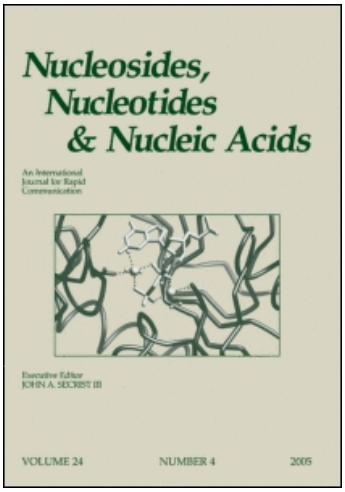

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S. Henrich[a]; S. Gez[a]; B. Crossett[a]; S. P. Mulligan[a]; R. I. Christopherson[a]
[a] School of Molecular and Microbial Biosciences, University of Sydney, New South Wales, Australia

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FLUDARABINE INDUCES DIFFERENTIAL EXPRESSION OF PROTEINS IN HUMAN LEUKEMIA AND LYMPHOMA CELLS

S. Henrich, S. Gez, B. Crossett, S. P. Mulligan, and R. I. Christopherson

School of Molecular and Microbial Biosciences, University of Sydney, New South Wales, Australia

□ *The purine analog fludarabine (FdAMP) is widely used for chemotherapy of B-lymphoid malignancies, and multiple mechanisms of action leading to apoptosis have been proposed. We examined changes at the protein level induced in the Raji cell line (Burkitt's lymphoma) by fludarabine nucleoside (FdA). Raji cells are sensitive to FdA. Raji cells treated with FdA (3 μM, 24 hours), accumulate multiple phosphorylated forms of p53 in the nucleus that in turn degrade to phosphorylated forms of p40. Using CD antibody microarrays to determine surface expression profiles for Raji cells treated with FdA, we found up-regulation of the following CD antigens: CD20, CD54, CD80, CD86, and CD95. FdA thus induces changes in the genetic program of the cells that might be exploited to obtain synergy with therapeutic antibodies.*

Keywords Fludarabine; mechanism; apoptosis; p53; phosphorylation; CD antigens

INTRODUCTION

The purine analogs, cladribine, fludarabine and clofarabine (Figure 1) are useful for chemotherapy of a variety of B-lymphoid malignancies and appear to have different and multiple mechanisms of action.[1,2] As depicted in Scheme 1, fludarabine is normally administered to patients as the nucleoside 5′-monophosphate (FdAMP), is degraded outside the cell by 5′-nucleotidase, and is then transported and phosphorylated within the cell to the triphosphate that is responsible for most of the likely cytotoxic effects of the drug.[1] *FdAMP* is used as a single drug for the treatment of cases of chronic lymphocytic leukemia (CLL).[3]

$$\text{FdAMP} \rightarrow FdA \rightarrow FdAMP \rightarrow FdADP \rightarrow FdATP \qquad (1)$$

Address correspondence to R. I. Christopherson, School of Molecular and Microbial Biosciences, University of Sydney NSW 2006, Australia. E-mail: ric@mmb.usyd.edu.au

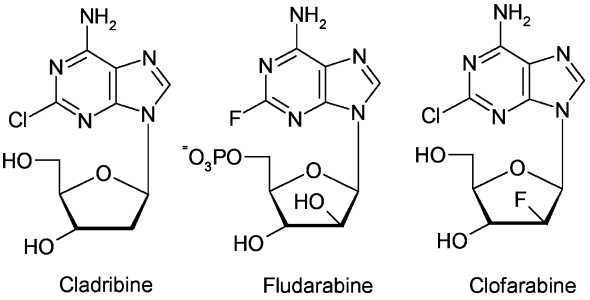

FIGURE 1 Purine analogs used in chemotherapy.

The following mechanisms of action have been proposed for these purine analogs (shown in Figure 1), with different combinations likely to predominate for each drug: stimulation of adenosine receptors, elevation of intracellular cAMP, inhibition of ribonucleotide reductase, inhibition of DNA and RNA polymerases, misincorporation into DNA, inhibition of DNA repair with strand breaks in DNA resulting in activation of protein kinases, accumulation of phosphorylated p53, and activation of a genetic program for cell cycle arrest, or apoptosis. Induction of apoptosis by DNA double strand breaks induced by FdA may occur through p53–dependent or independent mechanisms.[4]

In this paper, we have analyzed nuclear extracts for isoforms of p53 in Raji cells that have been treated with the nucleoside form of fludarabine (FdA) to gain further insight into the mechanisms of action of FdA in leukemia and lymphoma cells. The effects of FdA on CD antigens expressed on the surface of Raji cells have been determined and provide some explanation for the apoptotic effects of FdA and its synergy with therapeutic antibodies.

