A purine nucleoside phosphorylase (PNP) inhibitor induces apoptosis via caspase-3-like protease activity in MOLT-4 T cells

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Abstract

Children with congenital homozygous deficiency of purine nucleoside phosphorylase (PNP) have abnormalities in purine metabolism that result in T-cell selective immune deficiency. The mechanism of action for cell death has been attributed to intracellular accumulation of dGTP, a potent inhibitor of ribonucleotide reductase and subsequently DNA synthesis, in thymocytes and T-cells but not B-cells. However, the mode of cell death has not been determined to be either necrosis or apoptosis. To examine the involvement of apoptosis in T-cells following PNP inhibition, MOLT-4 cells, a human T-cell leukemia cell line, were co-treated with the PNP inhibitor, CI-1000 (2-amino-3,5-dihydro-7-(4-aminomethyl)4H-pyrido[3,2-d]pyrimidin-4-one HCl), and 2′-deoxyguanosine (dGuo) which resulted in a concentration-dependent loss of cell viability (trypan blue) and inhibition of initiated thymidine (3H)Tdr uptake. Staining of cells with the DNA dye Hoechst 33258 showed nuclear morphology characteristic of apoptosis. Western blots (24 h postexposure) were probed with antibodies against several proteins implicated in apoptosis. Anti-PARP revealed the presence of an 85 KD PARP breakdown product while, anti-α-spectrin revealed the accumulation a 120 KD breakdown product, both suggestive of cPFP52 cleavage (caspase-3; an ICE-like cysteine protease). Western blots also detected the loss of the intact 33 KD caspase-3 protein, a biochemical event associated with caspase-3 activation. Corresponding fluorometric activity assays detected a marked increase in caspase-3-like activity using the substrate Ac-DEVD-MCA. Lastly, a pan caspase inhibitor (Z-VAD-FMK) and 2′-deoxy-5-iodo-2′-deoxy-cytidine (GdCyd), which is known to prevent dGTP accumulation following PNP inhibition, were able to prevent cell death and all indicators of caspase-3-like activity in MOLT-4 cells co-treated with CI-1000 and dGuo. In summary, we provided several lines of evidence for the role of apoptosis and the contribution of caspase-3-like proteases in T-cell death following PNP inhibition. © 1997 Elsevier Science B.V.

1. Introduction

Purine nucleoside phosphorylase (PNP) deficiency is an inherited autosomal recessive disorder
resulting in the impairment of T-cell function with normal to elevated B-cell function in affected children (Kazmers et al., 1981; Martin and Gelfand, 1981; Parvis et al., 1981; Stockler et al., 1982; Sirac and Gilbertson, 1988; Kredich and Hershfeld, 1989; Carson and Carrera, 1990; Gilbertsen et al., 1991a). PN-P-deficient patients generally succumb to recurrent infections due to immune deficiency, and manifest neurological disorders including developmental delay and retardation. However, the mechanism of cell death of T-cells in PN-P deficient children has yet to be completely elucidated. The current accepted hypothesis for immune deficiency due to PN-P deficiency is via DNA accumulation and the preferential phosphorylation of dGTP to dGTP in replicating T-cells (Kazmers et al., 1981; Martin and Gelfand, 1981; Parvis et al., 1981; Stockler et al., 1982; Sirac and Gilbertson, 1988; Kredich and Hershfeld, 1989; Carson and Carrera, 1990; Gilbertsen et al., 1991a). Excessive increases in dGTP will result in inhibition of ribonucleotide reductase and depletion of the deoxyribonucleotide pool, particularly deoxyadenosine, severely disrupting DNA synthesis. In spite of these recent findings the mode of death which T-cells undergo following PN-P inhibition has yet to be established as either necrosis or apoptosis (also referred to as programmed cell death) (Nicholson et al., 1985; Sætveden and Dixit, 1995; Orti et al., 1996; Vaux and Strasser, 1996; Kerr and Harmon, 1991).

