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Modulation of pyridyl cyanoguanidine (CHS 828) induced cytotoxicity by 3-aminobenzamide in U-937 GTB cells

Henrik Lövborg[a,*], Petra Martinsson[a], Joachim Gullbo[a], Sara Ekelund[a], Peter Nygren[b], Rolf Larsson[a]

[a]*Division of Clinical Pharmacology, University Hospital, Uppsala University, S-751 85 Uppsala, Sweden*
[b]*Department of Oncology, Radiology and Clinical Immunology, University Hospital, Uppsala University, S-751 85 Uppsala, Sweden*

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Abstract

The role of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) and the ADP-ribosylation inhibitor 3-aminobenzamide (3-ABA) in the cytotoxicity induced by the novel antitumoral cyanoguanidine CHS 828 was investigated in the human lymphoma cell line U-937 GTB. Exposing cells to CHS 828 and 3-ABA in combination resulted in a 100-fold higher IC_{50} compared to exposure to CHS 828 alone. CHS 828 did not activate PARP, measured as PARP-activity and formation of poly(ADP-ribose). The ATP-levels and levels of extracellular acidification rate of cells exposed to CHS 828 in combination with 3-ABA were maintained for a longer period than for cells exposed to CHS 828 alone. To characterize the mode of cell death, caspase-3 activity and gross morphology were assessed. 3-ABA increased and delayed the caspase-3 activity in cells exposed to CHS 828. Cells exposed to high concentrations of CHS 828 showed a necrotic morphology, while high concentrations of CHS 828 in combination with 3-ABA switched the mode of cell death, generating an apoptotic morphology. The results indicate that the cytotoxicity and morphology induced by CHS 828 is not due to PARP activation but can be modulated by the ADP-ribosylation inhibitor 3-ABA.

Keywords: CHS 828; 3-Aminobenzamide; Apoptosis; ATP; PARP; U-937 GTB

1. Introduction

The novel antitumoral drug *N*-6-(4-chlorophenoxy)-hexyl-*N*′-cyano-*N*″-4-pyridylguanidine (CHS 828) has shown potent antitumor activity in a variety of human and animal tumor models both *in vitro* and *in vivo* [1–3]. CHS 828 belongs to a chemical class of drugs previously unknown to possess antitumor activity and its mechanism of action remains unclear. Studies using a panel of 10 human tumor cell lines showed low correlation with activity patterns of known anticancer drugs [1]. In the human lymphoma cell line U-937 GTB changes in protein and DNA synthesis are affected after 24 hr and morphological signs of toxicity is first apparent after about 44 hr. The cytotoxic effect of CHS 828 is inhibited by cycloheximide, indicating that protein synthesis is required for CHS 828 to induce cytotoxic effect. Cells exposed to CHS 828 for 48–72 hr show loss of membrane integrity without displaying the typical signs of apoptotic cell death, i.e. chromatin condensation, membrane blebbing and nuclear fragmentation [4]. *In vitro* studies indicate that CHS 828 stimulates the metabolism of a variety of cell lines and primary human tumor cells within minutes of exposure, measured as extracellular acidification rate in a Cytosensor microphysiometer. The increase in extracellular acidification rate could be explained by an inhibition of mitochondrial respiration and a subsequent increase in glycolysis. However, this effect does not seem to be sufficient to induce the cytotoxic effect induced by CHS 828 and structurally related cyanoguanidines [5].

Insight into the mechanism of action for new cytotoxic drugs is important both for generating novel substances affecting the same pathways in tumor cells, as well as for studies of mechanism of resistance to the drug. A recent unpublished screening of inhibitors of various signal transduction pathways and enzymes indicated that the inhibitor of ADP-ribosylation, 3-aminobenzamide (3-ABA), inhibited the cytotoxicity induced by CHS 828. One important

* Corresponding author. Tel.: +46-18-611-59-59; fax: +46-18-51-92-37.
E-mail address: henrik.lovborg@medsci.uu.se (H. Lövborg).

Abbreviations: PARP, poly(ADP-ribose) polymerase; 3-ABA, 3-aminobenzamide; FMCA, fluorometric microculture cytotoxicity assay; SI%, survival index; TCA, trichloroacetic acid; PKC, protein kinase C; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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ADP-ribosylation enzyme is PARP, a key enzyme in the response to DNA damage. It is activated by breaks in the DNA strand thereby consuming NAD^+ by forming chains of poly(ADP-ribose) [6]. The aim of this work was to study the role of PARP and 3-ABA in CHS 828 induced cytotoxicity with respect to morphological and metabolic effects, in an effort to reveal the mechanism of action for CHS 828.

2. Materials and methods

2.1. Compounds

3-ABA (Sigma Chemical Co) was dissolved in PBS. CHS 828 was provided by Leo Pharmaceutical Products and kept at -20° as stock solution in dimethyl sulphoxide. Stock solution of etoposide (Vepesid, Bristol–Myers Squibb) was maintained at room temperature. Compounds were diluted with PBS to desired concentrations.

