Role of Poly(ADP-ribose)ylation in the Killing of Chronic Lymphocytic Leukemia Cells by Purine Analogue

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ABSTRACT

Although the nucleoside analogues fludarabine and chlorodeoxyadenosine have become important therapeutic agents in chronic lymphocytic leukemia (CLL), their effectiveness is limited by drug resistance. Because such resistance is likely to result from impaired drug-induced apoptosis, it is clearly important to understand the mechanisms involved in this process. Whereas p53 can contribute to the nucleoside-induced killing of CLL cells, recent work from this laboratory and elsewhere has shown that such killing can also occur by p53-independent mechanisms. Because poly-(ADP-ribose) polymerase (PARP)-mediated NAD\(^+\)/ATP depletion has been implicated in the nucleoside-induced killing of normal resting lymphocytes, we postulated that this mechanism might account for the p53-independent component of nucleoside cytotoxicity in CLL. To address this question, we used 3-aminobenzamide (3AB) at a concentration (200 \(\mu M\)) known to produce selective inhibition of poly(ADP-ribose)ylation in intact cells and examined nucleoside-induced killing using a number of different end points (cell membrane disruption, cell shrinkage, mitochondrial depolarization, exposure of phosphatidyl serine, morphological changes, DNA fragmentation, and PARP-1 cleavage). In 27 of the 30 cases of CLL examined, 3AB delayed nucleoside-induced cell membrane disruption without inhibiting other manifestations of cytotoxicity. This indicates that PARP activity, rather than contributing to the induction of cell killing, was accelerating cell membrane disruption during the late stages of apoptosis. This novel observation has important implications for previous studies of PARP-mediated cytotoxicity. However, in cells from one CLL patient, 3AB inhibited all manifestations of nucleoside cytotoxicity; this was the only case in the study known to have a p53 gene defect affecting both alleles. This indicates that PARP activity can occasionally be central to nucleoside-induced killing and that such PARP-mediated killing is p53 independent.

INTRODUCTION

There is currently much interest in the therapeutic role and mechanism of action of purine analogues in CLL, a malignancy of predominantly nondividing mature B-cell lymphocytes. It is generally accepted that the killing of resting lymphocytes by purine analogues requires nucleoside phosphorylation, involves the accumulation of nucleoside-induced DNA breaks, and occurs by apoptosis. However, there are several problems with this explanation of nucleoside cytotoxicity. For example, ATP depletion is associated with cellular necrosis, and although purine analogues induce apoptosis, in addition, PARP-mediated NAD\(^+\)/ATP depletion might be confined to cells that have already undergone apoptosis. To complicate matters further, in these early experiments, 3AB was used at a high concentration (5 \(\mu M\)) now known to inhibit mono(ADP-ribose)ylation.

In some but not other studies of nucleoside cytotoxicity in CLL, activation of the tumor suppressor protein p53 has been implicated as a link between nucleoside-induced DNA breaks and subsequent killing. Recent work from this laboratory has reconciled these apparently contradictory findings by demonstrating that the nucleoside-induced killing of mouse splenocytes (7) and CLL cells (8) can occur by both p53-dependent and -independent mechanisms.

Regarding possible p53-independent mechanisms of cell killing, activation of PARP has previously been implicated in nucleoside cytotoxicity (9). Before the recent demonstration that cells from PARP-knockout mice can synthesize poly(ADP-ribose) (10), it was assumed that PARP was a single gene product. However, several structurally unrelated PARP enzymes have now been identified (11). Among these, the original PARP (now known as PARP-1) is by far the most abundant and well characterized. This nuclear enzyme, in addition to being a target for proteolytic cleavage during apoptosis (12), is thought to play a role in DNA repair by signaling genomic damage. Thus, PARP-1 binds to DNA breaks, becomes activated, and, using NAD\(^+\) as a substrate, adds ADP-ribose polymers to a range of nuclear proteins including itself, histones, and DNA repair enzymes.

The idea that poly(ADP-ribose)ylation might be involved in the cytotoxic action of purine analogues is based on work showing that the nucleoside-induced killing of normal resting lymphocytes (detected as loss of vital dye exclusion) was preceded by NAD\(^+\)/ATP depletion and that these events were prevented by the PARP inhibitor 3AB (9). These and other findings have led to a proposed model of nucleoside action involving the following sequence of events: (a) inhibition of ongoing DNA repair; (b) accumulation of DNA breaks; (c) PARP activation; (d) NAD\(^+\) consumption; and (e) ATP depletion.