The study of apoptotic signaling pathways is currently an area of intense investigation. Apoptosis is a physiological form of cell death that has been shown to be involved in the elimination of cells during development and maintenance of the immune system including clonal selection of the T-cell repertoire (Kapppler et al., 1987; von Hoehner, 1994), maintenance of self tolerance and prevention of autoimmune diseases by elimination of self reactive T cells (Kapppler et al., 1987; von Hoehner, 1994), regulating cessation of normal immunological responses (Thompson, 1995), and as a mechanism by which cytotoxic T cells kill foreign cells, tumor cells, and virus-infected cells (June and Tschopp, 1988; Durmon et al., 1994). Furthermore, apoptosis has also been implicated in the death of other cell types such as neurons (Nath et al., 1996a,b) and in disease processes including amyotrophic lateral sclerosis (Alexieu et al., 1994; Troost et al., 1995; Mair, 1996), Alzheimer's disease (Nixon et al., 1994; Saour et al., 1995; Goedworth et al., 1996; Vinh et al., 1996; Yamasuzu et al., 1996), and ischemia (Linaki et al., 1995; Chen et al., 1996).

Apoptosis is referred to as programmed cell death because it often involves genetically regulated mechanisms which result in the elimination of cells. However, tight genetic control is not an absolute requirement for apoptosis as it can be triggered accidentally or experimentally (Fraser and Evan, 1996; Vaux and Strasser, 1996). Recently, a class of proteins which belong to the ICE/CED-3 family (recently renamed caspase) has been implicated in vertebrate apoptotic cell death (Green et al., 1996). This proapoptotic family consist of at least eleven different homologs including interleukin 1β converting enzyme (ICE, caspase-1), IAP-1 (nedd2), caspase 2, CIP32 (caspase-3), ICH-2 (caspase-4), ICE_cel (caspase-3), Mch-2 (caspase-6), Mch-3 (caspase-7), Mch-5 (caspase-8), Mch-6 (caspase-9), Mch-4 (caspase-10), and ICH-3 (caspase-11) have been implicated in the cell death machinery leading to apoptotic cell death (Fraser and Evan, 1996; Vaux and Strasser, 1996; Alsen et al., 1996; Muro et al., 1997). The overall common feature of this family of proteins is the conservation of the active site QACRG pentapeptide to induce apoptosis and the absolute requirement for proteolytic cleavage of proenzymes and substrates at conserved aspartate cleavage sites (Ceretti et al., 1992; Thomberny et al., 1992). As a result of the intense research in this area new diagnostic markers of apoptosis have been developed beyond the classical markers such as DNA laddering and chromatin condensation (Kerr and Harmon, 1991; Gasperi et al., 1992). For example, several studies have shown that the human CED-3 homolog, caspase-3, can cleave poly (ADP-ribose) polymerase (PARP), a nuclear enzyme believed to be involved in DNA repair (Levy-Tzedek et al., 1994; Nicholson et al., 1995), and the membrane skeletal protein α-spectrin which can be reduced to a 120 kD breakdown product after caspase-3 cleavage (Nath et al., 1996b; Muro et al., 1995; Vanags et al., 1996). Consequently, an understanding of the biochemical events that contribute to apoptotic cell death is important since the proteolysis of these substrates can potentially lead to cell death.
This research employed an in vitro T-cell model using MOLT-4 cells to study PNP inhibition induced cell death. This model includes the use of a novel PNP inhibitor (CI-1000) (Biocheneke et al., 1994; Gilbertson et al., 1992, 1993) in tandem with exogenous G6PD to produce a synergistic dose response that results in cell death. In this study, we established multiple lines of evidence for the role of apoptosis and the contribution of caspase-3-like proteases in T-cell death following PNP inhibition.

