c], Peter Nygren [d], Rolf Larsson [a]

[a] *Department of Medical Sciences, Clinical Pharmacology, University Hospital, Uppsala University, SE-751 85 Uppsala, Sweden*
[b] *Department of Genetics and Pathology, University Hospital, Uppsala University, SE-751 85 Uppsala, Sweden*
[c] *Leo Pharmaceutical Products, Industriparken 55, DK-2750 Ballerup, Denmark*
[d] *Department of Oncology, Radiology and Clinical Immunology, University Hospital, Uppsala University, SE-751 85 Uppsala, Sweden*

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Abstract

N-(6-(4-chlorophenoxy)hexyl)-*N′*-cyano-*N″*-4-pyridylguanidine (CHS 828), with promising antitumoral effects in vitro and in vivo, is currently in clinical Phase I and II studies. Its exact mechanism of action is unclear, but previous studies indicate that CHS 828 induces a controlled, delayed mode of cell death. The characteristics of the cell death process were investigated in vitro in the apoptosis-prone cell line U-937 GTB. Mitochondria showed hyperpolarization at 24 to 32 h and a subsequent late disruption of mitochondria membrane potential ($\Delta\psi_m$). Between 44 and 72 h of CHS 828 exposure, there was an increasing frequency of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) positive cells indicative of apoptosis, but caspase-3 was only modestly increased and caspases-8 and -9 showed no activation upon CHS 828 exposure. Furthermore, the morphology of exposed cells did not conform to classical apoptosis, and viability and morphology were unaffected by inhibition of caspases. Thus, CHS 828 induces several unexpected features in this system, suggesting a potentially novel mechanism of action.

Keywords: Human cell line; Cell death; Caspase; Mitochondria membrane potential

1. Introduction

Over the years, convincing evidence has accumulated showing that most cytotoxic drugs in clinical use exert their effect by inducing programmed cell death and apoptosis (Dive et al., 1992; Eastman, 1990; Fisher, 1994; Hannun, 1997; Kaufmann and Earnshaw, 2000; Kerr et al., 1994; Searle et al., 1975, and references therein). In addition, tumor cell inability to execute the apoptotic cell death pathway has been put forward as a possible reason of tumor resistance to treatment with antineoplastic agents (Fisher, 1994; Hannun, 1997).

Caspase proteases are key activators in the orchestration of apoptosis (Harvey and Kumar, 1998; Nunez et al., 1998; Thornberry and Lazebnik, 1998). One proposed pathway for induction of apoptosis involves mitochondria with opening of a permeability transition pore complex (Bernardi et al., 1999; Zamzami et al., 1997) or by other means disruption of the mitochondria membrane potential ($\Delta\psi_m$), the release of membrane-bound cytochrome *c* and apaf-1, and the subsequent activation of caspase-9 (Green and Reed, 1998; Kuida, 2000). Another involves death receptors (Fas/CD95, tumor necrosis factor receptor I) and the activation of caspase-8 (Salvesen, 1999), and both pathways eventually activate downstream caspase-3 (Porter and Jänicke, 1999) and/or caspases-6 and -7. Activation of the caspases involved can be inhibited by a specific peptide (DEVD-fmk for caspase-3) or pan-caspase inhibitors (e.g. Z-Asp-DCB (Z-Asp(CH_2-[2,6-dichlorobenzoyl)oxy]methane)). This commonly promotes a switch to necrotic cell death rather than cell survival (Kitanaka and Kuchino, 1999; McConkey, 1998), indicating that caspases are not responsible for active cell death per se, but for its characteristic features. Recently, it has been suggested that cell death in vitro should be classified only as caspase-dependent or -independent, where the former corresponds to apoptosis (Blagosklonny, 2000).