2.2. Cell lines

In all experiments the human lymphoma cell line U-937 GTB was used [7]. In the cytotoxicity experiments the 8226/S myeloma cell line [8] was also used. Cells were grown in RPMI-1640 culture medium supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 50 μg/mL streptomycin and 60 μg/mL penicillin (all from Sigma) at 37° in a humidified atmosphere containing 5% carbon dioxide. Cells in log growing phase were used for all experiments. The cell lines were subcultivated and checked for growth characteristics and morphology weekly twice.

2.3. Cytotoxicity

To study the cytotoxic effect of inhibitors and drugs, the fluorometric microculture cytotoxicity assay (FMCA) was used. The assay has previously been described in detail [9]. Briefly, 96-well plates were prepared by adding 20 μL drug solution, or combination of drugs, to each well and was then kept frozen at -70° for up to 4 weeks. For experiments, the plates were thawed and 180 μL cell suspension/well (110,000 cells/mL) were added and the plates were incubated at 37°, 5% CO_2 for 72 hr. Six wells with medium only served as background controls and six wells with cells but without drugs served as unexposed control. After incubation the plates were centrifuged (200 *g*, 5 min) and washed once with PBS and 100 μL 10 μg/mL fluorescein diacetate (Sigma) was added to each well. After 40 min incubation at 37° the generated fluorescence was measured at 538 nm (excitation 480 nm) using a 96-well scanning fluorometer (Fluoroskan II, Labsystems Oy). The fluorescence signal is proportional to the number of viable cells [10].

Data are expressed as survival index (SI%), calculated by the formula $(\text{fluorescence}_{\text{test}} - \text{fluorescence}_{\text{blank}})/(\text{fluorescence}_{\text{control}} - \text{fluorescence}_{\text{blank}}) \times 100$. Three independent experiments for each drug and concentration were performed. The concentration giving 50% cell viability compared to untreated control (IC_{50}) was calculated by substitution into the equation for the straight line through the SI% values above and below 50%.

2.4. Measurement of PARP-activity

PARP-activity was assayed using a slightly modified method described by Schraufstatter *et al*. [11]. Cells were incubated in cell culture flasks in culture medium as above with 1 μM CHS 828, PBS (control) or 1.5 mM hydrogen peroxide (H_2O_2, Sigma) with or without 3-ABA as positive control. At various time-points triplicates of 500,000 cells were pelleted in microcentrifuge tubes by centrifugation (500 *g*, 5 min) and the medium was removed. 500 μL reaction-buffer (pH 7.5) containing 56 mM HEPES, 28 mM NaCl, 28 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) digitonin (all from Sigma) and 100 nM radioactive ^{32}P-NAD^+ 30 Ci/mmol (NEN Life Science Products Inc.), was added to each cell pellet. After 5 min the reaction was stopped by adding 200 μL ice-cold 50% trichloroacetic acid (TCA, Sigma) followed by centrifugation (12,000 *g*, 10 min). The pellets were washed twice with 500 μL 10% TCA and finally solubilized overnight in 0.2 M sodium hydroxide. The incorporation of ^{32}P-NAD^+ into TCA-precipitate was measured by scintillation counting using a Tri-Carb 2200 CA scintillator (Packard). Obtained counts were divided by the specific activity of the ^{32}P-NAD^+ to yield the amount of NAD^+ incorporated. Data are presented as mean values with SE from three independent experiments. Statistical analysis was performed using unpaired Student's *t*-test.

2.5. Poly(ADP-ribose) formation

Levels of poly(ADP-ribose) were analyzed according to Burkle *et al*. [12]. Briefly, U-937 GTB cells (300,000 cells/mL) were incubated with 1 μM CHS 828 for various times, centrifuged onto object glasses by Cytospin 3 (Shandon Scientific Ltd.) and immediately fixed in ice-cold 10% TCA for 10 min. Cells exposed to 1.5 mM H_2O_2 for 5 min served as positive control and cells preincubated with 3-ABA for 30 min and subsequently exposed to H_2O_2 were used to indicate the specificity of the method. Slides were washed in 70, 90 and 96% ethanol for 3 min each and allowed to air dry. After rehydration in PBS for 5 min Cytospin areas were incubated with 5 μg/mL of the poly-(ADP-ribose)-binding 10H monoclonal antibody (Alexis Corp.), washed three times in PBS followed by incubation with FITC-conjugated goat anti-mouse secondary antibody diluted 1:60 (Alexis Corp.). Antibodies were diluted in PBS with 5% non fat dry milk (Santa Cruz Biotechnology) and 0.05% Tween 20 (Sigma). Slides were washed in PBS and mounted with Vectashield (Vector Laboratories Inc.)

and photographed using a Nikon ECLIPSE F400 fluorescence microscope with a CCD-camera.

2.6. Measurement of ATP

ATP was measured luminometrically using the commercially available ApoGlow™ kit (LumiTech Ltd.). Cells seeded in wells of an opaque 96-well microtiter plate were incubated with 1 μM CHS 828, 3 mM 3-ABA or the combination of the two for indicated time-points. The plate was incubated under standard cell culture conditions. Each drug or drug combination was tested in triplicate, three wells with medium only served as blank and two wells with untreated cells as control. The plate was loaded into a dispensing luminometer (Mediators Diagnostika) and ATP levels were analyzed by adding 100 μL mixture of nucleotide monitoring and releasing reagent to each well and immediately reading the relative light units at 540 nm. ATP levels are expressed as percentage of unexposed cells with blank values subtracted. Statistical analysis was performed using unpaired Student's *t*-test.

