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**New insights into the regulation of deoxycytidine kinase
activity *via* Ser-74 phosphorylation: toward improved
activation of anticancer nucleoside analogs**

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Abbreviations

A

ADP:	adenosine diphosphate
AI:	autoinhibitory sequence
ANP32a:	Acidic Nuclear Phosphoprotein 32a
APAF-1:	apoptotic protease activating factor-1
Ara-C:	1- β -D-arabinofuranosylcytosine or cytarabine
Ara-CTP:	1- β -D-arabinofuranosylcytosine triphosphate
ATM:	ataxia telangiectasia mutated
ATP:	adenosine triphosphate
ATR:	ATM-and Rad3-related
AZT:	3'-azido-2', 3'-dideoxythymidine or zidovudine

B

BAX:	Bcl-2-associated X protein
BAK:	Bcl-2 antagonist or killer
BBH:	CNB-binding helical domain
BCL-2:	B cell lymphoma 2

C

CAFdA:	2-chloro-2'-arabino-fluorodeoxyadenosine
CAFdATP:	2-chloro-2'-arabino-fluorodeoxyadenosine triphosphate
CaM:	calmodulin
CBD:	calcium-calmodulin-binding motif
CdA:	2-chloro-2'-deoxyadenosine or cladribine
CdATP:	2-chloro-2'-deoxyadenosine triphosphate
Cdk1:	cyclin-dependent kinase 1
Cdk2:	cyclin-dependent kinase 2
cDNA:	complementary DNA
Chk1:	checkpoint kinase-1
Chk2:	checkpoint kinase-2
CIP2A:	cancerous Inhibitor of PP2A
CLL:	chronic lymphocytic leukemia
CNA:	calcineurin A
CNB:	calcineurin B
CNT(s):	concentrative nucleoside transporter(s)
CTP:	cytidine triphosphate

D

dAdo:	deoxyadenosine
dADP:	deoxyadenosine diphosphate
dATP:	deoxyadenosine triphosphate
dCDP:	deoxycytidine diphosphate
dCK:	deoxycytidine kinase
dCMP:	deoxycytidine monophosphate
dCTP:	deoxycytidine triphosphate
dCyd:	deoxycytidine
DDR:	DNA damage response
dFdC:	2',2'-difluorodeoxycytidine or gemcitabine
dGDP:	deoxyguanosine diphosphate
dGK:	deoxyguanosine kinase
dGMP:	deoxyguanosine monophosphate
dGTP:	deoxyguanosine triphosphate
dGuo:	deoxyguanosine
dN(s):	deoxyribonucleoside(s)
DNA:	deoxyribonucleic acid
DNA-PK:	DNA-dependent protein kinase
dNDP(s):	deoxyribonucleoside diphosphate(s)
dNK(s):	deoxyribonucleoside kinase(s)
dNMP(s):	deoxynucleoside monophosphate(s)
dNTP(s):	deoxyribonucleosides triphosphate(s)
DR:	death receptor
DSB:	double-strand break
dTMP:	thymidine monophosphate
dTTP:	thymidine triphosphate

E

E:	enzyme
<i>E. coli</i> :	<i>Escherichia coli</i>
E2F:	eucaryotic transcription factor 2
EMA:	European Medicines Agency
ENT(s):	equilibrative nucleoside transporter(s)
ERK:	extracellular signal-regulated kinase

F/G

FADD:	FAS-associated death domain protein
F-Ara-A:	2-fluoro-9- β -D-arabinofuranosyladenine or fludarabine
F-Ara-ATP:	9- β -D-arabinofuranosyl-2-fluoroadenine triphosphate
FCP:	TFIIF-associating component of RNA polymerase II CTD phosphatase

FDA:	Food and Drug Administration
GFP:	Green fluorescent protein
GTP:	guanosine triphosphate

H

HEAT:	Huntingtin/elongation/A-subunit/TOR
HEK293T:	human embryonic kidney 293T
HIV:	human immunodeficiency virus
Hsp90:	heat shock protein 90

I/J/K/L

JAK:	janus kinase
K_{cat} :	turnover rate constant
kDa:	kiloDalton
K_i :	constant of inhibition
K_m :	Michaëlis-Menten constant
LCMT1:	leucine carboxyl methyltransferase 1

M

MAPK:	MAP kinase
Mcl-1:	myeloid cell leukemia 1
MOMP:	mitochondrial outer membrane permeabilization
mRNA:	messenger RNA
MRP:	multidrug resistant protein

N/O

5'-NT:	5'-nucleotidase
NA(s):	nucleoside analog(s)
NDP(s):	ribonucleoside diphosphate(s)
NDPK(s):	nucleoside diphosphate kinase(s)
NER:	nucleotide excision repair
NLS:	nuclear localization signal
NMPK(s):	nucleoside monophosphate kinase(s)
NT:	nucleoside-specific membrane transporter
OA:	okadaic acid