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

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Since the modified pyridyl cyanoguanidine CHS 828 was synthesized and described by Schou et al. (1997) at Leo Pharmaceutical Products, Denmark, several studies have indicated a promising antitumoral effect of this drug (Ekelund et al., 2000; Jonsson et al., 2001; Martinsson et al., 2001; Hansen et al., 2001; Vig Hjarnaa et al., 1999). These findings encompass a high degree of human tumor cell kill without toxic effects in animal models (Jonsson et al., 2001; Vig Hjarnaa et al., 1999) unusual metabolic response in cell lines (Ekelund et al., 2000) and signs of active cell death seemingly lacking several apoptotic key features (Martinsson et al., 2001; Hansen et al., 2001) In the present study, we investigate further the cell death mechanisms involved in CHS 828 induced cytotoxicity in vitro in the human histiocytic lymphoma cell line U-937 GTB. These cells have been shown to respond to CHS 828 with cell death (Vig Hjarnaa et al., 1999) with previously described kinetics (Martinsson et al., 2001). The cells remain intact for 24 h, and subsequently abruptly shut off macromolecular synthesis. Viability is exposure time dependent and is unaffected up to 32 h from start of exposure. The cells are protected from undergoing CHS 828 induced cell death by protein synthesis inhibition.

On the other hand, U-937 GTB are readily inducible to undergo active cell death with classical apoptotic features, in response to various cytotoxic and physical stimuli (Ashush et al., 2000; Bicknell and Cohen, 1995; Dini et al., 1996; Dubrez et al., 1996; Lai et al., 1998; Lennon et al., 1991; Liminga et al., 2000; Okuma et al., 2000; Shrivastava et al., 2000; Yang et al., 2000). Etoposide at 25 μM is one clear example (Garrido et al., 1999; Sun et al., 1999) and was used as a control for apoptotic features detectable in U-937 GTB, by the methods employed in the present study.

In order to characterize the cell death induced by CHS 828, we studied DNA fragmentation by performing terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) of fixed cells on slides and $\Delta\psi_m$, by detecting the fluorescent reagent 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) in whole cell cultures exposed to CHS 828. Caspase activity was investigated by colorimetric detection of cleavage products and caspase dependence was evaluated from morphology and cell survival data.

2. Materials and methods

2.1. Drugs and reagents

Leo Pharmaceutical Products provided CHS 828 (*N*-(6-(4-chlorophenoxy)hexyl)-*N*′-cyano-*N*″-4-pyridylguanidine), which was dissolved in 100% dimethyl sulfoxide (DMSO) and kept at −20°C as a stock solution of 10 mM. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used as positive control for disruption of mitochondria membrane potential ($\Delta\psi_m$). For the experiments on the effect of caspase inhibitors, the pan-caspase inhibitor Z-Asp-DCB (Z-Asp(CH_2-[2,6-dichlorobenzoyl)oxy]methane; Calbiochem, La Jolla, CA) and the selective caspase-3 inhibitor DEVD-fmk (ApoAlert™ caspase-3 inhibitor; Clontech Laboratories, Palo Alto, CA) were used. All reagents for caspase activity measurements were part of a "Caspse-8 Colorimetric Assay" or "Caspse-9 Colorimetric Assay" (R&D Systems, Minneapolis, MN), except DEVD-pNA (Clontech Laboratories). Etoposide, 25 μM, was used as a control of apoptotic features in most of the experiments. 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. Cells

The histiocytic lymphoma cell line U-937 GTB (Sundström and Nilsson, 1976), harvested in log phase was used for all experiments. Cells were maintained in RPMI 1640 complete medium (Sigma–Aldrich, Irvine, UK; without phenol red for Microculture Kinetics (MiCK) experiments), supplemented with 10% heat inactivated fetal bovine serum (Hy Clone, Cramlington, UK), 2 mM glutamin, 50 μg/ml streptomycin and 60 μg/ml penicillin (Hy Clone) in a controlled humidified atmosphere of 37°C, 5% CO_2. Cultures were passaged twice weekly, receiving fresh growth medium on the day before subcultivation.