2.7. Measurement of extracellular acidification

Acidification rate was investigated in the Cytosensor microphysiometer (Molecular Devices) as described earlier [5] using 1.5×10^5 cells/channel. The non-adherent U-937 GTB cells were immobilized in the cell capsule during the experiments. The capsules were installed into the sensor chambers and perfused by a modified RPMI-1640 culture medium (Swedish National Veterinary Institute) at a flow rate of 100 μL/min. The modified medium was a low buffering-capacity medium without HEPES and bicarbonate but supplemented with 6 mL/L 4 M NaCl to preserve osmotic balance, and also contained L-glutamine, penicillin and streptomycin. A repeated (120 s) pump cycle was used throughout the experiments where the rate of acidification was measured during a period of 30 s followed by 90 s perfusion. Medium without drug was used to establish a baseline value during 1 hr. CHS 828 and/or 3-ABA or PBS for control were added to the modified medium. The cell culture media containing drugs were continuously perfused through the sensor chambers containing the cells for up to 24 hr. The rate of acidification of the medium during the recording time was calculated by the Cytosoft program as −μV/s and was presented as percent change from the initial value at start of the experiment when metabolic activity was set to 100%.

2.8. Morphology

Microscope slides of cells were prepared using a Cytospin 3 centrifuge (Shandon Scientific Ltd.). Slides were prepared after 72 hr incubation for control cells and cells exposed to 1 μM CHS 828 and/or 3 mM 3-ABA. The slides were stained according to May–Grünewald–Giemsa. Cells with disintegrated membrane were classified as necrotic and cells with condensed, fragmented nucleus but intact cell membrane as apoptotic. Cells in late stages of apoptosis, i.e. condensed, fragmented nucleus and disintegrated membrane, were also classified as apoptotic. A minimum of 300 cells in three–four areas per slide were assessed. The differences in mean values of the number of apoptotic cells in CHS 828 exposed cells compared to CHS 828 and 3-ABA in combination was tested using an unpaired Student's *t*-test.

2.9. Caspase-3 assay

U-937 GTB cells were incubated with 1 μM CHS 828 and/or 3 mM 3-ABA for 24, 32, 48, 56 and 72 hr. Cells incubated with 25 μM etoposide for 4 hr served as positive control. Duplicates of two million cells were collected from the cell cultures by centrifugation, supernatants were removed and the cell pellets frozen at −70° and were kept for up to 1 week. Caspase-3 activity in the cell pellets were measured using Caspase-3 Colorimetric kit (R&D Systems Inc.) measuring caspase-3-like dependent cleavage of DEVD-*p*-nitroanilide into the chromophore *p*-nitroanilide. The pellets were thawed and incubated on ice with 50 μL cell lysis buffer for 10 min followed by centrifugation at 10,000 *g* for 1 min. 50 μL supernatant from each sample was transferred to a flat-bottomed 96-well plates and 50 μL reaction buffer and 5 μL 4 mM DEVD-*p*-nitroanilide were added. The plates were incubated at 37° for 90 min and the absorbance at 405 nm was read in a Spectramax Plus spectrophotometer (Molecular Devices). Wells with reaction buffer and substrate only served as blank. Data are presented as mean absorbance with SE of the cleaved product at 405 nm. Statistical analysis was performed using unpaired Student's *t*-test.

3. Results

3.1. Modulation of cytotoxicity with 3-ABA

The data presented in Fig. 1 describe the concentration dependent cytotoxicity of CHS 828 as single drug and in combination with 3 mM 3-ABA after 72 hr incubation. The IC_{50} for CHS 828 was 0.42 nM ± 0.16 (mean ± SE) and 41 nM ± 17 for CHS 828 in combination with 3 mM 3-ABA. The increase in IC_{50} is maintained for at least 120 hr incubation (not shown). At the concentration used 3-ABA alone induced no toxic effect (not shown). Similar results were obtained with the 8226/S myeloma cell line (Fig. 1, inset).

3.2. Effects on PARP-activity and poly(ADP-ribose) formation

PARP-activity was assayed in cells exposed to 1 μM CHS 828 for 1, 4 or 27 hr, respectively and in cells exposed

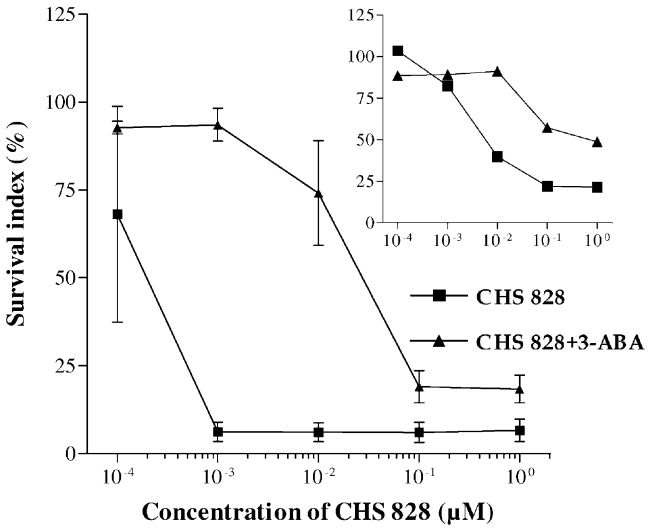


Fig. 1. Effect on cell survival of CHS 828 as single drug and in combination with 3 mM 3-ABA in the U-937 GTB cell line with continuous exposure for 72 hr. Data are presented as mean values ± SE from three independent experiments. Inset shows SI% for the myeloma cell line 8226/S, one experiment.

