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The combination of the antitumoural pyridyl cyanoguanidine CHS 828 and etoposide *in vitro* – from cytotoxic synergy to complete inhibition of apoptosis

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1 The present study was aimed at elucidating the apoptosis inhibitory properties of the cyanoguanidine CHS 828. CHS 828 exhibits impressive cytotoxic activity *in vitro* and *in vivo*. Apoptosis is not its main mode of cytotoxic effect, and we have previously proposed a dual mechanism, where CHS 828 inhibits its own cell death pathways.

2 Etoposide on the other hand, is a well-established anticancer agent with documented effect in a number of malignancies, induces apoptosis through extensively studied caspase dependent pathways.

3 Here we studied the combined effect of the two drugs in the human lymphoma cell line U-937 GTB. Cytotoxicity was evaluated as total viability measured by the fluorometric microculture cytotoxicity assay (FMCA). Caspase activity was assessed by colorimetric detection of specific cleavage products for caspases 3, 8 and 9, respectively. Morphology was evaluated in May-Grünwald/Giemsa stained preparations. Interaction analysis based on FMCA results of simple combination exposure revealed impressive synergistic effect on cell kill.

4 Detailed investigations of the kinetics involved showed that short pre-exposure (0–12 h) to CHS 828 enhanced caspase activation by etoposide, while longer pre-exposure (18–48 h) inhibited both caspase activation and apoptotic morphology otherwise induced by etoposide. The present results support the theory that CHS 828 block specific cell death pathways.

5 The synergistic results are promising for future combination trials in animals, however, different dosing schedules should be considered, in order to investigate whether the above findings translate into the *in vivo* setting.
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Abbreviations: CI, combination index; DMSO, dimethyl sulphoxide; FDA, fluorescein diacetate; FMCA, fluorometric microculture cytotoxicity assay; MiCK, microculture kinetics; PBS, phosphate buffered saline; pNA, p-nitroanilidine; SI, Survival Index

Introduction

CHS 828 (N-(6-(4-chlorophenoxy)hexyl)-N′-cyano-N″-4-pyridylguanidine) belongs to a new group of antitumoral substances, the pyridyl cyanoguanidines. It is presently in phase I and phase II clinical trials in solid tumour and haematological malignancies. *In vivo* animal studies have shown impressive cytotoxic activity, on both animal and human tumours, with acceptable toxicity in both rodents and dogs (Jonsson *et al*., 2001; Vig Hjarnaa *et al*., 1999). In addition, *in vitro* investigations on a human cell line panel have suggested a novel mechanism of action, together with low cellular cross-resistance to other cytotoxic compounds, as well as a differential pattern of induced resistance (Vig Hjarnaa *et al*., 1999). CHS 828 induces cell death features which to some extent resemble apoptosis (Hansen *et al*., 2000), but with several atypical features (Martinsson *et al*., 2001a,b). We have previously hypothesized that CHS 828 initiates a controlled cell death pathway, programmed cell death, but in addition inhibits factors further downstream, necessary for full-blown apoptotic features, such as morphological changes and caspase activation (Martinsson *et al*., 2001a). However, the exact target and mechanism of action is still largely unknown and its usefulness in the clinic remains to be proven.

Etoposide, on the other hand, was the first anticancer drug to be demonstrated to exert its antineoplastic effect through inhibition of topoisomerase II (Hande, 1998). The drug exhibits significant antitumour activity in many types of neoplasms, including germ-cell malignancies, lung cancer, non-Hodgkin's lymphoma, leukaemia, Kaposi's sarcoma, neuroblastoma, and soft-tissue sarcomas. Etoposide induces massive apoptosis, which has been described in detail for the human lymphoma cell line U-937 GTB (Martinsson *et al*., 2001c; Shrivastava *et al*., 2000; Sun *et al*., 1999) and involves and depends on activation of caspases 8, 9 and 3 and endonucleases.

Combination chemotherapy is commonly employed for several tumour types in contemporary clinical oncology, and

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is considered to provide numerous advantages over single agent treatments. The present study aimed at investigating the combination of etoposide and CHS 828 on malignant cells from two perspectives. First, the effects on cytotoxicity in an ordinary *in vitro* combination set-up, with an interaction evaluation. Secondly, to further elucidate the mechanisms behind the prolonged, and unusual kinetics of CHS 828 induced cell death features and its hypothesized apoptosis inhibitory properties.