2. Methods

2.1. Chemicals

The following were obtained from Sigma (St. Louis, MO) or Calbiochem (La Jolla, CA). ATP, GTP, dATP, dGTP, and dTTP were purchased from Boehringer Mannheim. Cell culture reagents were purchased from Life Technologies (Grand Island, NY). The PNP inhibitor CI-1000 (PDP: 141955-2; 2-amino-3,5-dihydro-7-(3-thiazolylmethyl)-4H-pyrrrole-[3,2-d]-pyrimidine-4-one HCl) and the pan caspase inhibitor Z-DEVD-CHO (Z-Asp-Glu-Val-Asp-CHO) were synthesized at Parke-Davis Pharmaceutical Research. Fluorescent peptide substrates, Ac-YVAD-MCA and Ac-IETD-MCA (where MCA represents 7-amido-4-methylcoumarin) were purchased from Bachem Biosciences (King of Prussia, PA). LDB CytoVue kit 96 was obtained from Promega (Madison, WI). Detergent compatible (DOC) protein assay was purchased from BioRad (Hercules, CA).

2.2. Cell lines and cell culture

These methods have been described in detail elsewhere (Kazmers et al., 1991; Siddiqui and Mitchell, 1994; Gilbertson et al., 1993). In brief, increasing dilutions of G6PD (10, 30 and 100 μM) were co-cultured with CI-1000 (10 μM) and 12 well plates containing 4 × 10⁴ MOLT-4 T-lymphoblastoid cells per well in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured for 48 h and labeled with [¹¹⁵]iodo-Tdr for the final six hours. Apoptotic positive controls were obtained by culturing MOLT-4 T-cells with staurosporine (0.5 μM final concentration) for 24 h under the identical culture conditions described above. Cell viability assessments using trypan blue were also conducted at 48 h. In all other studies, cells were cultured for 24 h. The ability of G6PD (10 μM), calpain inhibitor II (30 μM) and the pan caspase inhibitor Z-DEVD-CHO (50 μM) to protect T-lymphoblasts from cytotoxicity was determined by adding these reagents to cultures one hour prior to G6PD and CI-1000 exposure.

2.3. Cell viability measurement

MOLT-4 cell death was assessed by trypan blue staining. Values were expressed as the ratio of unstained cells/n of total cells counted (100 per well) × 100. The cell counts represent studies done in triplicate.

2.4. LDH measurement

Cell death was assessed by measuring the release of the cytosolic enzyme, lactate dehydrogenase, in the medium (25 μl of each sample) from co-cultures. Methods were identical to those described in recent studies (Kazmers et al., 1996a,b).

2.5. Hoechst staining of apoptotic nuclei

Cells were rinsed twice with phosphate buffer solution (PBS), 136 mM NaCl, 8.1 mM KCl, 1.6 mM Na₂HPO₄, and 14 mM KH₂PO₄, pH 7.4) and incubated with 2 μg/ml of DNA dye Hoechst 33258 stain in PBS for 5 min at room temperature. All photomicrographs were taken using a Leitz microscopic imaging system at a magnification of 320 x.

2.6. Protein extraction, SDS-PAGE, and western blot methodology

Cells were washed twice with TBS-EDTA (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) in
order to remove any residual serum. SDS—protein extraction of cell lysates was done in the presence of protease inhibitors and SDS as previously described (Neath et al., 1996a,b). The extraction procedure was completed by precipitating the proteins with tri- chloroacetic acid (TCA) and resolubilization of the pellet was achieved with 3 M Tris base. Protein determination of samples was performed using a modified Lowry assay. Protein samples containing 5—15 µg of protein were run on 4—20% acrylamide gradient gels employing a Tris glycerine running buffer. Following separation of proteins by SDS-PAGE, proteins were transferred onto a PVDF membrane using a Tris glycerine buffer (48 mM Tris base, 39 mM glycine, and 10% MeOH, pH 9.2) and semi-dry electrotransferring unit (BioRad) at 20 mA for 2 h. All lanes contained identical amounts of protein. To insures consistency of gel loading, we routinely per- form coomassie staining of polyacrylamide gels. Fol- lowing transfer of the proteins to PVDF membrane, non-specific sites were blocked with an incubation of 5% dry milk at 4°C. Incubation in a primary anti- body was performed with either anti-α spectrin (clone AA6; Affinity Research Products, Notting- ham), anti-PARP (Fasitene Systex Products, Santa Cruz, CA), or anti-caspase-3 (Transduction Labora- tories, Lexington, KY) monoclonal antibodies. Visu- alization of proteins was performed with a strepta- vidin—alkaline phosphatase kit using 5-bromo-4- chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) as the active chromagen.

