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NUCLEOSIDE ANALOGUES IN LEUKAEMIA

Our group was initially interested in purine metabolism, particularly adenine nucleotide metabolism, and its genetic defects. Thereafter, we expended our investigations on two therapeutic purine nucleoside analogues, 2-chlorodeoxyadenosine and fludarabine, which have revolutionized the treatment of indolent lymphoproliferative disorders. Despite their efficacy, clinical resistance to these drugs is frequently observed. The main objectives of our present studies are to unravel the mechanisms leading to resistance to nucleoside analogues and to find novel therapeutic strategies to counteract them, particularly in chronic lymphocytic leukaemia.

In 1997, a collaborative study of the anti-leukaemic purine nucleoside analogues (PNA), 2-chlorodeoxyadenosine (CdA) and fludarabine (Fig. 1), was started with the Department of Haematology of the University Hospital Saint-Luc. These two deoxyadenosine analogues display remarkable therapeutic properties in indolent lymphoid malignancies including hairy cell leukaemia and chronic lymphocytic leukaemia (CLL). Nevertheless, resistance is also observed, and PNA do not confer a survival advantage when compared to more conventional therapies such as alkylating agents. The aims of our studies are to understand the mechanisms that lead to resistance to nucleoside analogues, and to improve their therapeutic efficacy by searching for synergisms with other compounds.

CdA and fludarabine are prodrugs. To exert their antileukaemic effect, they have to be phosphorylated by deoxycytidine kinase (dCK) into nucleoside analogue monophosphates, followed by conversion into di- and triphosphate derivatives. The latter are the active metabolites. They have been shown to inhibit various processes involved in DNA and RNA synthesis. Moreover, they can be incorporated into newly synthesised DNA, causing chain termination. Together, these actions result in arrest of DNA synthesis (replication and repair) and in the progressive accumulation of DNA strand breaks, leading to apoptosis by mechanisms which are not yet entirely clear (1, for a review).

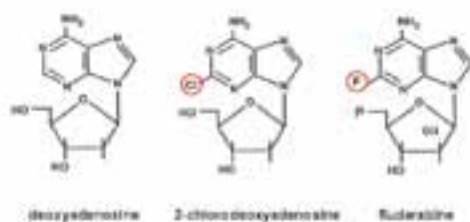


Fig. 1. Structures of deoxyadenosine and purine nucleoside analogues

MECHANISMS OF ACTION

F. Bontemps, C. Smal, L. Bastin-Coyette, E. de Viron, A. Arts, E. Van Den Neste

To improve our understanding of the mechanisms by which CdA induces apoptosis in CLL cells, we study its effects in EHEB cells, a continuous cell line derived from a patient with CLL. The EHEB cell line was found to be less sensitive (~10 fold) to CdA than other human lymphoblastic cell lines. This could be partly explained by a lower intracellular accumulation of CdATP, the active metabolite of CdA, due to a reduced dCK activity. In addition, analysis of the cell cycle showed that CdA accelerated the progression from G1 to S phase before inducing cell death (2). This cell response was unexpected because PNA are known to induce accumulation of p53, which typically results in the accumulation of its target p21, inhibition of cyclin-dependent kinase 2 (Cdk2) and G1/S phase arrest. This paradoxical result led us to examine the effects of CdA on the p53-p21 axis in this cell line. We clearly demonstrated that CdA, but also fludarabine and pyrimidine analogues induced p21 depletion, while p53 was upregulated (3). This p21 depletion resulted from an increased proteasomal degradation, which had already been reported after UV-irradiation, but never after nucleoside analogue treatment. In addition, we found that p21 depletion was associated with Cdk2 activation, which could explain the activation of the cell cycle by CdA in this cell line, and by

PCNA monoubiquitination, which promotes translesion DNA synthesis and favors DNA repair and cell survival. Further work is needed to determine whether PCNA monoubiquitination could play a role in the clinical resistance to PNA.