Methods

Cells

The histiocytic lymphoma cell line U-937 GTB (Sundström & Nilsson, 1976) maintained in RPMI 1640 complete medium (without phenol red for microculture kinetics (MiCK) experiments, Sigma-Aldrich, St Louis, MO, U.S.A.) was used in all experiments. The medium was supplemented with 10% heat inactivated foetal bovine serum (Hy Clone, Cramlington, U.K.), 2 mM glutamine, 50 μg ml^{-1} streptomycin and 60 μg ml^{-1} penicillin (Hy Clone). Cells were grown in culture flasks, kept under standard incubating conditions (humidified atmosphere of 37°C, 5% CO_2 in air). Cultures were monitored and passaged twice weekly, and harvested in log phase.

Drugs and reagents

Etoposide (Vepesid®, Bristol-Myers Squibb, Bromma, Sweden) as 20 mg ml^{-1} injection concentrate (ethanol solution) was diluted in sterile phosphate buffered saline (PBS). Total ethanol concentration was $<0.1\%$ in all experiments. CHS 828 was a kind gift from Leo Pharmaceuticals, Denmark, and 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 and PBS. Final DMSO concentration was $<0.04\%$ in all experiments.

Cell viability

FMCA The concept of 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 (FDA) to fluorescein by cells with preserved membrane integrity. Drug solution (or PBS for controls) was added at 1/10 of final cell suspension volume in 96-well microtitre plate. Seventy-two hours after the start of the experiment FDA was added as described previously (Larsson *et al*., 1992) and fluorescence measured after 40 min incubation. Results are expressed as Survival Indices (%SI) = (mean fluorescence of exposed triplicates − mean blank fluorescence)/(mean fluorescence of control triplicates − mean blank fluorescence) × 100. The assay has previously been shown to be highly sensitive being able to detect multiple logs (3–4) of cell kill in suspension growing cell lines (Larsson *et al*., 1990; 1992). Fluorescein-diacetate (FDA) used as a viability probe in the flow cytometric setting has previously shown good correlations with clonogenic assay when applied to suspension growing cells (Ellwart *et al*., 1988; Ross *et al*., 1989).

Interaction analysis based on the isobole method The isobole method is based on the sigmoid relationship that characterizes the response of many biological targets to increasing concentrations of a molecule. This is also valid for the response of such targets to a combination of agents maintained in constant proportion to each other (Gessner, 1995). The isobole method was used for evaluation of drug interaction between CHS 828 and etoposide since both drugs produced sigmoid concentration–response curves.

Concentration-response curves were created by non-linear regression analysis using the sigmoid concentration-response equation with variable slope (equation 1) (Workman *et al*., 1992) for CHS 828 and etoposide, respectively.

$$E_{predicted} = (E_{max} \times C^{slope})/(EC_{50}{}^{slope} + C^{slope}) \qquad (1)$$

The predicted effect at different drug concentrations (C) is calculated from the maximum effect (E_{max}) of the single drug, together with the slope of the dose–response curve (slope) and the concentration of the drug producing 50% effect (EC_{50}). The single drug concentration of one compound is plotted on the x-axis and the concentration of the second compound on the y-axis for a specified effect and by joining these two points one arrives at a straight diagonal line, denoted isobole. Isoboles for the effect levels 30, 40, 50, 60 and 70% were constructed in this way based on the single drug dose–effect curves and equation 1. The isoboles at the specified effect levels represent zero interaction between the two agents, i.e. simply the sum of the individual effects (Berenbaum, 1989) and is defined by equation 2. In equation 2, dx_a and dx_b are the concentrations of drug A and drug B, respectively, given in combination, and Dx_a and Dx_b are the concentrations of A and B which, when given as single drugs, induce the same effect level (X).

$$dx_a/Dx_a + dx_b/Dx_b = 1 \qquad (2)$$

Based on the five isoboles, five CHS 828/etoposide combinations were chosen from the midpoint on each isobole (see Table 1). These combinations were then tested using the FMCA and the observed effects were transformed to the corresponding single drug concentrations by use of equation 1. Thereafter, a combination index (CI) was calculated from equation 2 in order to assess the difference in observed and predictive concentrations (Berenbaum, 1989). In principle, when the CI <1 there is synergy, if CI $=1$ there is additivity, and if CI >1 there is antagonism. To account for the intra-assay variability, an interaction with a CI exceeding 1.2 was classified as sub-additive, under 0.8 as synergistic and for CIs between 0.8 and 1.2 the interactions were denoted additive (Jonsson *et al*., 1998; Kristensen *et al*., 1994; Larsson *et al*., 1993; Lepri *et al*., 1991).