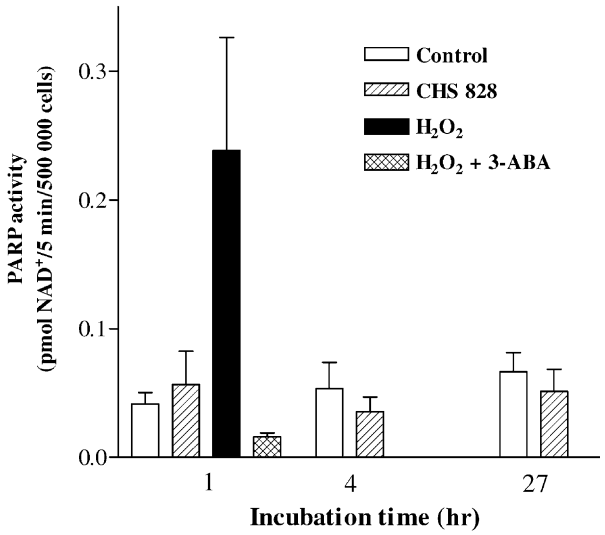


Fig. 2. PARP-activity, expressed as picamole ^{32}P-NAD$^+$ incorporated/5 min/500 000 cells, in unexposed U-937 GTB and cells exposed to 1 μM CHS 828 or 1.5 mM H_2O_2 with and without 3 mM 3-ABA for the indicated time periods. Data are presented as mean values + SE from two to four independent experiments.

to 1.5 mM H_2O_2 for 1 hr, with or without 3 mM 3-ABA, as positive control. There were no significant differences in PARP-activity, as measured by ^{32}P-NAD$^+$ incorporation into TCA-precipitated proteins, between unexposed cells and cells exposed to 1 μM CHS 828. Preliminary data indicated that this was also true for 0.1 μM CHS 828 and for incubation times in the range of minutes to about 32 hr. PARP activity was induced by H_2O_2, and this effect was inhibited by 3 mM 3-ABA (Fig. 2). Cells exposed to 1 μM CHS 828 for 1, 4 and 27 hr did not show any formation of poly(ADP-ribose) (Fig. 3A–C). Incubation with 1.5 mM H_2O_2 for 5 min induced a strong accumulation of poly-(ADP-ribose) (Fig. 3D), and this effect was inhibited by addition of 3 mM 3-ABA (Fig. 3E). Unexposed cells are shown as reference (Fig. 3F).

3.3. Effects on metabolic activity

In cells exposed to 1 μM CHS 828 ATP levels decreased rapidly after about 24 hr. After 48 hr no ATP was detectable.

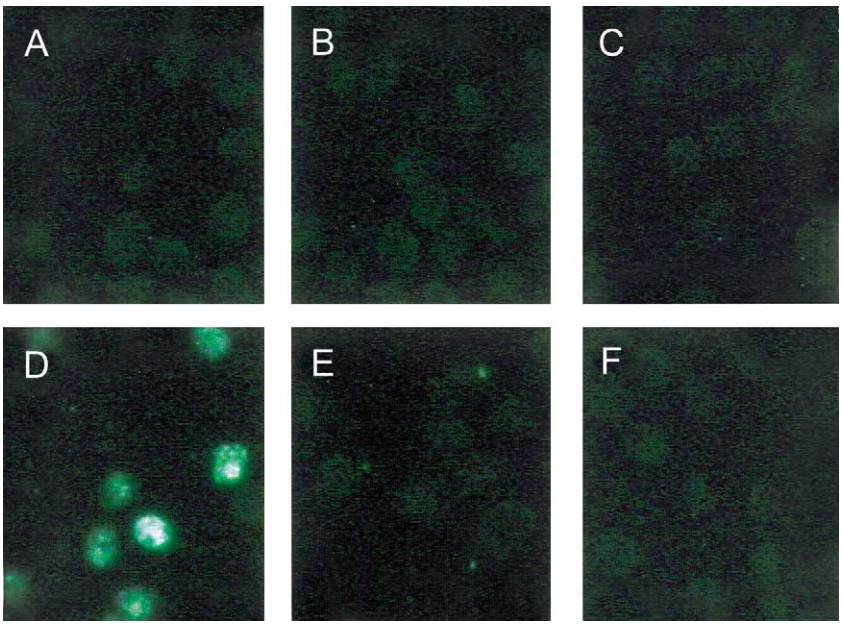
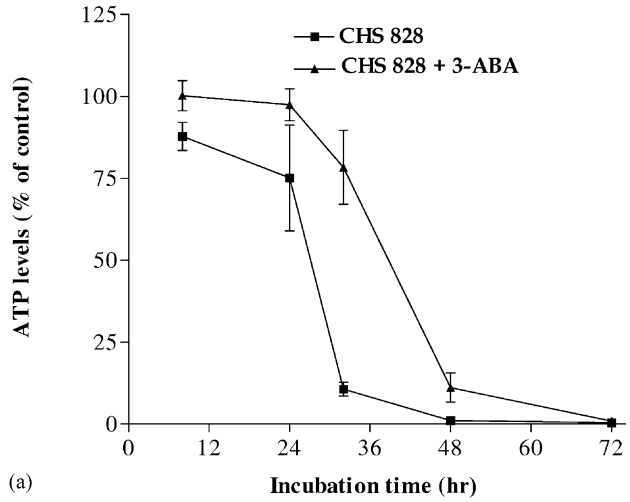