2.7. Trition protein extraction for assaying IEC and caspase-3 like activity

On completion of the experiment protocol MOLT-4 cells from three wells were collected in 15 µl centrifuge tubes and washed twice with 5 ml of TBS—EDTA buffer by centrifugation for 5 min at 4°C. Cell pellets were resuspended and used in a buffer containing 20 mM Tris—HCl (pH 7.4) at 4°C, 150 mM NaCl, 1 mM EDTA, 5 mM EGTA, and 1% Triton X-100 for 60 min. Following incubations of MOLT-4 cells in triton buffer, cell lysates were recovered after centrifugation and stored in 50% glycerol at −70°C. All cell lysates were assayed for protein concentration with a modified Lowry assay. IEC (caspase-1) and caspase-3 were assayed for proteolytic activity by using the fluoro- metric peptide substrates, Ac—YVAD—MCA and Ac—DEVD—MCA, respectively. Cell lysates were added to a buffer containing 100 µM peptide substrate, 100 mM HEPEs, 10% glycerol, 1 mM EDTA, 10 mM DTT and 10 mM of the general caspase inhibitor Z—D—D—C. Fluorescence excitation 380 ± 15 nm and emission 460 ± 15 nm was measured every 15—30 min up to 60 min with a millipore Cytosor 2000 fluorescence plate reader.

3. Results

3.1. PNP inhibition results in dose—dependent loss of cell viability and decline in thymidine uptake

The synergy between dGuo and CI-1000 pro- duced a concentration—dependent loss of MOLT-4 cell viability (trypan blue staining) 48 h after dGuo and CI-1000 exposure (Fig. 1A). CI-1000 alone did not produce any changes in cell viability (p < 0.05). Cells cultured with 10 and 30 µM of dGuo in the absence of CI-1000 did not demonstrate remarkable loss of cell viability (7.9% and 9.3%, respectively). However, the highest concentration of dGuo (100 µM) used in this study produced a net 38.3% loss of cell viability compared to untreated controls (p < 0.05). Cell viability counts on cells exposed to in- creasing concentrations of dGuo 10, 30, and 100 µM in the presence of 10 µM CI-1000 showed a marked decrease in viability from 94.7% in controls to 18.1% in cells exposed to 100 µM dGuo and 10 µM CI-1000 (p < 0.05). Determination of [3H]—Tdr incorporation into MOLT-4 cells was done in parallel with cell viability at 48 h following the addition of dGuo and CI-1000 (Fig. 1B). The reduction in uptake of [3H]— Tdr mirrored decreases in cell viability counts mea- sured by trypan blue staining. [3H]—Tdr uptake was unchanged at 10 µM dGuo alone, but at higher concentrations 30 µM and 100 µM dGuo produced a decrease in [3H]—Tdr uptake (69.335 cpm, and 9.244 cpm respectively) in comparison to untreated controls (160.150 cpm; p < 0.05). The up- take of [3H]—Tdr in the cells treated with dGuo and CI-1000 was almost completely blocked with the lowest used concentration of dGuo (10 µM) in the
presence of 10 μM CI-1000 remained reduced at 48 h (453 cpm and 351 cpm, respectively) in comparison to 10 μM CI-1000 controls (172, 974 cpm, (p < 0.05)).

LDH release was also performed at 48 h to examine cell viability following PNP inhibition (Fig. 1C). MOLT-4 cells co-cultured with 10 μM dGuo in the absence of 10 μM CI-1000 did not demonstrate remarkable increases in LDH release (p < 0.05). However, cells cultured with 30 μM and 100 μM dGuo in the absence of CI-1000 did produce an elevation in LDH release. MOLT-4 cells co-cultured with dilutions of dGuo in the presence of CI-1000 demonstrated a dose dependent increase in LDH release. Cells treated with staurosporine serving as a positive apoptotic control produced near maximal release.