The additive model In addition, the same set of data was analysed by the additive model, predicting the effect of a combination of two drugs to be equal to the product of the survival indices of the two drugs tested as single agents (Valeriote & Lin, 1975). A ratio between the observed survival index and that predicted by the additive model was calculated for all combinations. A ratio >1.2 was classified as a sub-additive interaction, <0.8 – a synergistic interaction and ratios between 0.8 and 1.2 – additive (Lepri *et al*., 1991), in accordance with the isobole method CI.

Table 1 Cytotoxic interaction of CHS 828 and etoposide

Expected effect level (%)	*CHS 828 concentration (μM) in the combination*	*Etoposide concentration (μM) in the combination*	*Observed effect level (%)*	*Combination Index (CI)*[a]
30	0.036	0.071	77	0.18[b]
40	0.039	0.088	76	0.22
50	0.041	0.105	77	0.24
60	0.044	0.126	78	0.27
70	0.047	0.153	79	0.30

[a]For the isobole method a combination index (CI) was calculated (see equation 3 in Methods). [b]Ratios $\leqslant 0.8$ were classified as synergistic and $\geqslant 1.2$ as antagonistic.

Morphology

Drug exposure Cells were exposed in cell culture flask under standard incubation conditions, at a cell density of 500,000 cells ml^{-1}. The drugs were added to make up one tenth of the final culture volume. CHS 828 at a final concentration of 1.0 μM was present from the start of the experiment. Etoposide at a final concentration of 25 μM was added to the CHS 828 exposed cultures after 0, 4 and 24 h, respectively. One flask received PBS only for control and one flask each of 1.0 μM CHS 828 and 25 μM etoposide as single drugs, respectively. Four hours after the addition of etoposide (standard apoptosis induction), cells exposed to the drug combination, or to etoposide alone, were collected by centrifugation. Control cells and cells exposed to CHS 828 as a single agent, were collected at 4, 8, 24, 32 and 48 h.

Staining Cytospin slides were prepared and stained with May-Grünwald/Giemsa for morphological evaluation. Apoptotic cells were microscopically identified by chromatin condensation and nuclear fragmentation (Darzynkiewicz & Traganos, 1998) and quantified by counting two visual fields ($\times$20,000 magnification) per slide from two separate preparations.

Microculture Kinetics (MiCK) assay The method measures changes in optical density in a cell culture due to early apoptotic changes in cell morphology, mainly cellular and nuclear condensation (Kravtsov *et al*., 1998). 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 demonstrated (Kravtsov *et al*., 1999). We have previously used this method to characterize etoposide induced apoptosis in U-937 GTB cells (Martinsson *et al*., 2001c). Flat-bottomed microtitre plates were prepared with 10 μl per well etoposide, at 500 μM (final concentration 25 μM), and kept at -70°C. Cells were preincubated with 1.0 μM CHS 828 at an initial cell density of 555,000 cells ml^{-1}. The drug was added to separate cultures 48, 24, 8 or 4 h before the start of the MiCK analysis. An-etoposide-prepared microtitre plate was thawed and seeded with 190 μl of the pre-exposed cell suspensions and the microtitre plate 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) to prevent evaporation and contamination, and the microtitre plate placed in a SpectraMax™ Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.). Absorbance at 600 nm was measured automatically every 14 min for 24 h. During this monitoring microtitre plates were kept at 37°C, protected from light.

Caspase activity

Activation of caspase 3 has been put forward as an obligatory event in apoptosis (Blagosklonny, 2000) and in this study it was used as a definite biochemical marker for etoposide induced apoptosis in CHS 828 preexposed U-937 GTB cells. Activity of caspases 8 and 9 representing the two separate induction pathways of apoptosis, *via* FasR/CD95R or mitochondria, respectively, were measured in parallel with caspase 3. Caspase activity was assayed by colorimetric detection of p-nitroanilidine (pNA) after cleavage of the peptide substrates DEVD-pNA (Asp-Glu-Val-Asp), IETD-pNA (Ile-Glu-Thr-Asp) or LEHD-pNA (Leu-Glu-His-Asp) specific for caspases 3, 8 and 9 respectively. All reagents were part of commercial 'Caspase Colorimetric Assays' (R&D Systems Inc., Minneapolis, MN, U.S.A.) for the three caspases. Cells were exposed continuously to 1.0 μM CHS 828 in culture flasks at a cell density of 2.5×10^5 cells ml^{-1}. After 0, 4, 24 and 48 h etoposide was added to separate cultures (final etoposide concentration was 25 μM). As controls, cells exposed to CHS 828 as a single agent for corresponding periods, cells not exposed to any drug, and cells exposed to etoposide as a single drug, were used. Four hours after etoposide addition, aliquots of 2×10^6 cells were collected in triplicates by centrifugation and washed once in RPMI 1640 complete medium. Supernatants were removed by decanting and the pellets were frozen and kept in -70°C until analysis. The assay was performed according to the commercial protocol and has been described previously (Martinsson *et al*., 2001a). Absorbance was measured at 405 nm in an ELISA reader (SpectraMax™Plus) after 90 min of dark incubation in a humidified atmosphere of 5% CO_2 in air at 37°C, followed by 20 s shaking. Activity was calculated as (test absorbance)/(control absorbance) with average absorbance of blank wells subtracted and expressed as percentages.