However, there are several problems with this explanation of nucleoside cytotoxicity. For example, ATP depletion is associated with cellular necrosis, whereas purine analogues induce apoptosis. In addition, PARP-mediated NAD\(^+\)/ATP depletion is known to occur as a consequence of apoptosis. Given that the cell membrane requires ATP for many of its functions, it seems likely that PARP-mediated NAD\(^+\)/ATP depletion might contribute to the cell membrane disruption that occurs during the late stages of apoptosis. If so, the previously demonstrated inhibitory effect of 3AB on nucleoside-induced cell membrane disruption might be confined to cells that have already undergone apoptosis. To complicate matters further, in these early experiments, 3AB was used at a high concentration (5 \(\mu M\)) now known to inhibit mono(ADP-ribose)ylation.

Whereas the role of poly(ADP-ribose)lation in the nucleoside-induced killing of normal resting lymphocytes is unclear, its role in the nucleoside-induced killing of CLL cells is completely unknown. It was therefore the aim of the present study to address this important question.

MATERIALS AND METHODS

CLL Patients
Peripheral blood was obtained with informed consent. In all cases, the malignant lymphocytes were morphologically typical and expressed low levels of light-chain-restricted surface immunoglobulin, together with CD5 and CD23. All of the patients had a lymphocyte count greater than 100 \(\times\) 10\(^3\)/liter.

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2 To whom requests for reprints should be addressed, at Department of Haematology, University of Liverpool, Liverpool L69 3GA, United Kingdom.
3 The abbreviations used are: CLL, chronic lymphocytic leukemia; PARP, poly(ADP-ribose) polymerase; MART, mono(ADP-ribosyl) transferase; 3AB, 3-aminobenzamide; 4AN, 4-amino-1,8-naphthalimide; 2NP, 2-nitro-6(5)-phenanthridine; CaDA, 2-chloro-2'-deoxyadenosine; PI, propidium iodide; FSC, forward-angle light scatter; SSC, side-angle light scatter; D6OGR, 3,3'-diethoxyloxycarbocyanine iodide; PS, phosphatidyl serine.

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Recent work from this laboratory has implicated as a link between nucleoside-induced DNA breaks and subsequent killing. It is clearly important to understand the mechanisms involved in this process. Whereas p53 can contribute to the nucleoside-induced killing of CLL cells, recent work from this laboratory and elsewhere has shown that such killing can also occur by p53-independent mechanisms. Because poly-(ADP-ribose) polymerase (PARP)-mediated NAD\(^+\)/ATP depletion has been implicated in the nucleoside-induced killing of normal resting lymphocytes, we postulated that this mechanism might account for the p53-independent component of nucleoside cytotoxicity in CLL. To address this question, we used 3-aminobenzamide (3AB) at a concentration (200 \(\mu M\)) known to produce selective inhibition of poly(ADP-ribose)ylation in intact cells and examined nucleoside-induced killing using a number of different end points (cell membrane disruption, cell shrinkage, mitochondrial depolarization, exposure of phosphatidyl serine, morphological changes, DNA fragmentation, and PARP-1 cleavage). In 27 of the 30 cases of CLL examined, 3AB delayed nucleoside-induced cell membrane disruption without inhibiting other manifestations of cytotoxicity. This indicates that PARP activity, rather than contributing to the induction of cell killing, was accelerating cell membrane disruption during the late stages of apoptosis. This novel observation has important implications for previous studies of PARP-mediated cytotoxicity. However, in cells from one CLL patient, 3AB inhibited all manifestations of nucleoside cytotoxicity; this was the only case in the study known to have a p53 gene defect affecting both alleles. This indicates that PARP activity can occasionally be central to nucleoside-induced killing and that such PARP-mediated killing is p53 independent.
Cell Culture

Mononuclear cells were prepared from whole blood by centrifugation over Lymphoprep (Life Technologies, Inc., Paisley, United Kingdom) and cultured at 37°C in RPMI + 1% BSA in the presence of 5% CO₂. Culture vessels were precoated with poly(2-hydroxyethyl) methacrylate (Sigma, Poole, Dorset, United Kingdom) to prevent cell adhesion (20).