P

^{32}P :	$[^{32}\text{P}]$ orthophosphate
pI:	isoelectric point

PK:	protein kinase
PKA:	cyclic AMP-dependent protein kinase
PKC:	protein kinase C
PKR:	protein kinase RNA-activated
PNP:	purine nucleoside phosphorylase
PP:	protein phosphatase
λ -PP:	λ protein phosphatase
PP1:	protein phosphatase 1
PP2A:	protein phosphatase 2A
PP2Ac:	protein phosphatase 2A catalytic subunit
PP2A _D :	protein phosphatase 2A heterodimer
PP2A _T :	protein phosphatase 2A heterotrimer
PP2B:	protein phosphatase 2B
PP2C:	protein phosphatase 2C
PP4:	protein phosphatase 4
PP4c:	protein phosphatase 4 catalytic subunit
PP5:	protein phosphatase 5
PP6:	protein phosphatase 6
PP6c:	protein phosphatase 6 catalytic subunit
PP7:	protein phosphatase 7
PPEF:	protein phosphatase with EF-hand
PPM:	metal-dependent protein phosphatase
PPP:	phosphoprotein phosphatase
pRb:	retinoblastoma tumor suppressor protein
PSK:	protein serine/threonine kinase
PSP:	protein serine/threonine phosphatase
PTK:	protein tyrosine kinase
PTP:	protein tyrosine phosphatase

Q/R

R :	regulatory
RNA:	ribonucleic acid
RR:	ribonucleotide reductase

S/T

SAP:	Sit4-associated protein
SCP:	small CTD phosphatase
Ser-74:	Serine 74
SET:	Su(var) 3-9/Enhancer of zeste/Trithorax
Sp1:	specificity protein 1
SSB:	single-strand break
Thd:	thymidine

TK:	thymidine kinase
TK1:	cytosolic thymidine kinase
TK2:	mitochondrial thymidine kinase
TPR:	tetratricopeptide repeat
TS:	thymidylate synthase

U

UDP:	uridine diphosphate
USF:	upstream stimulatory factor
UTP:	uridine triphosphate
UV:	ultraviolet

V/W/X/Y/Z

V _{max} :	maximal velocity
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Summary

Deoxycytidine kinase (dCK) catalyzes the phosphorylation of deoxycytidine, deoxyadenosine and deoxyguanosine, which constitutes the first and rate-limiting step in the synthesis of DNA precursors by the deoxynucleoside salvage pathway. In addition, dCK phosphorylates and activates numerous chemotherapeutic nucleoside analogs. As low dCK activity is often associated with reduced nucleoside analog efficacy, it is of particular interest to gain more insights into the mechanisms that regulate its activity. Prior to this thesis project, research conducted in our laboratory had demonstrated that dCK activity is regulated by post-translational modification, and more precisely that its activity can be increased *in vivo* through Ser-74 phosphorylation, a finding that could be exploited to improve therapeutic efficacy of nucleoside analogs.

The first objective of this thesis was to investigate the impact of Ser-74 phosphorylation on nucleoside analog activation and efficacy in cancer cells. We started by a kinetic study using recombinant dCK in which Ser-74 phosphorylation was mimicked by a S74E mutation. Although it was well demonstrated that S74E mutation increases dCK activity toward deoxycytidine and pyrimidine analogs, such as gemcitabine, its effect toward purine analogs had not yet been investigated. We found that S74E mutation enhanced the catalytic activity (K_{cat}) of dCK toward cladribine and clofarabine, but not fludarabine, with ATP or UTP as phosphoryl donor. However, K_m values were also increased so that the catalytic efficiencies (K_{cat}/K_m) of dCK for cladribine and clofarabine were not, or only slightly increased, and even decreased for fludarabine. In addition, increase of endogenous dCK activity, which was easily detected with deoxycytidine or gemcitabine as substrates after *in vivo*-induced increase of Ser-74 phosphorylation, was not observed with deoxyadenosine or purine analogs. Accordingly, treatment of CLL (chronic lymphocytic leukemia) cells with aphidicolin, which induces dCK activation through Ser-74 phosphorylation, did not enhance the conversion of cladribine or fludarabine into their active triphosphate form. Nevertheless, the same treatment induced higher activation of gemcitabine in CLL as well as in colon cancer cells and produced synergic cytotoxicity. From this study, it was concluded that increasing phosphorylation of dCK on Ser-74 might constitute a valuable strategy to enhance the clinical efficacy of particular nucleoside analogs, like gemcitabine.

The second objective of my PhD work was to identify the protein phosphatase (PP) responsible for Ser-74 dephosphorylation. Studies in intact cells with cell-permeable PP inhibitors suggested that this PP could be the phosphoprotein phosphatase 2A (PP2A). Investigation of the dephosphorylation of dCK by purified PP as well as experiments performed in whole or PP2A-immunodepleted cell lysates confirmed this hypothesis. Use of siRNA allowed definitively concluding that PP2A constitutively dephosphorylates dCK at Ser-74 and negatively regulates dCK activity *in vivo*. This identification adds new information about the signaling pathways that control dCK activity and provides a new potential target for combination therapy.

Foreword

Nucleoside analogs (NAs) are chemotherapeutic agents widely used in the treatment of cancer, and particularly in hematological malignancies. However, their clinical use is limited by primary and acquired resistance. Interest in deoxycytidine kinase (dCK) comes from the key role of this enzyme in the activation and efficacy of NAs, and hence from the hypothesis that increasing its activity could reinforce their therapeutic action and counteract resistance.

This manuscript starts by introducing NAs used in our study, and by explaining their mechanisms of action and potential causes of resistance. Next comes a review of dCK, its main characteristics and the mechanisms that control its activity, focusing on the phosphorylation of the Ser-74 residue. The third section concerns phosphoprotein phosphatases because of our interest for their role in the control of Ser-74 phosphorylation and dCK activity. Objectives of the thesis close the first part.

The second part of the manuscript presents personal work under the form of two publications. The first one investigates the consequences of Ser-74 phosphorylation on the kinetic properties of dCK and the activation of NAs in cancer cells. The second is devoted to the identification of the protein phosphatase that dephosphorylates Ser-74.