Fig. 3. Poly(ADP-ribose) staining in U-937 GTB cells exposed to 1 μM CHS 828 for 1, 4 or 27 hr (A, B and C, respectively) or 1.5 mM H_2O_2 for 5 min (D). Cells were grown in culture bottles, fixed on slides and immunostained with anti-PAR 10H monoclonal antibody and a secondary FITC-conjugated antibody. To indicate the specificity of the staining for poly(ADP-ribose), cells were preincubated with 3-ABA for 30 min before treatment with H_2O_2 (E). Unexposed cells are shown as reference (F). One representative experiment out of three.

However, cells exposed to 1 μM CHS 828 in combination with 3 mM 3-ABA retained ATP levels for about 32 hr ($P < 0.05$, Student's *t*-test vs. 1 μM CHS 828, 32 hr) with no detectable ATP after 72 hr (Fig. 4a). 3-ABA alone did not affect ATP levels compared to control cells (not shown).

As a measure of the metabolic activity the extracellular acidification rate was determined using the Cytosensor microphysiometer. Within the first hour 10 μM CHS 828 induced an increase in extracellular acidification rate of about 65% (Fig. 4b). The same level of activation was reached when CHS 828 was combined with 3 mM 3-ABA but the acidification rate was maintained at a higher level for an extended time period. 3-ABA alone did not affect the acidification rate compared to control cells in repeated experiments.



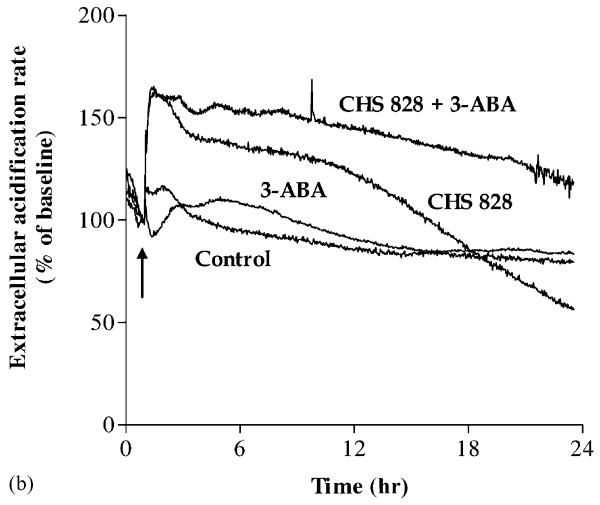


Fig. 4. ATP-levels in U-937 GTB cells exposed to 1 μM CHS 828 with or without 3 mM 3-ABA for the indicated time periods. Data presented as mean percent of control ± SE of three independent experiments (a). Extracellular acidification rate from U-937 GTB cells after exposure to 10 μM CHS 828 with or without 3 mM 3-ABA. Curves for control cells and cells exposed to 3-ABA only are included as reference. Arrow indicates addition of drugs. One representative experiment out of three (b).

3.4. Morphology

Morphological changes in cells exposed for 72 hr in cell culture bottles with 1 μM CHS 828, and CHS 828 in combination with 3-ABA are shown in Fig. 5a. Cells exposed to 1 μM CHS 828 for 72 hr showed a predominantly necrotic morphology with disrupted cell membranes and debris. However, cells exposed to CHS 828 in combination with 3-ABA showed marked increase in the typical signs of apoptosis, i.e. cell shrinkage, chromatin condensation and nuclear fragmentation with less necrosis. The same qualitative pattern was observed for cells exposed for 56 hr (not shown). Manual count of cells exposed for 72 hr is shown in Fig. 5b. Cells exposed to CHS 828 and 3-ABA for 72 hr showed significant increase in the rate of apoptosis compared to cells exposed to CHS 828 alone ($P < 0.01$, unpaired Student's *t*-test). Addition of the inhibitor of caspse-3-like activity, Z-DEVD-fmk, did not change the morphology of cells exposed to neither CHS 828 alone nor CHS 828 in combination with 3-ABA (not shown).

3.5. Caspase-3 activity

In cells exposed to 1 μM CHS 828 there was an increase in caspase-3-like activity at 48 and 56 hr. Cells exposed to both CHS 828 and 3-ABA showed a strong activation at 72 hr ($P < 0.05$ compared to CHS 828 and control, Student's *t*-test). Etoposide was included as a positive control (Fig. 6).