Pharmacological Agents

The purine analogues CdA and 9-β-D-arabinosyl-2-fluoroarabinoside (fludarabine) monophosphate were kind gifts from Janssen-Cilag (High Wycombe, Buckinghamshire, United Kingdom) and Schering (Burgess Hill, West Sussex, United Kingdom), respectively. CdA and fludarabine were used at the pharmacologically relevant concentrations of 0.2 and 2 μM, respectively (21, 22). These low nucleoside concentrations are not acutely toxic to CLL cells but instead produce a delayed killing response (8, 23).

The PARP inhibitors 3AB, 4AN, and 2NP were obtained from Sigma. 3AB was used at a concentration (200 μM) known to inhibit poly(ADP-ribosyl)ation but not mono(ADP-ribosyl)ation in intact cells (19), whereas 4AN and 2NP were used at approximately 1, 10, and 100 times their respective IC₅₀ values for PARP (24).

Measurement of Cell Killing

Loss of Membrane Integrity. Cultured cells were gently resuspended and added to an equal volume of PBS containing 10 μg/ml PI (Sigma). After incubating the cells on ice for 10 min, cells were analyzed by flow cytometry. Cells with an intact plasma membrane do not stain with PI (a red DNA-binding fluorochrome). In contrast, cells that have lost their membrane integrity take up the fluorochrome and therefore fluoresce bright red (25).

Cell Shrinkage. Cell death can be detected flow cytometrically as a change in FSC and SSC. Thus, dead cells have a lower FSC and higher SSC than do live cells (25).

Mitochondrial Depolarization. Cultured cells (50 μl) were gently resuspended and added to 150 μl of PBS containing 40 nM DiOC₆ (Sigma). After a 15-min incubation at 37°C, the cell suspension was added to an equal volume of PBS containing 10 μg/ml PI. After an additional 30 min of incubation on ice, cells were analyzed by flow cytometry. DiOC₆ is a cell-permeable green fluorochrome that is selectively taken up by charged mitochondria and therefore stains live cells but not apoptotic cells (26, 27). Dual staining with PI enables the identification of early apoptotic cells that have undergone mitochondrial depolarization but have not yet lost their membrane integrity (25).

Exposure of PS. Cultured cells were gently washed in PBS and resuspended in annexin V-FITC (PharMingen, Cowley, Oxford, United Kingdom) diluted 1:20 in a buffer consisting of 10 mM HEPES/sodium hydroxide (pH 7.4), 140 mM sodium chloride, and 2.5 mM calcium chloride. After a 15-min incubation at room temperature, the cell suspension was diluted in 300 μl of the same buffer containing 10 μg/ml PI. After an additional 30 min of incubation on ice, cells were analyzed by flow cytometry. Annexin V binds specifically to PS, a phospholipid that becomes exposed on the surface of cells undergoing apoptosis (28). Apoptotic cells therefore bind FITC-labeled annexin V and, as a result, fluoresce bright green. Dual staining with PI enables the identification of early apoptotic cells that have not yet lost their membrane integrity (25).

DNA Fragmentation. This was determined by the method of Nicoletti et al. (29). Briefly, 50 μl of CLL cells were gently centrifuged and resuspended in 400 μl of a solution containing 0.1% Triton X-100, 0.1% citrate, and 10 μg/ml PI. Cells were incubated on ice for at least 60 min before being analyzed.

Table 1. Effect of 3AB on nucleoside-induced cell membrane disruption in six cases

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Table 2. Effect of 3AB on nucleoside-induced cell shrinkage in six cases

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Fig. 1. Determination of cell viability by PI staining and FSC/SSC. Results are shown from a representative case (case 6). Cells were cultured for 4 days and analyzed as described in “Materials and Methods.” Live cells are denoted by the marker M1 on the PI histograms and by the region R1 on the FSC versus SSC dot plots.
by flow cytometry. During apoptosis, endonuclease activity produces low molecular weight DNA (30) that is lost from the cell after permeabilization. Permeabilized apoptotic cells therefore have a lower residual DNA content and stain less intensely with PI than do live or necrotic cells.

**Morphological Analysis.** Cytocentrifuge preparations of cultured CLL cells were stained with May-Grunwald-Giemsa and examined microscopically.