The last part of the manuscript is a general discussion that points out the different perspectives coming out from this work.

In the Annex Section, a paper on the role of casein kinase I δ in the control of dCK activity, which was not directly related to the initial objectives of the thesis, but to which I contributed, is presented.

CHAPTER I:

General introduction and research objectives

1. Nucleoside analogs

1.1. Overview

Nucleoside analogs (NAs) are synthetic molecules developed to mimic natural deoxynucleosides (dNs). Belonging to the class of antimetabolites, these compounds are broadly used in the treatment of hematological malignancies, solid tumors and viral infections. NAs are prodrugs that require intracellular activation to exert their therapeutic action. The active form of NAs is most often their triphosphate derivative that acts on different cellular targets, leading to the inhibition of nucleic acid synthesis, induction of DNA damage response and apoptosis (Jordheim *et al.*, 2005; Van Den Neste *et al.*, 2005; Jordheim *et al.*, 2007; Ewald *et al.*, 2008b; Parker, 2009; Robak *et al.*, 2009; Jordheim *et al.*, 2013).

There are currently nine Food and Drug Administration (FDA)- and European Medicines Agency (EMA)- approved cytotoxic anticancer NAs (Jordheim *et al.*, 2013). In this thesis, we will focus on five anticancer NAs that are activated by deoxycytidine kinase (dCK): fludarabine (F-Ara-A), cladribine (CdA) and clofarabine (CAFdA), which are purine analogs, and cytarabine (Ara-C) and gemcitabine (dFdC), which are pyrimidine derivatives. Although the mechanisms of action of these NAs are quite similar, some differences exist in their metabolism and interactions with target enzymes, which give unique properties to each of them and results in unique clinical activity (Parker, 2009).

Despite great success of NAs in cancer treatment, and mainly in leukemia, a major obstacle to successful treatment is the development of resistance. Understanding the mechanisms underlying resistance to NAs is essential to develop new therapeutic strategies to overcome them (Bergman *et al.*, 2002; Galmarini *et al.*, 2002; Mini *et al.*, 2006; Jordheim *et al.*, 2007).

1.2. Structure and clinical use

The three purine NAs investigated (fludarabine, cladribine and clofarabine), are derived from 2'-deoxyadenosine, while the pyrimidine NAs (cytarabine and gemcitabine) are derived from 2'-deoxycytidine. Their chemical structures are shown in Figures 1 and 2.

Fludarabine (9- β -D-arabinosyl-2-fluoroadenine-5'-phosphate, F-Ara-AMP) is an arabinosyl compound with a fluorine atom at the 2-position of the adenine ring and a phosphate group at the 5'-position of the sugar, added to improve its solubility (Danhauser *et al.*, 1986; Parker, 2009; Lee *et al.*, 2013). Because of its structure, fludarabine is protected from the action of catabolizing enzymes, which extends its half-life (Montgomery *et al.*, 1969). Indeed, the presence of a fluorine atom at the 2-position of the adenine ring prevents fludarabine from catabolism by adenosine deaminase. Moreover, the presence of a hydroxyl group (β instead of α) at the 2'-position of the sugar reduces cleavage of the glycosidic bond by bacterial purine nucleoside phosphorylase (PNP), which increases its oral bioavailability (Huang *et al.*, 1987; Pugmire *et al.*, 2002). Fludarabine is mainly used in the treatment of chronic lymphocytic leukemia (CLL) in monotherapy or in combination with alkylating agents, like cyclophosphamide, and monoclonal antibodies, like rituximab. It is also indicated for low-grade non-Hodgkin's lymphomas and acute myeloid leukemia (Ricci *et al.*, 2009; Badoux *et al.*, 2011).

Cladribine (2-chloro-2'-deoxyadenosine, CdA) differs from 2'-deoxyadenosine only by a chlorine atom at the 2-position of the purine ring. Due to the presence of this chlorine atom, CdA is, like fludarabine, resistant to deamination by adenosine deaminase. However, CdA has a poor oral bioavailability because it is not resistant to degradation by bacterial PNP (Lotfi *et al.*, 2003). CdA is the treatment of choice for hairy cell leukaemia, but is also active in CLL, low-grade non-Hodgkin's lymphomas, acute myeloid leukemia and Waldenström macroglobulinemia (Hellmann *et al.*, 1999; Johnson *et al.*, 2002; Evens *et al.*, 2004). In addition, this analog has been considered for the treatment of multiple sclerosis (Leist *et al.*, 2011).

Clofarabine (2-chloro-9-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-adenine, CAFdA) distinguishes from CdA by the presence of a fluorine atom, in the arabino configuration, at the 2'-position of the sugar. This NA combines the most advantageous pharmacokinetic properties of fludarabine and cladribine (Lindemalm *et al.*, 2004; Bonate *et al.*, 2006). Like CdA, CAFdA is resistant to the action of adenosine deaminase and like fludarabine, it is resistant to cleavage by bacterial PNP, which increases the stability and oral bioavailability of the drug (Montgomery *et al.*, 1992). CAFdA shows good efficacy in the treatment of either pediatric or adult lymphoid and myeloid leukemias (Parker, 2009; Lee *et al.*, 2013).