Statistical analysis

Student's *t*-test was employed for statistical evaluation of differences. All calculations were performed in the GraphPad Prism® 3.02 software (GraphPad Software, Inc., San Diego, CA, U.S.A.).

Results

Cytotoxic interactions of CHS 828 and etoposide

To obtain the concentration range of interest, preliminary concentration–response studies were first carried out for CHS 828 and etoposide as single drugs. Concentrations for each drug were determined using isoboles for the 1 : 1 combinations at five different effect levels (30, 40, 50, 60 and 70% cell kill). The effect levels and the concentrations chosen are listed in Table 1.

Using the isobole method, cytotoxic synergy was demonstrated for all effect levels tested, with a tendency for a more pronounced synergy at lower effect levels. The CI ranged from 0.18 for an effect level of 30% to 0.30 for an effect level of 70%, results which both are well within the limit for synergy. This trend was also confirmed with the additive model, with a ratio between observed and predicted SI values ranging from 0.27 to 0.30 (not shown).

Morphology

U-937 GTB cells exposed to 25 μM etoposide for 4 h exhibited massive apoptosis with condensation of cytoplasm and chromatin, and nuclear fragmentation (Figure 1A,B). Mere co-exposure to the two drugs for 4 h, or co-exposure of cells pre-exposed to CHS 828 for 4 h, resulted in apoptotic morphology indistinguishable from that of etoposide exposure alone (Figure 1A,C and A,D, respectively). However, after 24 h of CHS 828 pre-exposure, the addition of etoposide for 4 h co-exposure failed to induce apoptotic morphology (Figure 1A,E). Cells exposed to 1.0 μM CHS 828 alone were still fully viable after 24 h continuous exposure, without apoptotic signs (Figure 1A,F).

MiCK This method for optical monitoring of the kinetics of early apoptotic morphology confirmed the visual impression of conventional end point morphology. Cell cultures pre-exposed to CHS 828 responded with an apoptotic pattern when etoposide was added after 0 h (continuous co-exposure) to 4 h. However, pre-exposure to CHS 828 for 24 h largely attenuated the apoptotic response to etoposide. After 48 h of CHS 828 exposure, addition of etoposide resulted in an obvious and typical necrotic pattern (Figure 2).

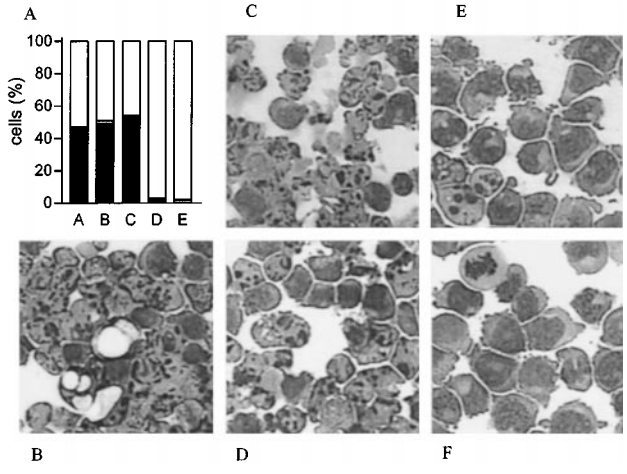

Figure 1 Representative photos of May-Grünwald/Giemsa stained U-937 GTB cells. (A) Graphic representation of the proportion of apoptotic (black bar), necrotic (grey bar) and viable (open bar) cells in preparation (B–F). Cells were induced to undergo apoptosis by 4 h exposure to 25 μM etoposide (B). (C) 4 h co-exposure to 1.0 μM CHS 828 and 25 μM etoposide. (D) 4 h pre-exposure to CHS 828, before addition of 25 μM etoposide. (E) 24 h pre-exposure to 1.0 μM CHS 828, before addition of 25 μM etoposide. (F) control 24 h 1.0 μM CHS 828, without etoposide.