3.2. PNP inhibition results in the production of chromatin condensation and DNA laddering characteristic of apoptosis

In an attempt to provide evidence for the role of apoptosis following PNP inhibition, we used Hoechst 33258 staining in MOLT-4 cells 24 h after co-culturing with dGuo and CI-1000 (Fig. 2). MOLT-4 cells challenged with dGuo in the absence of CI-1000 did not produce features of the classic apoptotic response, including chromatin condensation on the periphery of the nuclear envelope, except at the highest level of dGuo (100 μM) employed. The initiation of chromatin condensation was observed in the lowest concentration-dependent synergy between dGuo and CI-1000 (10 μM dGuo and 10 μM CI-1000). An increase in chromatin condensation and the appearance of apoptotic bodies was noted with increasing amounts of dGuo in the presence of 10 μM CI-1000. No apoptotic bodies were observed in cells cultured with 10 μM CI-1000 alone.

3.3. DNA fragmentation occurs following PNP inhibition

To confirm that apoptotic cell death occurs following PNP inhibition we employed DNA laddering. DNA laddering is a biochemical hallmark of apoptosis believed to be produced by activated endonuclease-
ases at vulnerable sites 180–200 base pair (bp) apart (Kerr and Harrison, 1991; Gavrieli et al., 1992). MOLE-4 cells challenged with dGuo in the absence of CI-1000 did not produce DNA laddering except at the highest concentration of dGuo (100 μM) used in this study (data not shown). The appearance of DNA

Fig. 2. Hoechst stained MOLE-4 cells were examined following co-culturing with dilutions of dGlut in the presence and absence of CI-1000. Photomicrographs of cells cultured with 0, 10, 30, and 100 μM of dGlut in the absence (A, B, C, and D, respectively) and presence of 30 μM CI-1000 (E, F, G, and H, respectively). Untreated controls (A) and cells treated with CI-1000 alone (E) were void of any evidence of apoptotic nuclei. Photomicrographs of cells co-cultured with 20 μM and 100 μM of dGlut in the presence of CI-1000 (100 μM) revealed large numbers of condensed and apoptotic nuclei (G, H, respectively). Bar = 25 μm.
ladder was detected in all the concentration-dependent synergies between dGlu and CI-1000 (10, 30 and 100 μM dGlu in the presence of 10 μM CI-1000, Fig. 3). Although DNA fragmentation was detected in the above described experimental conditions, the resolution of DNA laddering was not as evident to that seen in other types of T-cells (i.e., Jurkat) undergoing apoptosis (Chow et al., 1993). However, previous apoptosis studies using MOLT-4 cells have also described the difficulty of obtaining high resolution 200 by DNA laddering (Falcieri et al., 1993).

3.4. Cell loss via PNP inhibition is associated with the cleavage of PARP and α-spectrin

We employed SDS–PAGE and Western blotting methodology on protein extracts to determine if cleavage of PARP (Fig. 4A) and the appearance of the 120 Kd α-spectrin breakdown product (Fig. 4B), two markers of the caspase-3-like cysteine protease, occur following PNP inhibition. Cleavage of PARP from a 110 Kd band to an 85 Kd fragment was only observed at the highest concentration of dGlu (100 μM) when cultured in the absence of CI-1000 (Fig. 4A). CI-1000 (10 μM) alone did not result in marked PARP cleavage, but a dose dependent synergy between dGlu and CI-1000 did result in PARP cleavage at all concentrations employed in this study. Staurosporine, an established inducer of apoptosis, produced the 85 Kd PARP cleavage product characteristic of caspase-3-like proteolysis (Fig. 4A, lane 9).