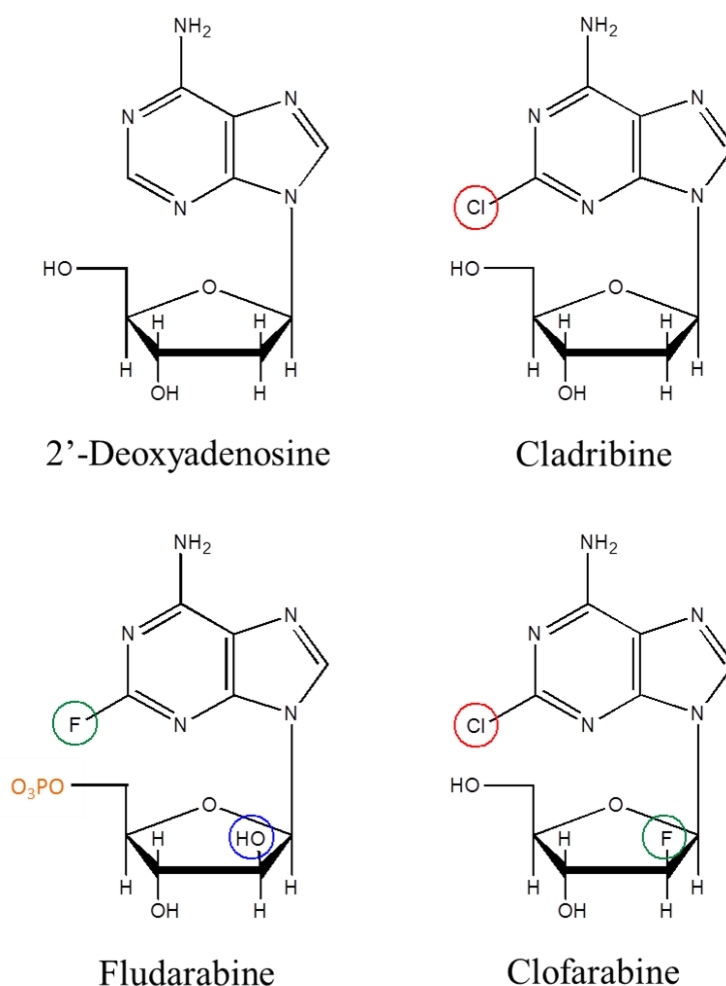


Figure 1: Structures of deoxyadenosine and deoxyadenosine analogs.

Cytarabine (1- β -D-arabinofuranosylcytosine, Ara-C) differs from deoxycytidine by the presence of a hydroxyl group, which is in the arabinose configuration, at the 2'-position of the sugar moiety. Ara-C is the cornerstone treatment of acute myeloid leukemia (Johnson, 2001; Jordheim *et al.*, 2005), but can also be used in acute lymphocytic leukemia and non-Hodgkin's lymphomas (Jordheim *et al.*, 2013).

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a deoxycytidine analog with two fluorine atoms at the 2'-position of the pentose. Unlike Ara-C, dFdC is active on solid tumor cells. It is used in the treatment of non-small lung cancer, pancreatic cancer, bladder cancer and breast cancer (Milano *et al.*, 2002; Jordheim *et al.*, 2005; Jordheim *et al.*, 2013).

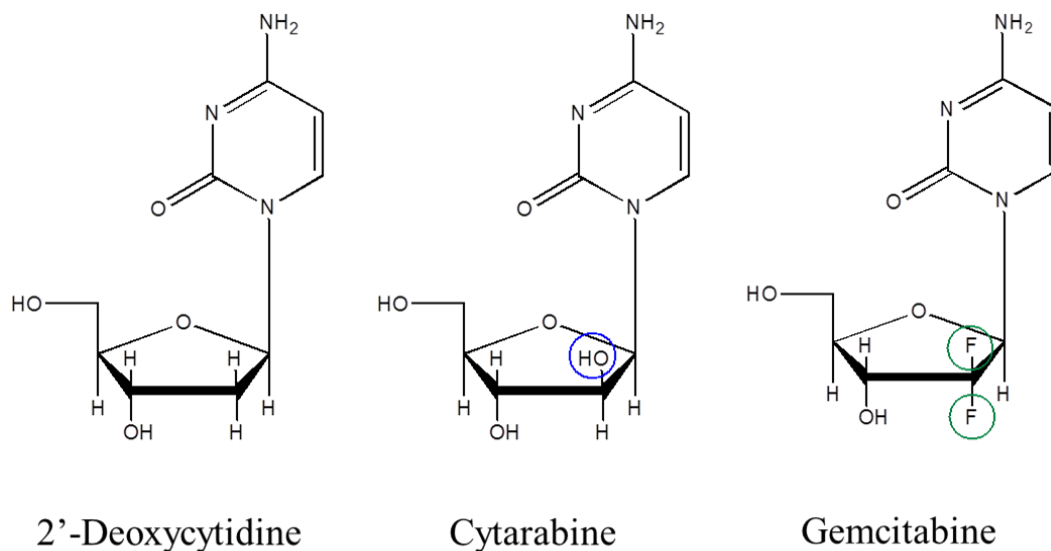


Figure 2: Structures of deoxycytidine and deoxycytidine analogs

1.3. Metabolism

Metabolism of nucleoside analogs depicted above is very similar to that of the natural nucleosides from which they derive. NAs enter the cells *via* nucleoside-specific transporters and are then converted into their active form by the successive actions of dCK and deoxynucleotide kinases (Figure 3).