Caspase activity

CHS 828 alone did not activate caspases to any major extent in U-937 GTB cells, while single agent etoposide induced significant activation of caspases 3, 8 and 9. Shorter pre-exposure times (0–12 h) to 1.0 μM CHS 828, significantly potentiated the activation of caspase 3 induced by etoposide (Figure 3). However, in accordance with the morphological changes, after 24 h of pre-exposure the activation of all three caspases by etoposide was significantly attenuated towards complete inhibition.

Total viability

The effect of pre-exposure to 0.1 μM CHS 828 on etoposide induced cell death was also investigated. Etoposide was added at a final concentration of 0.1 μM. CHS 828 alone resulted in a SI of 30% in these experiments. Etoposide added at 0, 4 or 8 h, alone or in combination with CHS 828 produced near total cell kill (SI: 0.4–2.0%). However, cells pre-exposed to CHS 828 for 24 h exhibited a significantly higher survival rate to 0.1 μM etoposide than did unexposed cells (Table 2).

Discussion

Most contemporary protocols for drug treatment of malignant diseases consist of several substances administered periodically according to a more or less arbitrarily derived time schedule, mainly restricted by patient side effects. The present *in vitro* study suggests that combining drugs with vastly different effect kinetics demands careful consideration of the temporal aspects of combined administration. Here we present an example of impressive synergy of two unrelated

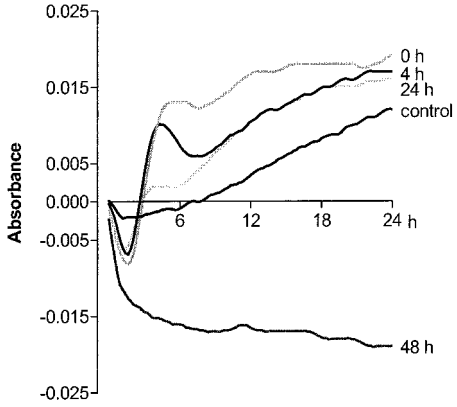

Figure 2 One representative MiCK experiment out of three. U-937 GTB response to 25 μM etoposide after indicated times of pre-exposure to 1.0 μM CHS 828.

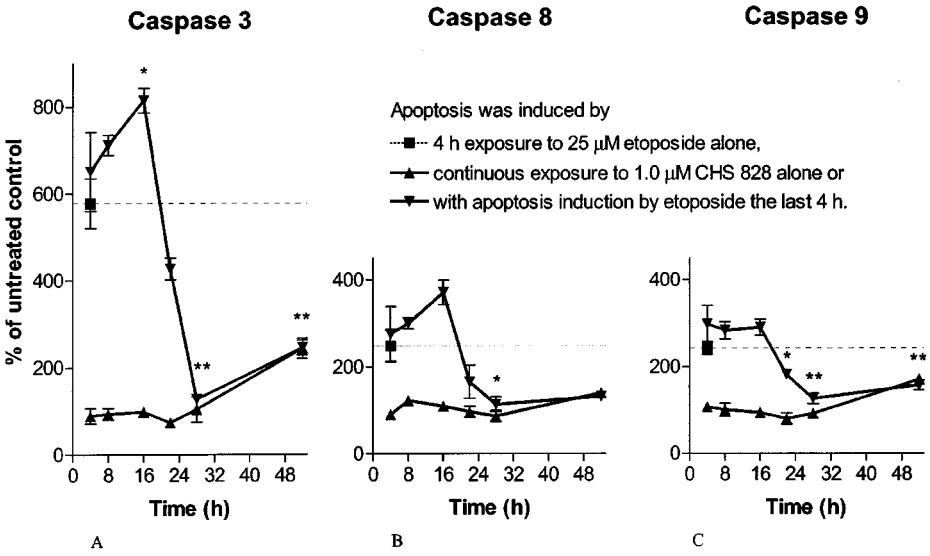

Figure 3 Substrate specific activities of caspase 3 (A), caspase 8 (B) and caspase 9 (C) in U-937 GTB cells. Cultures were exposed as indicated and results are presented as means of three separate experiments ± s.e. $*P<0.05$; $**P<0.01$, compared to 4 h etoposide exposure alone.