4. Discussion

The antitumoral activity of cyanoguanidines in general was discovered in an *in vivo* routine screening program [3]. Among the cytotoxic cyanoguanidines investigated so far, CHS 828 has shown the greatest promise as an anticancer drug in preclinical models and is presently in phase I/II trials. To further characterize and develop cyanoguanidines in general, and CHS 828 in particular, for cancer therapy it is important to elucidate their mechanisms of action. In this work, we present and evaluate the finding that 3-ABA has major effects on the cytotoxic effect of CHS 828 in U-937 GTB cells.

The nuclear ADP-ribosylation enzyme PARP is involved in the repair of DNA strand-breaks [13]. Upon activation of PARP, chains of ADP-ribose are synthesized with NAD^+ as substrate. This depletes the pool of NAD^+, and subsequently ATP [14]. An overactivation of PARP depletes the ATP stores and results in necrotic cell death [15]. Pharmacological inhibition of PARP has been shown to prevent depletion of ATP and induction of necrosis in renal epithelial cells exposed to hydrogen peroxide [16]. The same phenomenon has been observed in PARP deficient mouse fibroblasts exposed to DNA damaging agents

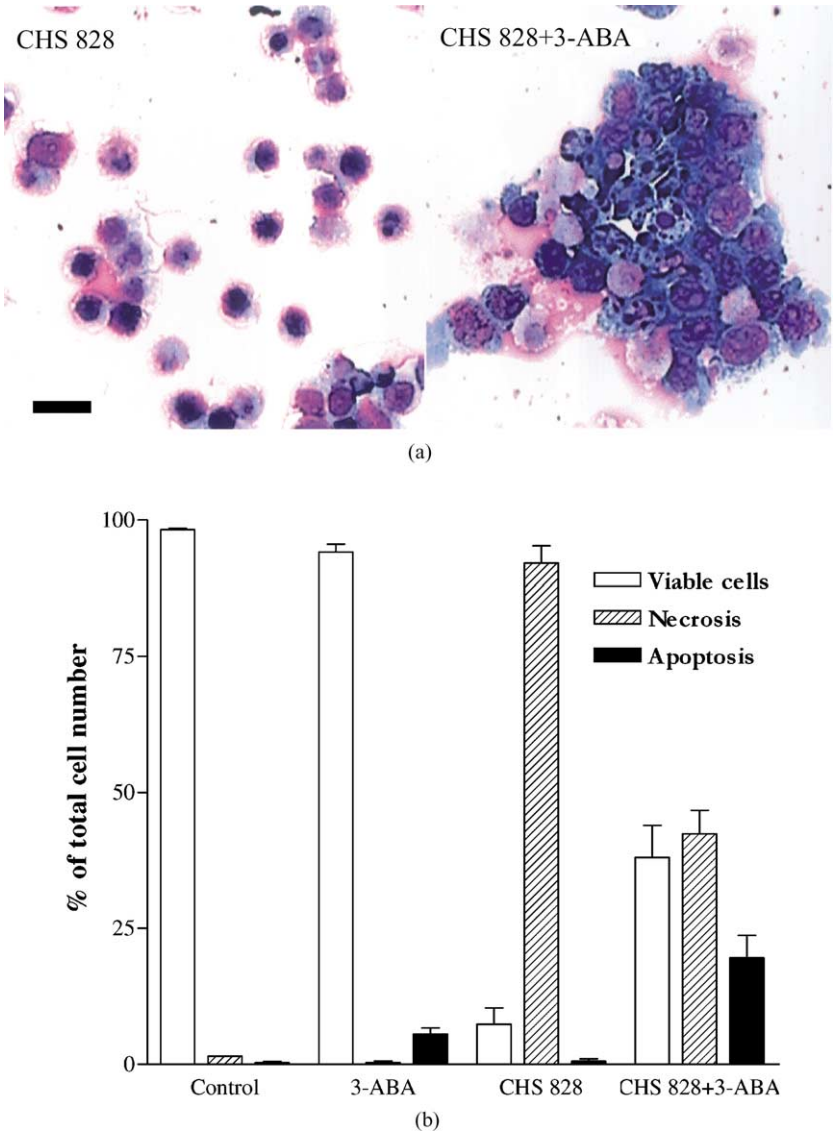


Fig. 5. Microphotographs of May–Grünewald–Giemsa stained U-937 GTB cells exposed as indicated with 1 μM CHS 828 with and without 3 mM 3-ABA for 72 hr. Bar equals 20 μm (a). A quantitative measure of the morphology (b) was obtained by counting the cells in three–four different areas on each slide (>300 cells per slide counted). Data are presented as mean values + SE of three independent experiments.

[15]. The observation that CHS 828 induce a cell death with necrotic features [17] and that 3-ABA partly inhibited cytotoxicity induced by CHS 828 led us to believe that PARP was activated in cells exposed to CHS 828. To study direct or indirect activation pathways of PARP that might lead to necrotic cell death, the activity of the enzyme itself and the level of its product was studied and found not activated by CHS 828 exposure. Since CHS 828 does not activate PARP and basal activity of PARP is very low [6] it is unlikely that PARP is involved in mediation of CHS 828 cytotoxicity, and that the effect of 3-ABA on cytotoxicity and morphology is mediated by a direct inhibition of PARP. The observation that 3-ABA modulates cytotoxicity of an anticancer drug in a PARP-independent way is however not novel. It has been shown that 3-ABA induce a PARP-independent *potentiation* of carmustine effect in human glioma cell lines [18]. This also underlines the fact that 3-ABA can both inhibit and potentiate the effect of cytotoxic drugs.