2.3. Measurement of DNA fragmentation

The TUNEL assay was performed stringent to the commercial protocol (in situ cell death detection kit, fluorescein; Boehringer Mannheim, Mannheim, Germany). In brief, cytospin preparations of cells cultured in 96-well microtiter plates continuously exposed to CHS 828 0.1 μM, were fixed in 4% formaldehyde for 30 min, washed repeatedly with phosphate-buffered saline (PBS) and permeabilized in a solution of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min. After rinsing twice with PBS, the labeling reaction was allowed to take place under Parafilm M® for 60 min at 37°C, in a humidified atmosphere with 5% CO_2, protected from light. Slides were rinsed and air-dried before covering with antifade mounting medium (Vectashield®. Vector Laboratories, Burlingame, CA) and coverslips. Corresponding cell cultures continuously exposed to etoposide (25 μM) were used as a positive controls and slides were also made from untreated, negative controls. Two visual fields (40 × magnification) from slides from three separate experiments were analyzed visually in a confocal microscope. For every TUNEL slide, a corresponding cytospin preparation was air-dried and stained with May-Grünwald/Giemsa (MGG) for morphological evaluation.

2.4. Measurement of mitochondria membrane potential ($\Delta\psi_m$)

Changes in mitochondria membrane potential was assessed using JC-1, a lipophilic cation that accumulates and is retained in mitochondria, showing green fluorescence at low concentrations, but exceeding a concentration of 0.16 nM inside mitochondria it forms J-aggregates producing red fluorescence (Reers et al., 1991). Cells were suspended in medium at a concentration of 5×10^5 cells/ml and CHS 828 to a final concentration of 0.1 μM or CCCP to a final concentration of 10 μM was added. One corresponding culture received only PBS and served as control. The samples were incubated in a standard incubator and at 8, 24, 32, 48 and 72 h, 0.5×10^6 cells were collected by centrifugation and resuspended in 1 ml of PBS. A 10.1-μl JC-1 at 50 μg/ml was added (final concentration of 0.5 μg/ml) followed by incubation for 10 min and centrifugation ($200 \times g$, 5 min). Cells were resuspended in fresh PBS and 200 μl/well was dispensed into a microtiter plate. The plate was read in the Fluoroskan II, spectrofluorometer (Labsystems OY, Helsinki, Finland), with excitation and emission wavelengths set at 540 and 585 nm (red fluorescence) and 485 and 530 nm (green fluorescence), respectively. Results were calculated after fluorescence values had been corrected for cell density (as assessed in a Bürcher chamber) as (red fluorescence of test triplicates − red fluorescence of blanks)/(green fluorescence of test triplicates − green fluorescence of blanks) for each experiment. These mean fractions were averaged together and divided by mean control fractions and expressed as percentages.

2.5. Detection of caspase activity

Caspase activity was assayed by colorimetric detection of *p*-nitroanilidine (pNA) after cleavage of the peptide substrates DEVD-pNA, IETD-pNA or LEHD-pNA for caspases-3, -8 and -9, respectively. Cells were continuously exposed to 0.1 μM CHS 828, or 25 μM etoposide for positive control, in culture flasks at a cell density of 2.5×10^5 cells/ml. Cells from a flask not exposed to CHS 828 served as negative control. Aliquots of 2×10^6 cells were collected in duplicates by centrifugation at the indicated time points (Fig. 3). The supernatant was removed by aspiration, and pellets frozen and kept in −70°C until analysis. Pellets were thawed and cell lysis was induced by addition of 100 μl Cell Lysis Buffer followed by incubation on ice for 10 min. After 3 min of centrifugation at $12\,000 \times g$, 50 μl of the protein containing cell lysate was transferred to microtiter plate wells with flat bottoms, and 50 μl of Reaction Buffer with 1% dithiothreitol was added, together with 5 μl of the pNA-substrates (yielding final concentrations of 48 μM of DEVD-pNA or 190 μM of IETD-pNA and LEHD-pNA). Blank wells received 100 μl Reaction Buffer and 5 μl substrate. Absorbance was measured at 405 nm in a SpectraMax Plus plate reader after 90 min of dark incubation at 37°C. Activity was calculated as mean values from three consecutive experiments as (mean absorbance of duplicate CHS 828 exposed wells − mean absorbance of blank wells)/(mean absorbance of control wells − mean absorbance of blank wells) and expressed as percentages.