To provide another line of evidence of protein cleavage by caspase homologs following PNP inhibition we probed for the appearance of a 120 Kd α-spectrin breakdown product that is exclusively produced by caspase-3 (Fig. 4B). Western blots revealed the loss of the parent subunit and a significant accumulation of the 120 Kd BDP at only the 100 μM concentration of dGlu cultured in the absence of CI-1000. Western blots containing protein extracts from cells exposed to increasing concentrations of dGlu in the presence of 10 μM CI-1000 revealed a dose dependent decrease in the parent α-spectrin subunit and a concomitant increase in the 120 Kd BDP. Conditions resulting in the appearance of the 120 Kd spectrin BDP corresponded identically to those that demonstrated PARP cleavage. MOLT-4 cells cultured only with CI-1000 did not exhibit a significant increase in the 120 Kd spectrin BDP. Staurosporine positive controls similarly produced

![Fig. 3. DNA laddering in MOLT-4 cells following co-culturing with dGlu in the absence and presence of CI-1000. Lane 1: 113 bp ladder, lane 2: 2.9 μM dGlu, lane 3: 9 μM dGlu/10 μM CI-1000, lane 4: 19 μM dGlu/10 μM CI-1000, lane 5: 30 μM dGlu/10 μM CI-1000, lane 6: 100 μM dGlu/10 μM CI-1000.](image)

![Fig. 4. PARP and α-spectrin immunoblotting following PNP inhibition. PNP inhibition produces the accumulation of the 95 Kd PARP cleavage product (A) and 120 Kd α-spectrin BDP (B) in MOLT-4 cells suggestive of caspase-3-like substrate proteolysis. Western blot analyses were performed on cell lysates from cultures incubated with dilutions of dGlu (0, 10, 30, and 100 μM) in the absence and presence of 10 μM CI-1000. The data shown is a representative blot from three independent experiments.](image)
the 120 kD spectrin BDP characteristic of caspase-3-like proteolysis (Fig. 4B, lane 9).

3.5. PNP inhibition produces the cleavage of caspase-3 and an increase in caspase-3-like activity

In an attempt to establish more direct evidence for the role of caspase-3-like proteases in this process, we employed SDS-PAGE and Western blotting to determine if cleavage of caspase-3 had occurred (Fig. 5A). Cleavage of the 32 kD caspase-3 subunit is a biochemical event necessary for the initiation of caspase-3 activation (Gerritti et al., 1992; Darmon et al., 1994; Nicholson et al., 1995). Western blots revealed a significant decrease of caspase-3 at the highest concentration of dGluo alone (100 μM), and a progressive decrease of the caspase-3 band with increasing amounts of dGluo co-culturing with 10 μM CI-1000. The mature 17 kD caspase-3 form was not detected in these cells by the anti-caspase-3 antibody.

To further confirm caspase homolog activation (i.e. caspase-3) in PNP inhibition induced cell death, we extracted cell lysates at 24 h using Triton 100. Two fluorogenic peptide substrates, Ac–DEVD–MCA and Ac–YVAD–MCA, were used to examine the activity of caspase-1 and caspase-3-like proteases in MOLT-4 cells following PNP inhibition (Fig. 5B). Ac–DEVD–MCA was used to infer caspase-3-like activity because it is more readily preferred over Ac–YVAD–MCA (Dutta et al., 1996; Talanian et al., 1997). Caspase-1 can potentially cleave both substrates at a similar rate (Nicholson et al., 1995). We observed very low levels of Ac–YVAD–MCA hydrolytic activity at all concentrations of dGluo in the absence and presence of CI-1000 employed in this study. However, Ac–DEVD–MCA hydrolytic activity demonstrated a remarkably different profile.

A progressive increase in hydrolytic activity was detected with increasing concentrations of dGluo alone which became maximal at 100 μM (0.76 activity units ±0.150) and was significantly different from untreated controls (0.27 activity units ±0.005), (p = 0.05). Similar to other assays providing lines of evidence for the role of caspase-3 in PNP inhibition-induced cell death, there was a statistically-significant increase in Ac–DEVD–MCA hydrolytic activity in lysates from MOLT-4 cells cultured with 10 μM dGluo plus 10 μM CI-1000 (0.75 ± 0.042 activity units) versus 10 μM CI-1000 controls (0.263 activity units ± 0.089, p < 0.05). A sustained increase in hydrolytic activity was also observed in cell lysates co-culturing with 30 μM dGluo and 10 μM CI-1000 (0.768 ± 0.051 activity units). Interestingly, there was a marked drop off in Ac–DEVD–MCA hydrolysis activity at 180 μM dGluo plus 10 μM CI-1000 (0.279 ± 0.009 activity units).