MATERIALS AND METHODS

Cell Culture and Nuclear Protein Fractionation

Human Raji cells were grown in RPMI 1640 and 10% (v/v) fetal calf serum at 37°C. Cells were incubated with FdA for specified times and concentrations. Nuclei were isolated and nuclear proteins extracted as previously described.[5]

Apoptosis Detection

Raji cells were stained with the Apoptosis Detection Kit I (BD Biosciences, USA) where Annexin V-PE binds to externalised phosphatidylserine on apoptotic cells and 7-AAD stains the DNA in necrotic cells. Assays were performed in triplicate with untreated controls.

Gel Electrophoresis

For two-dimensional polyacrylamide gel electrophoresis (2DE), nuclear protein samples were loaded onto 11 cm pH 4–7 linear gradient IPG gel strips (Bio-Rad, Hercules, USA) and 2DE was carried out as previously described.[5] Protein in nuclear fractions was quantified in triplicate using the two-dimensional Quant Kit (GE Healthcare, USA).

Western Blotting

Following 2DE, Western blotting was performed by established procedures[5] using a monoclonal antibody against p53 (mouse monoclonal IgG_{2a}, 200 μg/mL diluted to 0.8 μg/mL, Santa Cruz Biotechnology Inc., USA), and a secondary IgG-alkaline phosphatase conjugate (final concentration 0.04 μg/mL) detected using AttoPhos (Promega, USA).

Immunophenotyping Cells Using Dotscan Microarrays

Live Raji cells were captured on DotScan CD antibody microarrays (Medsaic Pty Ltd., Australia) as previously described.[6] Briefly, a 300 μL aliquot of the cell suspension (3×10^6 cells) was incubated for 30 minutes on the microarray, then unbound cells were removed by gentle washing. Captured cells were fixed and imaged using a scanner (Medsaic Pty., Ltd.) and dot intensities were quantified for each antigen in duplicate, using data analysis software on an 8-bit pixel greyness scale from 1 to 256 that reflects the level of expression of a particular antigen and the proportion of cells expressing that antigen.[6]

RESULTS

Analysis of Raji cells treated with FdA (3 μM; 0, 24, 48 hours) using the Apoptosis Detection Kit showed an increasing proportion of cells entering apoptosis, indicated by positive staining with Annexin V and negative for 7-AAG (data not shown). At 24 hours, more than 40% of cells are viable with only ~10% remaining viable at 48 hours.

Effects of FdA on p53

Nuclear fractions were analyzed by 2DE and Western blotting to determine the effects of FdA on isoforms of p53 and their levels. As seen in Figure 2, FdA induced a substantial accumulation of nuclear p53 from 24 to 48 hours. Multiple species of p53 are apparent at 24 and 48 hours with pI values decreasing from the unphosphorylated form (pI = 6.4). These isoforms have similar molecular weights. In addition to multiple isoforms of