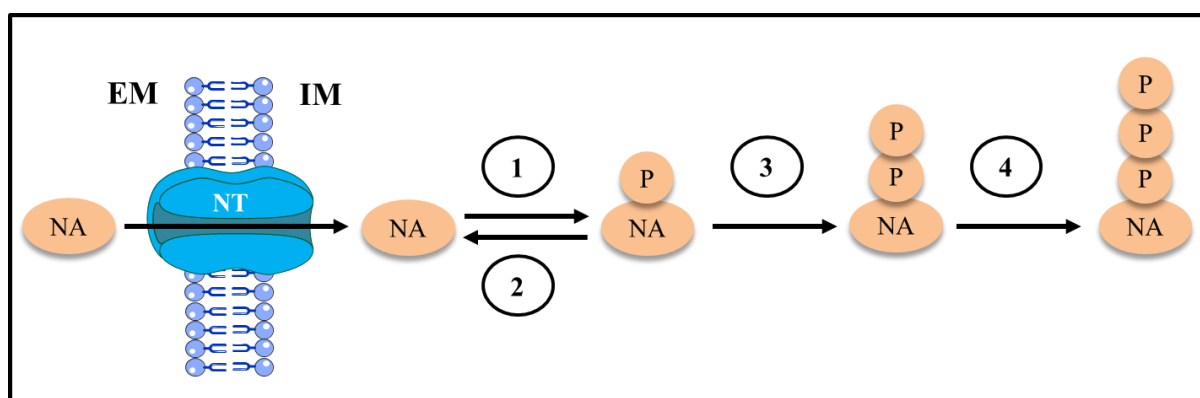


Figure 3: Metabolism of nucleoside analogs

1: deoxycytidine kinase; 2: 5'-nucleotidase; 3: nucleoside monophosphate kinase; 4: nucleoside diphosphate kinase; NA: nucleoside analog; NT: nucleoside transporter; EM: extracellular medium, IM: intracellular medium; P: phosphate group.

1.3.1. Transport

NAs, like natural nucleosides, are hydrophilic molecules that show low or negligible permeability across cell membranes. They are thus translocated via specific membrane proteins: the nucleoside transporters (NTs). Several NTs have been identified and can be divided into two categories: the equilibrative nucleoside transporters (ENTs) and the concentrative nucleoside transporters (CNTs) (Damaraju *et al.*, 2003; Baldwin *et al.*, 2004; Kong *et al.*, 2004; Pastor-Anglada *et al.*, 2004; Jordheim *et al.*, 2007; Leung *et al.*, 2007). Both types of NTs only accept dephosphorylated forms of nucleosides, so that fludarabine, which, unlike other NAs, is administered as a monophosphate compound, enters the cells only after its dephosphorylation by serum phosphatases and the membrane-bound ecto-5'-nucleotidase (CD73) (Mackey *et al.*, 2005).

NTs are widely distributed, from the plasma membrane to membrane of organelles such as mitochondria, and their distribution differs depending on the tissue (Leung *et al.*, 2007). ENTs mediate facilitated nucleoside transport across the plasma membrane according to the concentration gradient until equilibrium is reached. They are characterized by low affinity for their substrates and by low specificity, accepting both purine and pyrimidine substrates. In contrast, CNTs mediate concentrative high-affinity nucleoside influx coupled to the trans-membrane sodium gradient. Also, CNTs show distinct specificities for purine and pyrimidine nucleosides. In human, four ENTs (hENT1 to 4) and three CNTs (hCNT1 to 3) have been described and characterized in terms of substrate specificity and sensitivity to inhibitors (Jordheim *et al.*, 2007). F-Ara-A, CdA and CAFdA are translocated via hENT1, hENT2, hCNT2 and hCNT3, whereas Ara-C and dFdC are transported via hENT1, hENT2, and hCNT1. In addition, dFdC can be translocated by hCNT3. NTs are the first actors of the metabolism of NAs and may thus contribute to the cytotoxicity of these drugs.

1.3.2. Conversion into the active form

Once inside the cell, several NAs are converted by dCK into their monophosphate form, which is trapped within the cell due to its negative charge. Although dCK is the primary enzyme involved in this first phosphorylation reaction, other deoxynucleosides kinases (see more details in section 2.1.2.) can also play a certain role: the mitochondrial deoxyguanosine

kinase for the activation of purine analogs and the mitochondrial thymidine kinase for the activation of pyrimidine analogs (Munch-Petersen *et al.*, 2007).

Following phosphorylation by dCK, the monophosphate form of NA is converted to its triphosphate form by the successive action of nucleoside monophosphate (NMPK) and diphosphate kinases (NDPK). The reactions catalyzed by NMPKs and NDPKs are reversible. Among human NMPKs, adenylate kinase and UMP-CMP kinase seem to be important for purine and pyrimidine NAs activation, respectively (Van Rompay *et al.*, 2000). As for NDPKs, which are eight in humans (from A to H) (Seifert *et al.*, 2005), only the forms A and B would be implicated in the activation of NAs (Krishnan *et al.*, 2002). Although dCK activity is rate-limiting for the accumulation of triphosphate derivatives of F-Ara-A, Ara-C and dFdC, this is not the case for CdA and CAFdA, whose accumulation of triphosphate form is additionally determined by the rate of conversion of monophosphate into diphosphate form by adenylate kinase.