2.6. Effect of cell permeable caspase inhibitors

Microtiter plates with flat or V-shaped bottomed wells were prepared with CHS 828 in triplicates at 20 times the desired final concentration and kept in −70°C. For each experiment, one microtiter plate with flat bottom wells and one with V-shaped wells were thawed and caspase inhibitors at 20 times the desired final concentration were added (as indicated in Fig. 4). Cells were seeded at a density of 1.1×10^6/ml and 1.1×10^5/ml in the two plate types, respectively. Due to the high cell density required for the MiCK assay a higher concentration of CHS 828 (1 μM) was used in this setup. MiCK assay was performed on the flat-bottomed microtiter plate. The V-bottomed microtiter plate was placed in a standard incubator, cytospin slides were prepared from designated wells at indicated time points, and were stained with MGG, and Fluorometric Microculture Cytotoxicity Assay (FMCA; see below) performed after 72 h. The slides were microscopically examined for the presence of apoptosis, based on morphologic criteria previously established (Levin, 1998). Ten high power fields (40×) were examined in each sample.

2.7. The total cell kill FMCA

This non-clonogenic total cell kill assay has been described in detail elsewhere (Larsson et al., 1992) and is based on measurement of fluorescence generated by the hydrolysis of fluorescein diacetate to fluorescein by cells with preserved membrane integrity. Cells in triplicate wells in microtiter plates were exposed to the desired drug for 72 h before analysis. When testing single drugs, 20 μl/well was added at 10 times the desired final concentration and when testing combinations of two drugs 10 μl/well at 20 times the desired final concentration for each drug was added. Six wells receiving 20 μl PBS were used as controls. Results are expressed as Survival Indices (%) = (mean fluorescence of exposed triplicates − mean blank fluorescence)/(fluorescence of control triplicates − mean blank fluorescence) × 100.

2.8. The microculture kinetic (MiCK) assay monitors morphology

The MiCK assay detects early apoptotic cellular changes corresponding to morphologically detectable membrane

blebbing (Kravtsov et al., 1999). In brief, flat-bottomed microtiter plates were prepared with 10 μl/well of 20 μM CHS 828 in triplicates (20 times the desired final concentration) and 10 μl/well caspase inhibitors were added at 20 times desired final concentration upon thawing of plate, or 20 μl CHS 828 at 10 times desired final concentration (when tested as single drug) and kept at −70°C. U-937 GTB cells were washed and resuspended in complete medium without phenol red and cell density adjusted to 1.1×10^6 cells/ml. Aliquots of 180 μl of cell suspension were seeded into the wells and the plate was then placed in a standard incubator for 30 min of gas and temperature equilibration. Subsequently each well was layered with 50 μl of sterile mineral oil (Sigma–Aldrich, Stockholm, Sweden) and the microtiter plate placed in a Spectramax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). Absorbance at 600 nm was measured automatically every 14 min for 48 h. During this monitoring, the microtiter plate was kept at 37°C and protected from light. Data from the Spectramax Plus was analyzed by the SOFTmax® PRO (Molecular Devices).

3. Results

3.1. DNA degradation occurs late, in an exposure time-dependent fashion

When investigating DNA degradation in U-937 GTB by TUNEL assay, untreated controls showed a frequency of TUNEL positive cells of approximately 1% at all time points (0–72 h) whereas exposure to 25 μM etoposide for 8 h results in 99% of the cells staining positive. In response to CHS 828, TUNEL staining was indistinguishable from that of untreated controls up to 32 h of continuous exposure, at which time the percentage of stained nuclei increased to 5% of the total cell population, and the increase escalated further over time (Fig. 1). The increase in TUNEL positivity paralleled the appearance of cells with disrupted plasma membrane integrity as judged from MGG-stained control slides (not shown).