In parallel studies, we have analysed the mechanisms of cell death induced by 2-chloroadenosine (2-CAdo) in various types of leukaemic cells. This analogue is not activated by dCK, like CdA, but by adenosine kinase. We found that 2-CAdo was efficiently converted into 2-chloroATP, resulting in ATP depletion, as well as in inhibition of DNA, RNA and protein synthesis. 2-CAdo also caused activation of the intrinsic pathway of apoptosis, which was p53-independent and associated with a decline in the antiapoptotic protein Mcl-1 protein. Prevention of ATP loss by inhibition of AMP deaminase reduced apoptosis, indicating that ATP depletion plays a role in 2-CAdo-induced apoptosis (4).

More recently, we have initiated microarray analyses to identify survival or death pathways that are activated in response to CdA and fludarabine. We intended to compare genes induced or repressed by these PNA in sensitive and refractory CLL patients. We found that, in chemosensitive samples, PNA predominantly increased the expression of p53-dependent genes, among which PLK2 (polo-like kinase 2) was the most highly activated at early time points. Conversely, in chemoresistant samples, p53-dependent and PLK2 responses were abo-

lished. Using qPCR, we confirmed that PNA dose- and time-dependently increased PLK2 expression in chemosensitive but not chemoresistant CLL samples. Analysis of a larger cohort of CLL patients showed that cytotoxicity induced by PNA correlated well with PLK2 mRNA induction. In conclusion, we propose that testing PLK2 activation after a 24-h incubation with PNA could be used to investigate the functional integrity of DNA-damage response pathways in CLL cells, and predict clinical sensitivity to these drugs (5). This study was performed in collaboration with Dr L. Knoops from the Ludwig Institute for Cancer Research (Brussels). The following step was to investigate the role of PLK2 during PNA-induced apoptosis. However, PLK2 could not be detected at the protein level in CLL cells, precluding a role of this kinase in PNA-induced apoptosis. We are currently investigating whether PLK2 expression could be regulated by microRNAs. This study is performed in collaboration with Dr G. Bommer (de Duve Institute).

SEARCH FOR POTENTIATION OF ANTILEUKAEMIC EFFECT OF 2-CHLORODEOXYADENOSINE

E. Van Den Neste, C. Smal, F. Bontemps

Some years ago, we have shown that combination of CdA with DNA-damaging agents, such as cyclophosphamide (CP) derivatives (6) or UV-light, resulted in synergistic cytotoxicity in CLL lymphocytes, due to inhibition of DNA repair. The *in vitro* synergy between CdA and CP derivatives has provided the rationale for a clinical trial of this combination, which gives encouraging results.

Lately, we have explored the possibility that CdA interacts with the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway. We have shown that CdA, at concentrations close to the IC₅₀, activated the ERK pathway in EHEB cells. Because activation of this pathway is assumed to exert anti-apoptotic effect, we combined CdA

with inhibitors of the ERK pathway. The latter were found to enhance CdA-induced apoptosis. These results suggest that the efficacy of CdA could be strengthened by combination with inhibitors of the ERK pathway (7).

REGULATION OF DEOXYCYTIDINE KINASE ACTIVITY

C. Smal, R. Amsailale, A. Arts, E. Van Den Neste, F. Bontemps

Deoxycytidine kinase (dCK) activates numerous nucleoside analogues used in anti-cancer and antiviral therapy. Studies of the mechanism(s) that control the activity of this enzyme are thus of particular interest. As literature data suggested that CdA could induce dCK activation through reversible phosphorylation, we decided to investigate this hypothesis. We overexpressed dCK in HEK 293T cells and observed that the enzyme was labelled after incubation with [³²P]orthophosphate, confirming that dCK is a phosphoprotein. Tandem mass spectrometry performed by Dr D. Ver-tommen and Prof. M.H. Rider from the Horm-Phos unit (de Duve Institute) allowed the identification of four *in vivo* phosphorylation sites, Thr-3, Ser-11, Ser-15 and Ser-74 (Fig. 2). Site-directed mutagenesis demonstrated that Ser-74 phosphorylation was crucial for dCK activity in HEK 293T cells, whereas phosphorylation of other identified sites did not seem essential (8). Phosphorylation of Ser-74 was also observed on endogenous dCK in CCRF-CEM cells and in lymphocytes from CLL patients, in which this phosphorylation was increased by several genotoxic agents (CdA, UV, etoposide...) that enhanced dCK activity, and decreased by sorbitol that diminished dCK activity. Moreover, interindividual variability in dCK activity in CLL lymphocytes could be related to its phosphorylation level on Ser-74 (9). To conclude, our work has demonstrated that dCK activity in leukaemic cells largely depends on the phosphorylation state of Ser-74.