Intracellular accumulation of the active triphosphate form of NAs is limited by the activity of cytosolic 5'-nucleotidases (5'-NT) that dephosphorylate the monophosphate form of NAs. So, a high ratio of dCK to 5'-NT, like in lymphocytes, will favor activation of NAs. Conversely, high expression of the cytosolic 5'-NT has been negatively correlated with NA efficacy (Jordheim *et al.*, 2007). In addition to 5'-NT, deaminases, especially cytidine deaminase and dCMP deaminase, can reduce the activation of pyrimidine analogs by converting them or their monophosphate form to uridine derivatives (Bianchi *et al.*, 2003; Galmarini *et al.*, 2003a; Hunsucker *et al.*, 2005). Moreover, it has been described that Ara-C and dFdC could be removed from cell by ATP-dependent efflux pumps such as multidrug resistant protein (MRPs) 5 and 7 (Chen *et al.*, 2011). Therefore, the overall activation of NAs will depend on the balance between activating and inactivating enzymes (Bianchi *et al.*, 2003).

1.4. Mechanisms of action

Once activated, NAs are able to interact with diverse intracellular proteins, particularly enzymes that are implicated in the metabolism of natural nucleosides and/or in the synthesis of nucleic acids. Consequences of these interactions will vary depending on the NA, due to

different affinities for their targets. NAs mainly interfere with DNA synthesis, which leads to DNA damage (Figure 4) and eventually induction of apoptosis (Jordheim *et al.*, 2005). In addition, some of them inhibit RNA synthesis, which contributes to their cytotoxicity.

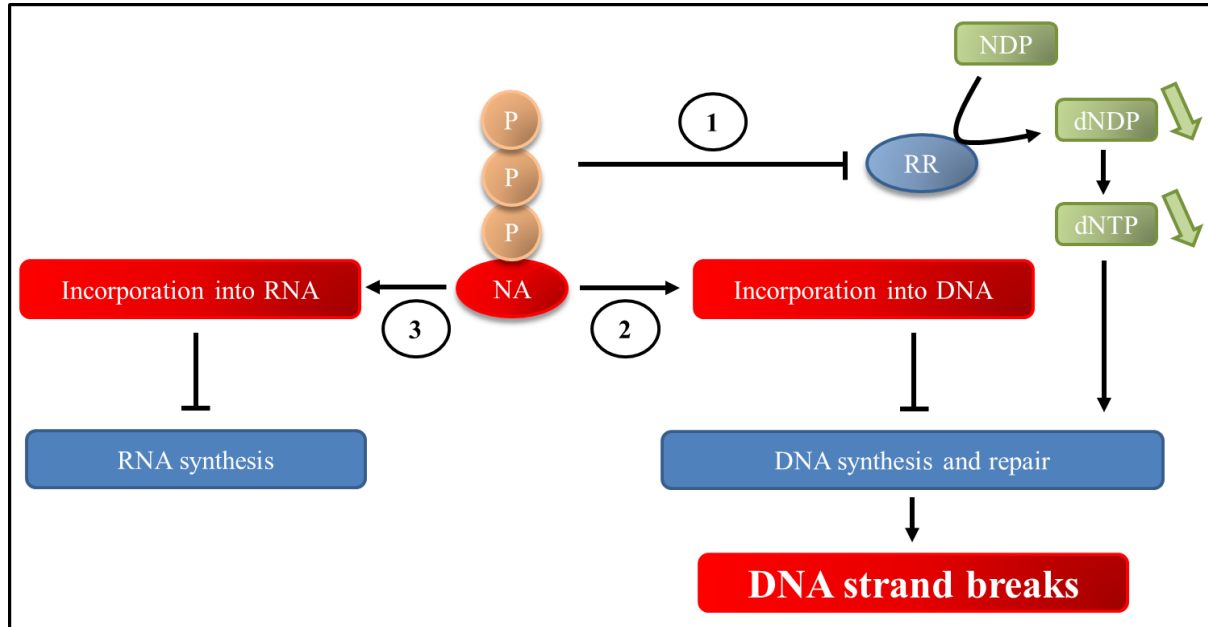


Figure 4: Inhibition of nucleic acid synthesis by NAs

NAs can inhibit DNA synthesis by two major mechanisms: (1) inhibition of ribonucleotide reductase (RR), an enzyme involved in the synthesis of DNA precursors, and (2) incorporation into DNA and chain termination, leading to the formation of DNA strand breaks. In addition, certain NAs are incorporated into growing RNA chain (3), resulting in arrest of its synthesis. dNDP: deoxyribonucleoside diphosphate; dNTP: deoxyribonucleoside triphosphate; NDP: ribonucleoside diphosphate.

1.4.1. Effect on nucleic acid synthesis

1.4.1.1. Inhibition of replicative DNA synthesis

Inhibition of DNA replication is a major cause of NA cytotoxicity (Parker *et al.*, 1988; Griffing *et al.*, 1989; Chunduru *et al.*, 1993; Huang *et al.*, 1995; Galmarini *et al.*, 2002; Van Den Neste *et al.*, 2005). This inhibition implicates several mechanisms.