**PARP-1 Cleavage.** Cultured cells (2.5 × 10^6) were gently washed in PBS and lysed in 100 μl of buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 6 M urea, 5% β-mercaptoethanol, 10% glycerol, and 0.00125% bromphenol blue. Lysates were sonicated for 15 s and heated at 65°C for 15 min before being subjected to SDS-PAGE and Western blotting. Membranes were sequentially reacted with an anti-PARP-1 mouse monoclonal antibody (clone A6.4.12; Insight Biotechnology Ltd., Wembley, Middlesex, United Kingdom) and a peroxidase-conjugated antimouse second layer antibody (Transduction Laboratories, Lexington, KY). The reactive protein bands were visualized using the enhanced chemiluminescence system (Amersham, Buckinghamshire, United Kingdom). During apoptosis, intact Mr 113,000 PARP-1 is cleaved by caspases, producing a characteristic Mr 89,000 COOH-terminal fragment (11, 12).

**Determination of p53 Status**

The p53 status of all 30 cases had been determined previously and will be reported in detail elsewhere. Briefly, each case was tested by Western blotting for radiation-induced up-regulation of p53 and the p53-dependent protein p21^{CIP1/WAF1}. Cases in which such up-regulation was impaired were subsequently examined for p53 gene mutations.

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**RESULTS**

**Effect of 3AB on Nucleoside-induced Cell Membrane Disruption and Cell Shrinkage.** To examine the role of poly(ADP-ribose)lation in the nucleoside-induced killing of CLL cells, six cases with typical disease were incubated with CdA or fludarabine in the presence or absence of the PARP inhibitor 3AB. Cell death was measured as an increase in PI staining (measures cell membrane disruption) or as a reduction in FSC (measures cell shrinkage) (Fig. 1). As expected, CdA and fludarabine induced extensive killing, as detected by both parameters (Tables 1 and 2). 3AB delayed nucleoside-induced membrane disruption in all six cases (Table 1) but inhibited cell shrinkage only in case 5 (Table 2). Because it appeared that 3AB was producing two different effects, case 5 and two of the other cases (cases 2 and 4) were examined in more detail to further define these two effects. To do this, cell death was measured using several additional parameters (Figs. 2 and 3).

Mitochondrial depolarization is an early manifestation of apoptosis that can be detected flow cytometrically as a reduction in staining with DiOC₆, a cell-permeable fluorochrome that is concentrated in polarized mitochondria (26, 27). Exposure of PS is another early apoptotic event that can be detected flow cytometrically as increased staining with annexin V-FITC (28). These events precede membrane disruption in apoptosis and occur synchronously with it in necrosis. In contrast, DNA fragmentation and PARP-1 cleavage are specific manifestations of apoptosis (12, 30).
Effect of 3AB on Other Parameters of Nucleoside-induced Killing. In cases 2 and 4 (Fig. 2), fludarabine-induced cell membrane disruption (increased PI staining) was accompanied by extensive mitochondrial depolarization (decreased DiOC₆ staining) and exposure of PS (increased annexin staining). Importantly, the number of early apoptotic (DiOC₆-dim/PI-dim and annexin-bright/PI-dim) cells as well as late apoptotic/necrotic (PI-bright) cells was increased. Fludarabine also induced extensive DNA fragmentation (decreased PI staining of permeabilized cells) and PARP-1 cleavage, confirming that cell death was occurring by apoptosis. The majority of fludarabine-treated cells had a smeared morphology reflecting cell membrane disruption.

As expected, 3AB produced a marked decrease in the number of late apoptotic/necrotic (PI-bright) cells and a corresponding increase in the number of early apoptotic (DiOC₆-dim/PI-dim and annexin-bright/PI-dim) cells. Importantly, the inhibitor did not increase the proportion of live (DiOC₆-bright/annexin-dim) cells. Furthermore, there was no diminution in the extent of DNA fragmentation or PARP-1 cleavage, confirming that cell death was occurring by apoptosis. The majority of fludarabine-treated cells had a smeared morphology reflecting cell membrane disruption.