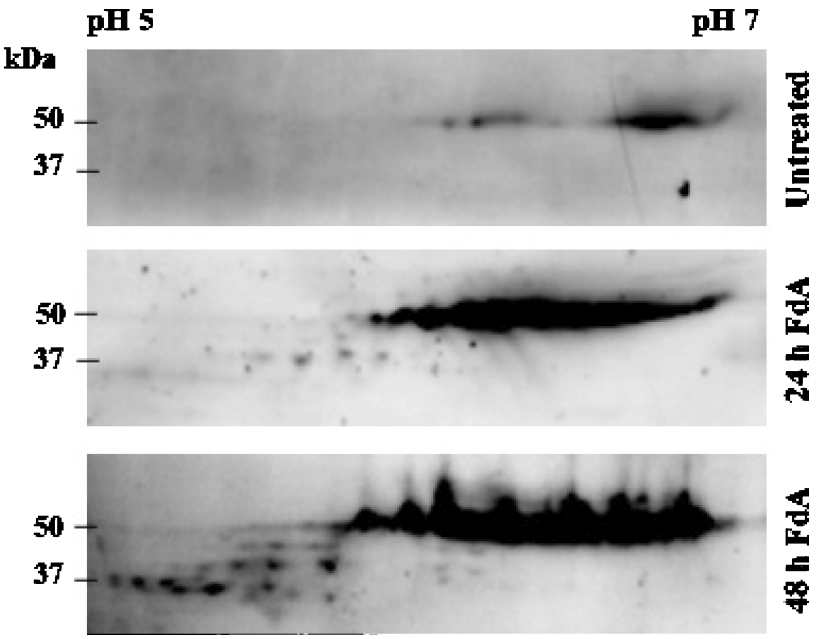

FIGURE 2 Effect of FdA on p53 phosphorylation and accumulation in Raji cells. Cells were treated with FdA (3 μM; 0, 24, 48 hours) and nuclear protein extracts (60 μg) were separated by 2DE. Proteins were then transferred to a PVDF membrane for Western blotting against p53. Further details are provided in Materials and Methods.

p53 with molecular weights of ~53 kDa, there are three series of proteolytic derivatives with lower molecular weights apparent after 48 hours with FdA (Figure 2).

Effects of FdA on Expression of CD Antigens

These major changes in the levels of p53 isoforms and proteolytic derivatives would be expected to change the genetic program of the cells. A change in the pattern of gene expression should be reflected in a change in expression of CD antigens on these cells. To test this proposal, Raji cells were treated with FdA (1 μM, 24 hours) and then analyzed by cell capture on CD antibody microarrays (Figure 3). The results obtained showed up-regulation of CD20, CD54, CD80, CD86, and CD95 when the dot pattern for FdA-treated cells was compared with the untreated control. Thus, FdA induces a significant change in the surface expression profile of Raji cells.

DISCUSSION

The increasingly acidic isoforms of p53 (Figure 2) are consistent with multiple states of phosphorylation of p53 with progressively lower pI values. Induction of apoptosis by FdA appears to occur in a p53-dependent manner,

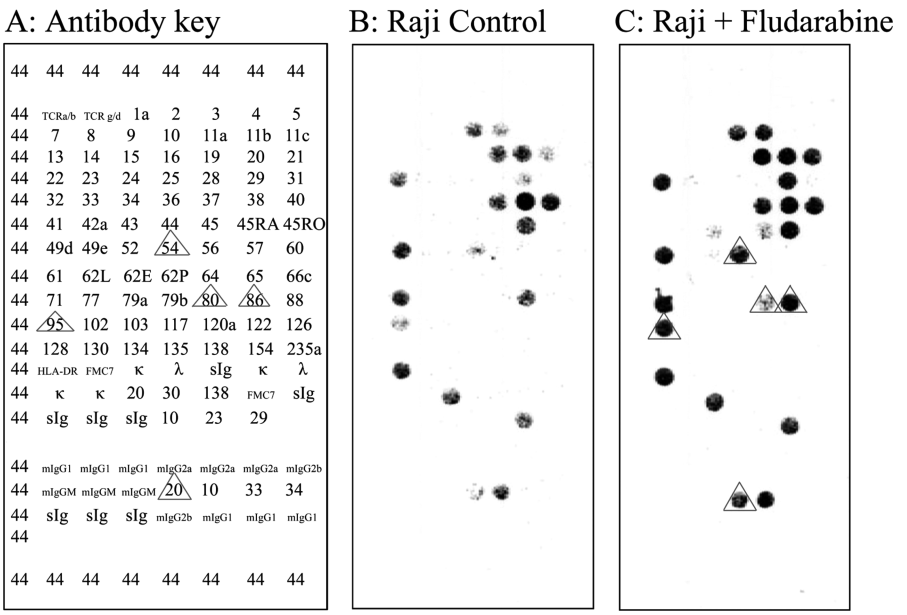

FIGURE 3 Effect of FdA on the immunophenotype of Raji cells. A) Positions of CD antibodies in the DotScan microarray, B) untreated Raji cells, C) Raji cells treated with FdA (1 μM, 24 hours). CD antigens upregulated by FdA are indicated by upward triangles. Details of the analysis are provided in Materials and Methods.