Fig. 5. Examination of protein activity following PNP inhibition in MOLT-4 cells. The role of caspase-like enzymes in MOLT-4 cells following PNP inhibition was evaluated by (A) examining caspase-3-like activity using Western blotting and (B) measuring the hydrolysis of fluorometric substrates for either caspase-1 (Ac–YVAD–MCA) or caspase-3 (Ac–DEVD–MCA) cells pretreated with or without dGluo (identical to those described in Fig. 4) in the absence or presence of 10 μM CI-1000. Data are expressed as the mean ± SEM (n = 3). Turkey-ANOVA was performed to determine group differences (p < 0.05). Indicates significant differences from 0 μM dGluo control.
3.6. Pan caspase inhibitor Z-D-DCB and dCyd each reduce LDH release, PARP and co-spectrum cleavage following PNP inhibition

To determine if PNP inhibition indeed induces apoptotic cell death via caspase activity, we examined the ability of two different types of caspase inhibitors to attenuate LDH release. Caspase inhibitor 2 (a potent aldehyde peptide inhibitor of both μ-calpain and m-calpain) (Wang and Yeen, 1994) and Z-D-DCB (a non-specific caspase inhibitor) (Dodici et al., 1994) were employed to differentiate the contribution of proteases in MOLT-4 cell death following PNP inhibition. In addition, we also wanted to determine if CI-1000-induced apoptosis could be prevented by the coadministration of dCyd, which is known to prevent PNP inhibitor plus dGlu-induced MOLT-4 cell death (Gilbertson et al., 1991b). Consequently, we cultured MOLT-4 cells with 10 μM or 100 μM dGlu in the presence of 10 μM CI-1000 and 10 μM dCyd.

Cell death as measured by LDH release detected a dose-dependent synergy between increasing concentrations of dGlu and 10 μM CI-1000 in MOLT-4 cells (Fig. 6). Effects of protease inhibitors and decycteclinid amine inhibition on LDH release in MOLT-4 cells following PNP inhibition. MOLT-4 cells were cultured with 10 μM CI-1000 in the presence of 0 μM (lane 1), 10 μM (lanes 2, 4, 6, 8) or 100 μM (lanes 3, 5, 7, 9) dGlu in addition with either calpain inhibitor 2 (lanes 4, 5), Z-D-DCB (lanes 6, 7) or 10 μM dCyd (lanes 8, 9). (A) SDS-PAGE and Western blot analysis were performed on cell lysates 24 h following the addition of dGlu and CI-1000 using anti-PARP. (B) Both Z-D-DCB and calpain inhibit PARP cleavage following PNP inhibitor-induced apoptosis. Immunoblot as shown above were analyzed quantitatively for the accumulation of the 85 kDa PARP cleavage product by computer-assisted scanning densitometry. Data are normalized and expressed as the percentage of maximum accumulation of the 85 kDa cleavage product for each MOLT-4 cell line. The data shown are the means of three independent experiments (mean ± SEM). Table-1005 ANOVA was performed to determine group differences (p < 0.05). *Indicates significance in comparison to 100 μM dGlu/10 μM CI-1000 cultures (lane 3).
4. Discussion