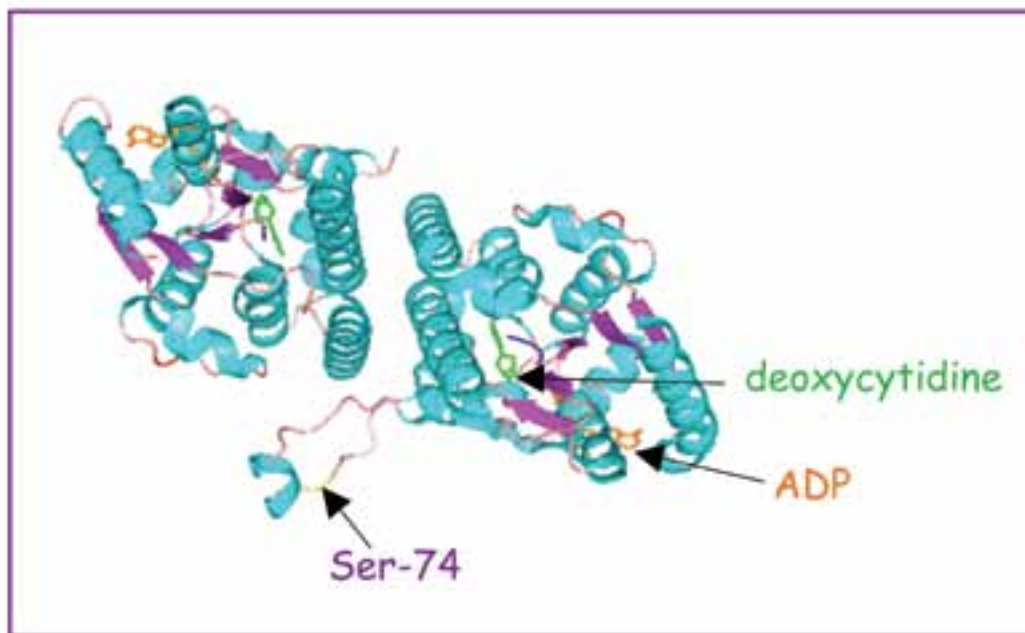


Fig. 2. Three-dimensional structure of deoxycytidine kinase in complex with ADP and deoxycytidine. The Ser-74 phosphorylation site is located at a very flexible 15-residue insert (Ser-63-Asn-77) easily accessible to cellular protein kinases. The N-terminal extremity of dCK (residues 1-19) was flexible and could not be solved. Therefore, the Thr-3, Ser-11 and Ser-15 phosphorylation sites cannot be exactly located.

We are now attempting to identify the protein kinase(s) and the protein phosphatase(s) that control Ser-74 phosphorylation and the signalling pathways that lead to dCK activation following treatment with DNA damaging agents. We showed that casein kinase 1 d (CKI d) could phosphorylate dCK on Ser-74 and increase its activity (10). However, neither CKI d inhibitors nor CKI d siRNA-mediated knock-down modified Ser-74 phosphorylation or dCK activity in cultured cells, precluding a role of CKI d in the regulation of dCK activity *in vivo*. Besides searching for the signalling pathways that control dCK activity, we are investigating whether increase of Ser-74 phosphorylation could enhance efficacy of nucleoside analogues in various types of cancer cells.

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