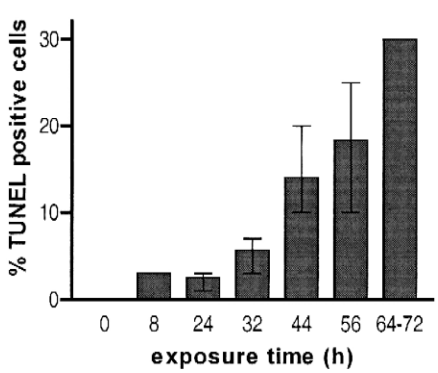

Fig. 1. Effect of 0.1 μM CHS 828 on frequency of TUNEL positive U-937 GTB cells at indicated exposure times. Means of two to four experiments, error bars indicate standard error of the mean (S.E.M.).

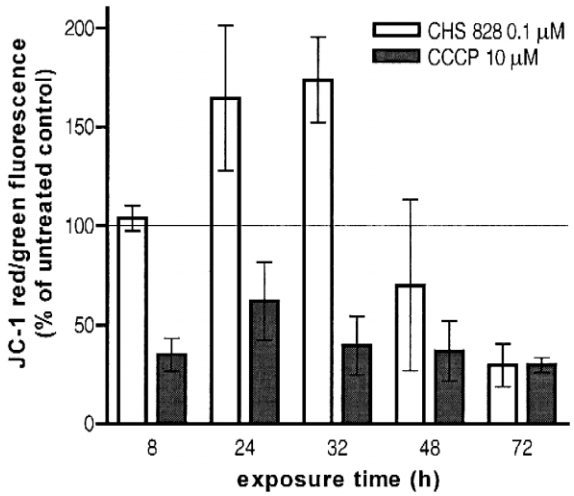

Fig. 2. Measurement of $\Delta\psi_m$, using the fluorescent probe JC1, after indicated times of exposure to CHS 828, and for positive control cell cultures exposed to CCCP. The results are presented as the ratio between JC1 red and green fluorescence in percentage of the ratio of untreated control cultures. Mean values (±S.E.M.) of three to four experiments.

3.2. Mitochondria are hyperpolarized as a primary response to CHS 828 exposure

When $\Delta\psi_m$ of U-937 GTB cells was estimated using the probe JC-1, a significant hyperpolarization of mitochondria occurred, evident after 24 h of CHS 828 exposure (Fig. 2), rather than the primary decrease in $\Delta\psi_m$ typical

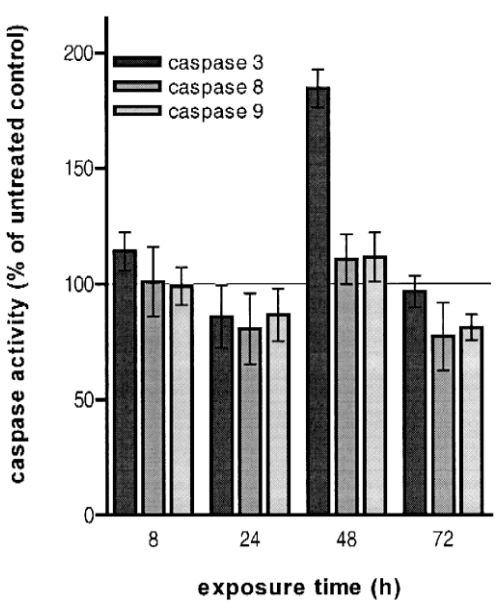

Fig. 3. Activity of indicated caspases in U-937 cells exposed to 0.1 μM CHS 828, shown as percentage of the activity in untreated control cultures. Mean values (±S.E.M.) of three experiments.

of apoptosis. After 48 h, disruption of $\Delta\psi_m$ followed. The electron transport uncoupler CCCP, used as a positive control, produced a decrease of 40–60%.

3.3. Caspase activities are low or absent

The activities of caspases-3, -8 and -9 were measured by detecting the cleavage product of their respective specific pNA substrates. Results are expressed as percentages of the activity in untreated controls and revealed that during 72 h no CHS 828 induced activation of any caspase was evident, with the exception of a modest increase of caspase-3 activity to 184% ($P < 0{,}001$) at 48 h (Fig. 3). This is in sharp contrast to etoposide-treated cells where the top value (at 4 h) exceeded 500% (not shown).