preceded by accumulation of multiple phosphorylated forms of p53. The first stage of this process would be the phosphorylation of FdA (equation 1 above) with incorporation of FdATP into DNA and development of strand breaks that activate ATM and ATR kinases[8] that in turn phosphorylate p53. Accumulated phosphorylated p53 then induces a subset of genes responsible for apoptosis with rapid induction of cell death. By contrast, the cell line MEC1 derived from B-cell CLL, was relatively resistant to FdA, requiring treatment with FdA (100 μM, 24 hours) for similar apoptotic effects (S. Henrich and R.I. Christopherson, unpublished experiments). However, even at this high concentration of FdA, p53 was not phosphorylated and did not accumulate, suggesting that the mechanism of resistance might be related to p53.

After FdA treatment, derivatives of p53 with a lower molecular weight of ~47 kDa and ~40 kDa appeared, and became pronounced after 48 hours (Figure 2). These proteins may be C-terminally cleaved derivatives of p53 (Figure 4), that may contain the same phosphate groups added originally to p53. Fragmentation of p53 following DNA damage in cells has been reported,[9] and the cleavages have been correlated with the type of DNA damage. Okorokov and Milner[10] have shown that these p53 breakdown products are involved in cell cycle arrest and/or induction of apoptosis, especially a nuclear N-terminal 40 kDa breakdown product (p40) of p53.

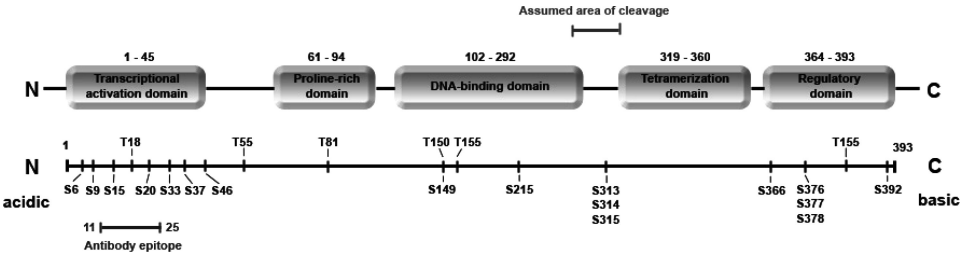

FIGURE 4 The functional domains of human p53. Sites of phosphorylation that have been identified in vitro on serine and threonine residues are shown with the likely region for proteolytic cleavage, and the retained N-terminal epitope (amino acids 11–25) recognized by the p53 monoclonal antibody (modified from Toledo and Wahl[7]).

Sadji-Ouatas et al.[9] proposed that p40 is a novel transactivation factor that may play a significant role in the regulation of multiple p53 signaling pathways and induction of apoptosis.

FdA also induces a 40 kDa breakdown product of p53 that is recognized by an antibody against an epitope from the acidic N-terminus of p53 (Figure 4). The p40 seems to be due to a C-terminal truncation that occurs after p53 phosphorylation, consistent with the p40 reported in other cases of DNA damage.[9] p40 could play a crucial role in the apoptotic response to FdA in lymphocytes. Further information on the role of p40 in induction of apoptosis will help to explain the mechanisms of action of FdA against cancer cells expressing normal p53.

The significant changes induced by FdA in the surface expression profile of Raji cells (Figure 3) would be likely to result from changes in gene expression. There is up-regulation of CD20, CD54, CD80, CD86, and CD95. These effects of FdA should be considered in terms of the functions of these CD antigens. CD20 is a tetraspanin calcium ion channel, CD54 is an intracellular adhesion molecule (ICAM-1), CD80 and CD86 are co-activators of T-cells, and CD95 mediates signaling for apoptosis. CD95 is known to be up-regulated by accumulated phosphorylated p53 and contributes to the consequent apoptotic effects on cells. The up-regulation of a particular epitope of CD20 by FdA is of particular interest because it may explain the reported synergy between FdA and Rituximab (anti-CD20).[11] These effects of FdA on the immunophenotype of cells have been shown to 'translate' to similar effects observed on primary CLL cells obtained from patients (S. Gez and R.I. Christopherson, unpublished experiments).

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