To try to further understand the mechanism of action for CHS 828 one may speculate about other possible signal transduction pathways that might be affected by 3-ABA. It has been proposed that 3-ABA, beside PARP-inhibition, interferes with protein kinase C (PKC) activity [19]. It is, however, less likely that the proposed PKC inhibitory activity of 3-ABA explains the observed effects since other PKC inhibitors do not seem to affect the cytotoxic effect of CHS 828 (unpublished data).

CHS 828 has been proposed to inhibit the respiratory function of the mitochondria leading to a compensatory increase in metabolic activity, measured as extracellular acidification rate, in the cells, probably in an effort to maintain ATP levels and membrane integrity. The increase in acidification rate is likely to be due to an increase in

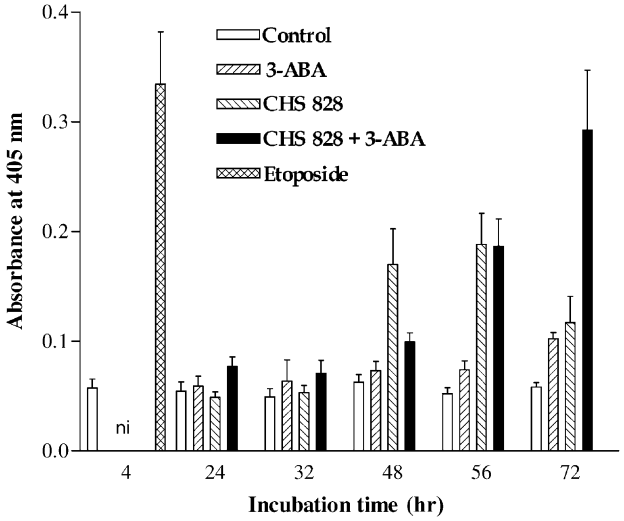


Fig. 6. Caspase-3 activity in U-937 GTB cells exposed to 1 μM CHS 828 with or without 3 mM 3-ABA for the indicated time periods. Activity was measured as the absorbance at 405 nm of the colored product formed by cleavage of the caspase-3 substrate DEVD-*p*-nitroanilide. Exposure to 25 μM etoposide for 4 hr served as positive control. Data are presented as mean values + SE of four independent experiments (ni: not investigated).

glycolysis, since the increase was abolished by removal of glucose from the perfusion medium [5]. In our experiments, we observed the same initial increase in extracellular acidification rate. After 10–12 hr of CHS 828 exposure the rate decreased, perhaps an early sign of a metabolic collapse eventually leading to cell death with a necrotic morphology. However, exposing the cells to both CHS 828 and 3-ABA maintained the extracellular acidification rate, interpreted as increased glycolytic activity, well beyond this time-point. The maintained glycolytic activity could explain both the higher survival of the cells at 72 hr and the maintenance of ATP levels observed. Colussi *et al*. [20] have shown that ADP-ribosylation of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) may inhibit glycolysis in U-937 GTB cells exposed to oxidative stress, and that this effect is inhibited by 3-ABA. ADP-ribosylation of glycolytic enzymes might be a specific process involved in the shutdown of glycolysis in cells exposed to CHS 828. The assumption that the inactivation of GAPDH is mediated by the transfer of one or a few ADP-ribose units to the enzyme may explain why no poly(ADP-ribose) was detected in CHS 828 exposed cells.

Cells exposed to CHS 828 show a loss of viability after about 32–44 hr of continuous exposure. As previously described this cell death does not show the typical signs of apoptosis [4,17]. Exposure to high concentrations of CHS 828 and 3-ABA in combination resulted in a switch from necrotic morphology seen with CHS 828 alone, to an apoptotic morphology. This might be due to the maintenance of ATP levels with CHS 828 and 3-ABA compared to CHS 828 alone, since ATP seems to be important in determining the mode of cell death, i.e. necrosis or apoptosis [21,22]. In Jurkat cells an early ATP-depletion of more than 50% of control is sufficient to switch the mode of cell death from apoptosis to necrosis [23]. This should be compared with the observed ATP-value of 11% of control after 32 hr of CHS 828 treatment preceding the morphological changes observed in both this and previous studies [4]. Treatment with CHS 828 and 3-ABA in combination yielded the same level of ATP depletion but this occurred instead after 48 hr. Even though this time delay is not reaching the time-point of caspase-3 activation it may give the necessary energy and time for the apoptotic process to be completed since ATP-dependent steps in the apoptotic cascade have been proposed both up-stream and down-stream of caspase-3 activation [24]. It has previously been shown that CHS 828 induce a non-apoptotic cell death without the major biochemical and morphological signs of apoptosis [4,17]. However, the functional relevance of caspase-3 activity in the cell death process induced by CHS 828 in combination with 3-ABA is unclear, since the inhibitor of caspase-3-like activity, Z-DEVD-fmk, did not alter the morphology.