In this study we have explored the role of apoptosis and the involvement of caspase-3-like processes in MOLT-4 T cell death induced by PNP inhibition and dGuo treatment. The data in this study represent the first evidence for apoptotic cell death following PNP inhibition in a T-cell line. We have observed several features unique to apoptotic cell death including chromatin condensation using Hoechst staining and DNA laddering following PNP inhibition (Figs. 2 and 3). In addition we demonstrated the cleavage of the 110 kD PARP protein into a signature 85 kD apoptotic fragment (Fig. 5A). Lazebnik et al., 1994; Nicholson et al., 1995). One mechanism of action for apoptotic cell death is the involvement of the cysteine CED-3/ICE family (caspase) of proteases. Caspase-3, a member of the caspase family, is synthesized as a 32 kD precursor protein that is proteolytically cleaved to active forms of 17 kD and 13 kD (Nicholson et al., 1995; Tewari et al., 1995). Past studies have demonstrated the activation of caspase-3 in T-cells using the Jurkat cell line following anti-fas induced apoptosis (Nath et al., 1996a;b; Schlegel et al., 1996). Similarly we have detected the activation of caspase-3 in our current studies of PNP inhibition in MOLT-4 T-cells at 24 h following PNP inhibition. The loss of the 32 kD caspase-3 precursor protein was reliably detected in cells co-cultured with the PNP inhibitor Cl-1000 and dGuo (Fig. 5A). Of interest, the lower molecular weight 17 kD active form was not detected in our cell lysates. The inability of our selected antibody to detect this fragment at 24 h post PNP inhibition may be due to the fact that this fragment only reacts weakly with the antibody (Nath et al., 1996a;b). However, corresponding caspase-3-like activity data using the fluorometric substrate Ac-DEVD-MCA sufficiently substantiated the role of caspase-3-like processes following PNP inhibition induced cell death in MOLT-4 cells (Fig. 5B).

Moreover, even though we did not detect evidence
for the contribution of caspase-1 in our experimental protocols, we cannot exclude the possibility of its involvement in PNP inhibition induced cell death in MOLT-4 cells.

The detection of proteolytic fragments following PNP inhibition-induced cell death in MOLT-4 cell lysates provided an alternative strategy to identify the contributing role of specific proteases. In addition to PARP cleavage to an 83 kD fragment, the 120 kD α-spectrin break down product (BDP) (Fig 4B) produced by the membrane skeletal protein α-spectrin, (Naft et al., 1996a; Martin et al., 1995; Vintas et al., 1996) provided another diagnostic tool for examining the role of proteases in PNP inhibition induced cell death. α-spectrin can be degraded by several candidate proteases producing markedly dif-
ferent fragmentation patterns. In cells cultured with dGuo and CI-1000 there was a dose dependent in-
crease in the appearance of a 120 kD spectrin BDP suggestive of caspase-3 proteolysis. In addition there was the presence of a faint 150 kD BDP. The dramatic increase in the 150 kD spectrin BDPs fol-
lowing PNP inhibition in Z-D-DCB treated cells (Fig. 8A) is of potential interest because its presence can also infer proteolytic activity. The 150 kD frag-
ment can potentially be produced by caspase-3 (Martin et al., 1995; Naft et al., 1996a,b) or calpain proteolysis (Roberts-Lewis et al., 1994; Naft et al., 1996a,b) of the α-spectrin subunit.

Calpain is an intracellular cysteine protease that requires sustained elevations of intracellular calcium for maximal activation. The two most predominate isoforms found in most cell types are referred to as n-calpain and m-calpain (Dolle et al., 1994). The pathologic activation of calpain has been implicated in many diseases where disturbances in calcium homeostasis have been demonstrated such as Wiskott–Aldrich inherited platelet/T lymphocyte disease (Kennedy et al., 1994), Duchenne muscular dystrophy (Jagadeesh et al., 1993), acute brain injury (Yuen and Wong, 1996; Posmantur et al., 1997) and Alzheimer’s disease (Meksen et al., 1995; Vito et al., 1996). The involvement of proteases responsible for necrosis has been mostly attributed to calpain (Dolle et al., 1994; Roberts-Lewis et al., 1994; Posmantur et al., 1997; Meksen et al., 1995; Spencer et al., 1995; Atora et al., 1996; Seiffert, 1998) rather than cas-
pase-3-like proteases. However, calpain is not exclu-

vation of proteases and subsequent apoptotic cell death.

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