Case 5 differed from cases 2 and 4 in two major respects. First, although fludarabine-induced cell membrane disruption was again accompanied by extensive mitochondrial depolarization, exposure of PS, and cell smearing, the extent of DNA fragmentation and PARP-1 cleavage (two hallmarks of apoptosis) did not reflect the extent of killing as determined by the other parameters measured (Fig. 3). This indicates that killing was occurring in part by necrosis. Secondly, 3AB had a pronounced inhibitory effect on all manifestations of fludarabine-induced killing (Fig. 3). Similar results were obtained for Cda (Fig. 4).

To confirm that the observed effects of 3AB resulted from inhibition of poly(ADP-ribosyl)ation rather than inhibition of mono(ADP-ribosyl)ation, we examined the effect on nucleoside-induced killing of two additional PARP inhibitors, 4AN and 2NP. These compounds have IC₅₀ values for PARP of 0.18 and 0.35 μM, respectively, and values for MART of 200 and 83 μM, respectively (24). As expected, 4AN and 2NP reduced the extent of nucleoside-induced killing in case 5 and inhibited the transition from early-stage to late-stage apoptosis in case 2 (Fig. 5). Importantly, both of these inhibitory effects were demonstrable at concentrations of 4AN and 2NP close to their IC₅₀ values for PARP and considerably lower than their IC₅₀ values for MART. These experiments therefore confirm that the inhibitory effects observed in the present study are due to inhibition of poly(ADP-ribosyl)ation rather than inhibition of MART activity.

In summary, our findings therefore indicate that in case 5, nucleoside-induced killing was PARP dependent and partly necrotic, whereas in the other cases, killing was PARP independent and entirely apoptotic.

Frequency of PARP-mediated Killing. To determine the frequency of PARP-mediated killing in CLL, the effect of 3AB on nucleoside cytotoxicity (as measured by the PI/DiOC₆ method) was
examined in 24 additional cases. As expected, 3AB inhibited nucleoside-induced cell membrane disruption (Fig. 6A). However, the inhibitor did not prevent mitochondrial depolarization in any of these additional cases (Fig. 6B). Therefore, poly(ADP-ribosyl)ation made a major contribution to nucleoside-induced killing in only 1 of the 30 cases of CLL studied.

**Relationship between p53 Status and PARP-mediated Nucleoside Cytotoxicity.** We have shown previously that purine analogues kill resting lymphoid cells by both p53-dependent and -independent mechanisms (7, 8). It was therefore of interest to relate the mechanism of nucleoside-induced killing to p53 status. The results are presented in Table 3.

Of the 30 cases used in the present study, 7 had evidence of p53 dysfunction (detected as an impaired p53/p21 response to ionizing radiation). Three of these p53-dysfunctional cases (cases 5, 25, and 27) had a p53 gene mutation, and 1 of these (case 5) also had a deletion. This was the same case that underwent PARP-mediated killing.

**DISCUSSION**

CLL is the commonest adult leukemia, yet it remains incurable (1). Although the purine analogues represent a major therapeutic advance in the disease, their usefulness is limited by drug resistance (2). Because such resistance is likely to reflect defects in nucleoside-induced apoptosis, it is very important to understand the mechanisms involved in this process.

Although p53 gene defects in CLL predict for poor survival and failure to respond clinically to purine analogues (31, 32), recent work from this laboratory (8) and elsewhere (6) has shown that the *in vitro* killing of CLL cells by nucleosides can be p53 independent. Because PARP-mediated NAD<sup>+</sup>/ATP depletion has been implicated in the nucleoside-induced killing of normal resting lymphocytes (9, 15), it seemed important to determine whether or not this enzymatic activity contributed to the cytotoxic action of purine analogues in CLL. To do this, we used 3AB at a concentration known to produce selective inhibition of poly(ADP-ribosyl)ation in intact cells (19) and measured cell death by a number of different methods.

In keeping with its previously reported effects on normal lymphocytes (9), 3AB delayed nucleoside-induced killing (as detected by loss
of vital dye exclusion) in the great majority of CLL cases studied, indicating that PARP activity is indeed involved in such killing. However, when cell death was examined in more detail, it became clear that, in most cases of CLL, the major effect of 3AB was to delay the loss of membrane integrity of cells that had already undergone apoptosis. This indicates that PARP activity was accelerating the transition of cells from the early stage to the late stage of apoptosis (18) but was not contributing to the induction of this process. This observation illustrates the importance of using the appropriate analytical techniques in experiments involving inhibition of cell killing and cells into question the results of previous studies involving PARP inhibition in which cytotoxicity was detected as loss of vital dye exclusion.