3.4. Caspase activity is not responsible for the morphology of dying cells

The dependency on caspase activity was investigated by co-incubating cells with 1.0 μM CHS 828 and either of the cell permeable caspase inhibitors DEVD-fmk or Z-Asp-DCB and monitoring crude kinetics of cell morphology, total cell viability and end point morphology. Concentrations of the caspase inhibitors were chosen as to produce little or no toxicity on their own (survival indices as measured by the FMCA of 110–73%, with Z-Asp-DCB 50 μM responsible for the bottom value), yet effectively blocking apoptosis induced by etoposide (not shown).

Neither of the two inhibitors, at any concentration, were able to prevent the cell death induced by CHS 828 (Fig. 4), and survival indices remained near 15%.

Accordingly, monitoring of the kinetics of morphological change by means of the MiCK assay, provided no evidence of any effect on morphology of the caspase inhibitors (Fig. 5). Visual analysis of morphological changes over time in MGG stained cytospin preparations further established the impression that the caspase inhibitors lacked influence on the shape of CHS 828 induced cell demise (not shown).

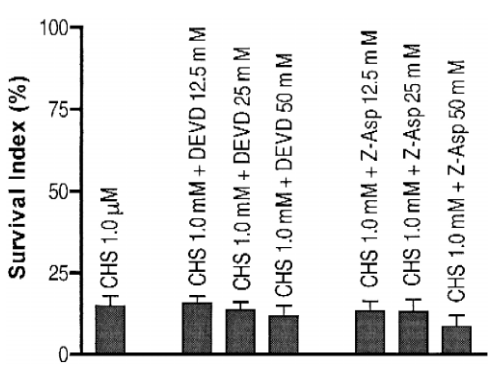

Fig. 4. The effect on cell viability after 72 h of continuous exposure as assessed in the FMCA of the caspase-3 inhibitor DEVD-fmk and the pan-caspase inhibitor Z-Asp-DCB at the indicated concentrations (μM) on U-937 GTB cells exposed to 1.0 μM CHS 828. Mean values (±S.E.M.) of three separate experiments.

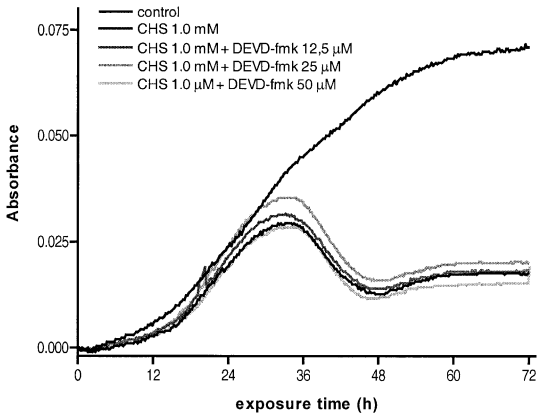

Fig. 5. Kinetics of crude morphology changes visualized by the MiCK assay. U-937 GTB cells were continuously co-exposed to 1.0 μM CHS 828 and the indicated concentrations of the caspase-3 inhibitor DEVD-fmk for 72 h.

4. Discussion

Active cell death with apoptotic features appears to be the main mechanism whereby chemotherapeutic agents induce tumor cell death (Dive et al., 1992; Hannun, 1997; Hickman, 1992, 1996; Huschtscha et al., 1995; Sachs and Lotem, 1993). This process requires active participation by the dying cell and, at least partly, the activation of an intact signaling pathway including the caspase cascade. This leads to endonuclease activation and ultimately to DNA cleavage, formation of apoptotic bodies and the typical apoptotic morphology (Depraetere and Golstein, 1998; Dubrez et al., 1996; McConkey, 1998; Nicotera et al., 1999).