The first one involves dNTP depletion and imbalance, through direct inhibition of ribonucleotide reductase (RR). This enzyme, which is essential for DNA replication, catalyzes the reduction of ribonucleoside diphosphates (NDPs) into deoxyribonucleoside diphosphates (dNDPs), which are subsequently converted into deoxynucleoside triphosphates (dNTPs), the

building blocks of DNA. RR is a multi-subunit enzyme consisting of a large subunit R1 (α) and a small subunit R2 (β). R1 subunit contains the catalytic site and two allosteric effector-binding sites: the specificity site that controls which nucleotide is reduced and the activity site that controls NDP reduction rates. R2 subunit houses a stable tyrosyl free radical required for NDP reduction. RR activity is stimulated by ATP and inhibited by dATP following their binding to the allosteric activity site of the R1 subunit. Activated purine analogs inhibit RR activity by binding to this site (Rofougaran *et al.*, 2006; Jordheim *et al.*, 2007; Fairman *et al.*, 2011; Jordheim *et al.*, 2011). CdATP and CAFdATP, the active forms of CdA and CAFdA, are among the most potent inhibitors of RR with IC_{50} around 60 nM (to compare with an IC_{50} of 45 μ M for dATP). F-Ara-ATP is less inhibitory with an IC_{50} of 0.6 μ M. No inhibitory effect of Ara-CTP has been reported, but the diphosphate form of gemcitabine, which is falsely recognized as a natural substrate, is a potent suicide inhibitor of RR (Baker *et al.*, 1991; Pereira *et al.*, 2004).

A second mechanism involved in inhibition of DNA synthesis is the misincorporation of NA triphosphates at the 3'-ends of growing DNA chains instead of natural dNTPs, which is facilitated by the decreased amount of natural dNTPs due to RR inhibition. Therefore, by depleting the dNTP pool, NAs exert a self-potentiating effect on their own incorporation into DNA. All the NAs have shown to be good substrates for replicative DNA polymerases. Once in DNA, they generally reduce the ability of DNA polymerase to add subsequent nucleotides, which leads to the stalling of replication forks. Alternatively, incorporation of two or more NAs in tandem leads to chain termination (Griffing *et al.*, 1989; Chunduru *et al.*, 1993).

Finally, triphosphate NAs may also inhibit other enzymes implicated in DNA replication like DNA primase and DNA ligase, as well as topoisomerase II and proof-reading exonucleases (Parker *et al.*, 1988; Kamiya *et al.*, 1996).

1.4.1.2. Inhibition of DNA repair synthesis

In addition to inhibition of DNA replication, activated NAs are also able to inhibit DNA repair occurring in basal conditions or after cell exposure to DNA-damaging agents (UV-C irradiation, alkylating agents). Majority of DNA repair mechanisms, like nucleotide excision repair (NER), include a step that allows the synthesis of a new patch of DNA to fill the gap left by the excision process. In resting cells, like CLL cells, this repair mechanism

represents the only opportunity for activated NAs to be incorporated into DNA (Seto *et al.*, 1985; Sandoval *et al.*, 1996; Van Den Neste *et al.*, 2002; Rao *et al.*, 2003).

1.4.1.3. Inhibition of RNA synthesis

Beside inhibition of DNA synthesis, cytotoxicity of NAs may also involve inhibition of RNA synthesis. Some of them, like F-ara-A (Huang *et al.*, 2000) and dFdC, can be incorporated into growing RNA chain inducing the premature termination of RNA transcript (Gandhi *et al.*, 2002), whereas others, like CdA, can interfere with binding of transcription factors and progression of RNA polymerases after their incorporation into double-stranded DNA (Hentosh *et al.*, 1995; Hartman *et al.*, 2004). In addition, CdA, CAFdA and fludarabine might inhibit polyadenylation (Chen *et al.*, 2008). Finally, the triphosphate form of dFdC provokes inhibition of CTP synthetase (the enzyme that produces CTP from UTP), which induces CTP depletion and potentiation of the incorporation of dFdC into RNA (Gandhi *et al.*, 2002; Mini *et al.*, 2006).

1.5. Induction of DNA damage response and cell cycle arrest

Incorporation of NAs into DNA and inhibition of DNA synthesis provoke DNA lesions that can induce DNA damage response similar to that observed with other DNA-damaging agents. Indeed, termination of DNA chain induced by NAs is like a DNA single-strand break (SSB) or abnormal DNA end. SSBs can also overlap, forming DNA double-strand breaks (DSBs) (Seto *et al.*, 1985; Hirota *et al.*, 1989; Genini *et al.*, 2000a; Sampath *et al.*, 2003). Moreover, incorporation of non-natural nucleotide might modify the structure of DNA by distortion of the DNA helix (Foley *et al.*, 2004).

DNA damage induced by NAs can be detected by three sensor kinases: ataxia telangiectasia mutated (ATM), ATM-and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) (Achanta *et al.*, 2001; Sampath *et al.*, 2003; Ewald *et al.*, 2008a). Once activated, the sensor kinases can induce cell cycle arrest *via* two different pathways (Figure 5). The first one, more rapid, implicates a phosphorylation cascade leading to inactivation of the cyclin-dependent kinase 2 (Cdk2), the enzyme responsible for the G1/S transition of the cell cycle (Falck *et al.*, 2001; Sampath *et al.*, 2002; Wang *et al.*, 2002; Ewald *et al.*, 2008a).

The second one, responsible for a delayed response, involves the tumor suppressor protein p53. After its phosphorylation by the sensor kinases, p53 is stabilized and can function as a transcription factor, activating transcription of numerous genes involved in cell cycle control, DNA repair or apoptosis. Among them is *CDKN1A* gene that encodes for p21, an inhibitor of Cdk2 (Mirzayans *et al.*, 2012). Both pathways end in cell cycle arrest, which might allow for DNA repair.