Table 2 Total viability of U-937 GTB cells as a result of etoposide addition after CHS 828 pre-exposure

Frames of exposure to 0.1 μM CHS 828 ■, 0.1 μM etoposide ▽ or a combination of the two ■.	% SI (±S.E.M)	Difference in viability	Fold SI difference
	29 (±0.8)	-	-
	1.3 (±0.2) 1.0 (±0.2)	$P = 0.25$	1.3
	1.1 (±0.1) 0.8 (±0.1)	$P = 0.14$	1.3
	1.2 (±0.1) 0.8 (±0.1)	$P = 0.036$	1.5
	13 (±0.6) 2.2 (±0.5)	$P < 0.0001$	6.0

0 4 8 24 72 h

Viability was assessed by the FMCA, 72 h after the start of drug exposure. % SI (Survival Index) is the mean fluoresence in test wells in per cent of control wells, with blank values subtracted. Etoposide was added to cultures pre-exposed to CHS 828, or to unexposed cultures, as indicated. Means of four consecutive experiments.

substances under certain circumstances, which turns into inhibited effector pathways under other conditions. Thus, care may have to be taken in the *in vivo* setting, to isolate the positive interactions.

Previous studies of the effect kinetics of the investigational CHS 828 (Ekelund *et al*., 2000; Martinsson *et al*., 2001a,b) have shown a prolonged path of action, with cells unaffected in growth and viability for 24 h or more with subsequent inhibition of DNA and protein synthesis. During the second and third day of continuous exposure, cells become visually disintegrated, and after 72 h, CHS 828 has effectively killed a large proportion of the cells, even at nanomolar concentrations. No major activity is detected in apoptotic pathways and the exact mechanism of action remains to be revealed.

Etoposide on the other hand, induces obvious apoptosis within 2–4 h of exposure, including massive caspase activation, mitochondrial changes and nuclear condensation and fragmentation (Martinsson *et al*., 2001c; Sun *et al*., 1999).

The present study shows that simultaneous exposure to CHS 828 and etoposide results in impressive synergy over a range of effect levels, measured as total viability after 72 h. Furthermore, supporting our *in vitro* findings, *in vivo* studies in tumour bearing animals have shown synergistic effects between etoposide and CHS 828 with respect to antitumour activity (P.J. Vig Hjarnaa *et al*., Leo Pharma Copenhagen, Denmark, unpublished results). This information, naturally, holds considerable promise for the role of CHS 828 as a new agent in the oncology clinic. However, the results also reveal additional information about the interplay between CHS 828 and etoposide. The proposed dual action of CHS 828 has implications for the effect of etoposide.

Mere co-incubation from time 0 resulted in synergistic effects on total cell viability and a tendency to increased caspase activation. A short pre-exposure to CHS 828 (4 h) further potentiated the response to etoposide with significantly higher levels of activity for caspase 3. These combinations also produced obvious apoptotic morphology. However, cells pre-exposed to CHS 828 for 24 h failed to respond to etoposide with morphological changes, as well as caspase activation. They also exhibited significantly better viability after etoposide addition than do previously unexposed cells. CHS 828 is a rare exception to the contemporary notion that all cytotoxic agents kill susceptible cancer cells by apoptosis (Martinsson *et al*., 2001a). Here we also show that the powerful apoptosis inducer etoposide, fails to produce an apoptotic response in cells exposed to CHS 828. This indicates that CHS 828 itself, after a prolonged incubation time of 24 h inhibits pathways necessary for apoptosis. This inhibition could have several explanations. CHS 828 exposed cells starts to run out of energy (measured as intracellular ATP concentrations) after 24 h (H Lövborg *et al*., 2002). This might be enough to prevent execution of the apoptotic machinery (Leist *et al*., 1997; Lelli *et al*., 1998). Indeed, co-incubation with 3-aminobenzamide, which delays the decrease in ATP, also delays the CHS 828 induced inhibition of apoptosis, supporting this notion. Alternatively,

CHS 828 may inhibit the apoptotic process at other levels, upstream of caspase activation. For example, it was recently observed that inhibition of the proteasome and hence the NFκB pathway prevents etoposide-induced apoptosis (Stefanelli *et al*., 1998; Watanabe *et al*., 2000).

In conclusion, the present study provides an interesting example of the response kinetics for apoptosis by anticancer drug treatment. Temporal considerations of future treatment protocols with CHS 828 might differentiate between successful treatment and failure of malignant cells to respond.

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