Importantly, we identified one case of CLL in which cell death occurred by a mixture of apoptosis and necrosis and in which all manifestations of nucleoside-induced killing were dramatically inhibited by 3AB. Interestingly, this was the only case among the 30 studied in which a biallelic p53 gene defect was detected. This indicates that poly(ADP-ribosyl)ation can occasionally be central to the loss of membrane integrity of cells that had already undergone apoptosis.

It is highly unlikely that the inhibitory effects of 3AB were due to inhibition of MART, given that 3AB inhibits this enzyme with an IC_{50} that is 20-fold higher than the concentration used in the present study (19). However, to exclude this possibility, we used two other PARP inhibitors, 4AN and 2NP. Both compounds produced a pattern of inhibition identical to that of 3AB. The inhibitory effects of 4AN and 2NP were not due to inhibition of MART because they were demonstrable at inhibitor concentrations 1000-fold (4AN) or 200-fold (2NP) lower than the IC_{50} values for this enzyme (24).

It is unclear which specific enzymes are responsible for mediating the PARP-dependent events demonstrated in the present study. However, owing to its relative abundance and known propensity for activation by DNA breaks (11), PARP-1 is likely to be the major enzyme involved in PARP-dependent killing and cell membrane disruption. Nevertheless, the possibility that other PARP enzymes might contribute to either or both of these events cannot be excluded.

PARP activation has been implicated in the cytotoxic action of DNA-damaging agents other than purine analogues. For example, 3AB has been shown to antagonize the cytotoxic effects of free radicals in myeloid leukemia cells (33), neurones (34), and pancreatic islet cells (35). Indeed, PARP-1 knockout mice are highly resistant to diabetes induced by the β-cell-specific genotoxic drug streptozocin (36). In contrast, thymocytes from such animals undergo a normal apoptotic response to γ-irradiation (37).

Therefore, whether or not DNA damage results in PARP-mediated cytotoxicity clearly depends on the cell type and/or the nature of the genotoxic agent. Because PARP-mediated NAD^+/ATP depletion is likely to require sustained PARP activation, this mechanism of killing may be restricted to genotoxic agents that induce persistent rather than transient DNA breaks. Furthermore, PARP-mediated killing is likely to be most prominent in cells in which other cytotoxic DNA damage response pathways (e.g., p53 activation) are blocked.

Taken together with previous work, our findings in CLL cells are consistent with a model of purine analogue cytotoxicity in which unrepaired DNA breaks result in the activation of two potentially cytotoxic pathways: (a) p53-mediated apoptosis; and (b) PARP-mediated NAD^+/ATP depletion. The fact that p53 dysfunction is associated with delayed nucleoside-induced killing (7, 8) suggests that p53-mediated apoptosis is the most rapid cytotoxic pathway activated by purine analogues and that PARP-mediated killing is therefore only likely to occur in cells with p53 dysfunction.

However, we have demonstrated in the present study that PARP activity is not always responsible for the nucleoside-induced killing of CLL cells with p53 dysfunction (detected as an impaired p53/p21 response to ionizing radiation). There are, we believe, two possible explanations for this observation. First, nucleosides might constitute a more potent stimulus for p53 activation than radiation. This seems plausible, given that purine analogues induce the progressive accumulation of DNA breaks (9), whereas radiation-induced DNA breaks are transient (38). Purine analogues might therefore be capable of triggering p53-mediated apoptosis in cases of CLL that are refractory to radiation-induced p53 activation but contain some wild-type p53 protein. Alternatively, nucleosides might activate a third cytotoxic pathway that kills cell more slowly than p53-mediated apoptosis but more quickly than PARP-mediated NAD^+/ATP depletion. PARP-mediated killing would then only occur in cells in which the other two pathways were blocked. Further work is in progress to identify which of these possibilities is correct.

In conclusion, the present study has identified two roles for PARP in the nucleoside-induced killing of CLL cells: (a) as a p53-independent mechanism of cell killing responsible for cytotoxicity in occasional cases; and (b) as a mediator of cell membrane disruption during the late stages of apoptosis.
REFERENCES

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