In conclusion, it is evident that both the cytotoxicity and the morphology induced by CHS 828 are modulated by the ADP-ribosylation inhibitor 3-ABA. It is likely that this effect is mediated by alterations in the metabolic processes in the cells rather than inhibition of PARP. The apparent non-apoptotic features of CHS 828 induced cell death may at least in part be explained by these metabolic alterations leading to decreased levels of ATP during critical phases of the cell death process. However, further work is needed to reveal the exact mechanism of action for CHS 828 and the possible role of ADP-ribosylation in CHS 828 induced cytotoxicity.

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References

[1] Hjarnaa PJ, Jonsson E, Latini S, Dhar S, Larsson R, Bramm E, Skov T, Binderup L. CHS 828, a novel pyridyl cyanoguanidine with potent antitumor activity in vitro and in vivo. Cancer Res 1999;59: 5751–7.

[2] Jonsson E, Friberg LE, Karlsson MO, Hassan SB, Nygren P, Kristensen J, Tholander B, Binderup L, Larsson R. In vivo activity of CHS 828 on hollow-fibre cultures of primary human tumour cells from patients. Cancer Lett 2001;162:193–200.

[3] Schou C, Ottosen ER, Petersen HJ, Björkling F, Latini S, Hjarnaa PJ, Bramm E, Binderup L. Novel cyanoguanidines with potent oral antitumor activity. Bioorg Med Chem Lett 1997;7:3095–100.

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

effects of the novel antitumour pyridyl cyanoguanidine (CHS 828) on human lymphoma cells. Eur J Cancer 2001;37:260–7.

[5] Ekelund S, Liminga G, Bjorkling F, Ottosen E, Schou C, Binderup L, Larsson R. Early stimulation of acidification rate by novel cytotoxic pyridyl cyanoguanidines in human tumor cells: comparison with *m*-iodobenzylguanidine. Biochem Pharmacol 2000;60:839–49.

[6] D'Amours D, Desnoyers S, D'Silva I, Poirier GG. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem J 1999;342:249–68.

[7] Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17: 565–77.

[8] Matsuoka Y, Moore GE, Yagi Y, Pressman D. Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma. Proc Soc Exp Biol Med 1967;125: 1246–50.

[9] Larsson R, Kristensen J, Sandberg C, Nygren P. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia, using a fluorometric microculture cytotoxicity assay (FMCA). Int J Cancer 1992;50:177–85.

[10] Larsson R, Nygren P. A rapid fluorometric method for semiautomated determination of cytotoxicity and cellular proliferation of human tumor cell lines in microculture. Anticancer Res 1989;9:1111–9.

[11] Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg RG, Cochrane CG. Oxidant injury of cells. DNA strand-breaks activate polyadenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. J Clin Invest 1986;77:1312–20.

[12] Burkle A, Chen G, Kupper JH, Grube K, Zeller WJ. Increased poly(ADP-ribosyl)ation in intact cells by cisplatin treatment. Carcinogenesis 1993;14:559–61.

[13] Durkacz BW, Omidiji O, Gray DA, Shall S. (ADP-ribose) participates in DNA excision repair. Nature 1980;283:593–6.

[14] Berger SJ, Sudar DC, Berger NA. Metabolic consequences of DNA damage: DNA damage induces alterations in glucose metabolism by activation of poly (ADP-ribose) polymerase. Biochem Biophys Res Commun 1986;134:227–32.

[15] Ha HC, Snyder SH. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. Proc Natl Acad Sci USA 1999;96:13978–82.

[16] Filipovic DM, Meng X, Reeves WB. Inhibition of PARP prevents oxidant-induced necrosis but not apoptosis in LLC-PK1 cells. Am J Physiol 1999;277:F428–36.

[17] Martinsson P, de la Torre M, Binderup L, Nygren P, Larsson R. Cell death with atypical features induced by the novel antitumoral drug CHS 828, in human U-937 GTB cells. Eur J Pharmacol 2001;417:181–7.

[18] Winter S, Weller M. Poly(ADP-ribose) polymerase-independent potentiation of nitrosourea cytotoxicity by 3-aminobenzamide in human malignant glioma cells. Eur J Pharmacol 2000;398:177–83.

[19] Ricciarelli R, Palomba L, Cantoni O, Azzi A. 3-Aminobenzamide inhibition of protein kinase C at a cellular level. FEBS Lett 1998;431:465–7.

[20] Colussi C, Albertini MC, Coppola S, Rovidati S, Galli F, Ghibelli L. H_2O_2-induced block of glycolysis as an active ADP-ribosylation reaction protecting cells from apoptosis. Faseb J 2000;14:2266–76.

[21] Leist M, Single B, Naumann H, Fava E, Simon B, Kuhnle S, Nicotera P. Inhibition of mitochondrial ATP generation by nitric oxide switches apoptosis to necrosis. Exp Cell Res 1999;249:396–403.

[22] Lelli Jr. JL, Becks LL, Dabrowska MI, Hinshaw DB. ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. Free Radic Biol Med 1998;25:694–702.

[23] Leist M, Single B, Castoldi AF, Kuhnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med 1997;185:1481–6.

[24] Eguchi Y, Srinivasan A, Tomaselli KJ, Shimizu S, Tsujimoto Y. ATP-dependent steps in apoptotic signal transduction. Cancer Res 1999; 59:2174–81.