The observed CHS 828-induced cytotoxicity did not conform to the typical morphology and biochemistry of classical apoptosis, even though U-937 GTB cells evidently have an intact apoptotic machinery, as shown by others (Dubrez et al., 1996; Liminga et al., 2000), and as confirmed by the results of our positive control etoposide. Although an increased frequency of TUNEL positive nuclei was observed after 44 h, this staining pattern may, at least to some degree, reflect slow cell death and non-specific postnecrotic DNA degradation since a significant proportion (20–30%) of the cells show collapsed membrane integrity at this time point (Martinsson et al., 2001). Furthermore, no major increase in caspases-8 or -9 activity was evident at, or prior to these events despite initiated cytotoxicity. Caspase-3 exhibits a modest, yet statistically significant, increase at 48 h, potentially representing a proper activation being diluted by the prolonged death process. However, the cell death process was unaffected by caspase-3 inhibition and pan-caspase inhibition by

DEVD-fmk and Z-Asp-DCB, respectively. Activation of the caspase cascade has been considered a key event in the apoptotic process, preceding changes in nuclear morphology (Kerr et al., 1972; Lennon et al., 1991; Levin, 1998) and a prerequisite to denominate the observed cell death apoptosis (Blagosklonny, 2000).

Active cell death with non-apoptotic features has been described previously (D'Herde et al., 1996; Nicotera et al., 1999; Piwocka et al., 1999; Schwartz et al., 1993; Umemura et al., 1996; Zakeri et al., 1993), and the induction of non-apoptotic active cell death in the presence of caspase inhibitors with broad specificity has suggested the existence of caspase-independent active cell death pathways (Borner and Monney, 1999; Kitanaka and Kuchino, 1999; Nicotera et al., 1999). A programmed series of events giving rise to cell death with necrotic appearance has been observed in different cell systems including fibroblasts, neuronal cells and hepatocytes (Nicotera et al., 1999, and references therein). These cell systems can all undergo cell death in an organized manner involving signaling and execution that results in cell morphology associated with necrosis. The term "slow cell death" has been suggested when this occurs in apoptosis-resistant cells or when the caspases have been inhibited (Blagosklonny, 2000).

Another possibility for an atypical cell death could be that the apoptotic program may be interrupted before its final steps are completed and cells may be terminated by an uncontrolled accumulation of lethal reactions (Ankarcrona et al., 1995; Nicotera et al., 1999). This might explain the fact that at least some of the cells exposed to CHS 828 show an early apoptotic appearance at the ultra structural level (Martinsson et al., 2001) and that CHS 828 exposed cells exhibit a slight increase in caspase-3 activity at 48, but not at 72 h. In the present study, cells treated with CHS 828 responded with hyperpolarization of mitochondria at 24–32 h and a subsequent late disruption of $\Delta\psi_m$ at 48 h. In this context, it is interesting to note that the cellular level of ATP may be crucial for determining the mode and morphology of cell death, with low levels during critical time periods leading to a switch of an expected apoptotic response to that of necrosis (Lelli et al., 1998; Nicotera et al., 1999; Zakeri et al., 1993). In fact, the increased metabolic stimulation induced by CHS 828 appears to be due to increased glycolysis, secondary to inhibition of mitochondrial function (Ekelund et al., 2000). Although the mitochondrial effects appear neither necessary nor sufficient to account for CHS 828-induced cytotoxicity, it may thus contribute to the features of CHS 828-induced cell death. Indeed, an early drop in ATP levels at 24–30 h of CHS 828 exposure has recently been observed, supporting this notion (data not shown).

The present study, in addition to previous studies indicate that CHS 828 induce cell death requiring an extended time frame and active participation by the cell. Furthermore, caspases are either not involved, or their effect is inhibited downstream of their activation, and mitochondria seem a likely center of action. This excludes active cell death with classical apoptosis, as well as immediate oncotic, or "ultra-fast" necrosis, as the mode of CHS 828 induced cell death. A plausible explanation for this, in this apoptosis-prone cell line, would be a dual action of CHS 828, both inducing a cell death pathway and inhibiting its executioners, potentially by an early depletion of energy stores.

The results of this study suggest that further development of CHS 828 as an anticancer agent might be worthwhile. Previous studies have indicated its low cross-reactivity with other anticancer drug targets and low correlation to known mechanisms of drug resistance (Vig Hjarnaa et al., 1999). Furthermore, CHS 828 may be active in tumour cells that are resistant to apoptosis, a newly suggested mechanism of resistance, as an intact system for apoptosis is not required for CHS-induced cell death.

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