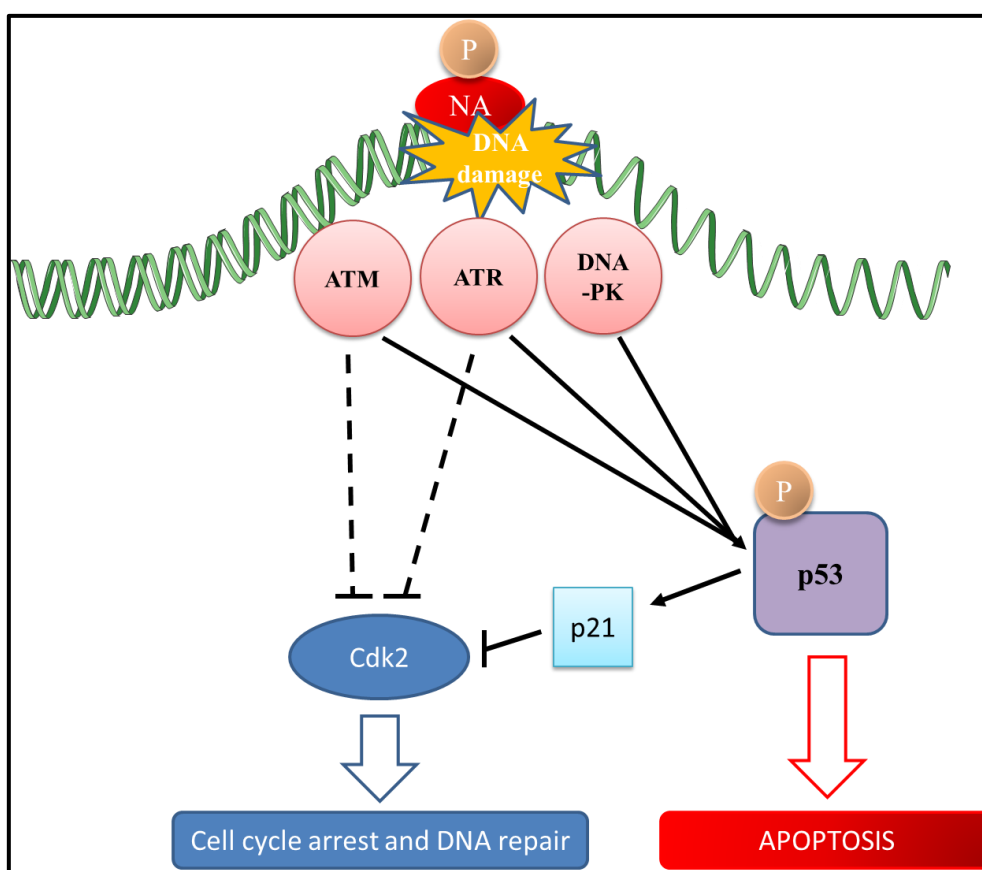


Figure 5: Molecular and cellular response to DNA damage induced by NAs.

As explained in the text, DNA damage induced by NAs can be recognized by sensor proteins such as ATM, ATR and DNA-PK. Activation of these kinases leads, by two different pathways, to inhibition of Cdk2, arrest of the cell cycle and potentially to DNA repair. If DNA lesions are irreparable, apoptosis is induced. NA: nucleoside analog; ATM: ataxia telangiectasia mutated; ATR: ataxia telangiectasia-related kinase; DNA-PK: DNA-dependent protein kinase; Cdk2: cyclin-dependent kinase 2.

1.6. Induction of apoptosis

1.6.1. Apoptotic pathways

If the extent of DNA damage induced by NAs is too big to be repaired, the cell can induce its suicide through a programmed cell death called apoptosis (Sampath *et al.*, 2002;

Sampath *et al.*, 2003). Apoptosis is characterized by the activation of different members of a conserved family of cysteine proteases, the caspases (cysteiny **asp**artate-specific protease) (Chowdhury *et al.*, 2008b; Logue *et al.*, 2008). Once activated, these caspases provoke the cleavage of different proteins essential for cell survival, which results in apoptotic cell death (Cohen, 1997). Two pathways (Figure 6) can lead to caspase activation: the extrinsic pathway and the intrinsic pathway.

The extrinsic pathway is initiated by the binding of distinct ligands, such as TNF- α , to a specific family of transmembrane receptors called death receptors (DR). This binding allows interaction of DRs with adaptor molecules such as FAS-associated death domain protein (FADD) that recruit then the pro-caspase 8. After dimerization and proteolytic cleavage, the initiator caspase 8 can directly cleave and activate the effector caspases 3 and 7, leading to apoptosis (Lavrik *et al.*, 2005; Tait *et al.*, 2010).

The intrinsic pathway can be induced by different cellular stresses, including serum deprivation, oxidative stress, γ - or UV-irradiation, ischemia or DNA lesions. The intrinsic pathway, also known as the mitochondrial pathway, is characterized by mitochondrial outer membrane permeabilization (MOMP) (Kroemer *et al.*, 2007). This process occurs after activation or inhibition of members of B cell lymphoma 2 (BCL-2) protein family, which are proteins that tightly control the integrity of the mitochondrial outer membrane. Activation of the pro-apoptotic proteins Bcl-2-associated X protein (BAX) and Bcl-2 antagonist or killer (BAK) (Kroemer *et al.*, 2007) results in the formation of membrane pores, which induces MOMP and the release of apoptotic factors from the intermembrane space. One of these factors, the cytochrome c, interacts in the cytosol with the apoptotic protease-activating factor 1 (APAF1) and dATP to form a structure termed the apoptosome responsible for the activation of caspase 9. Caspase 9 activates then the executioner caspases 3 and 7, which provokes apoptosis (Riedl *et al.*, 2007).

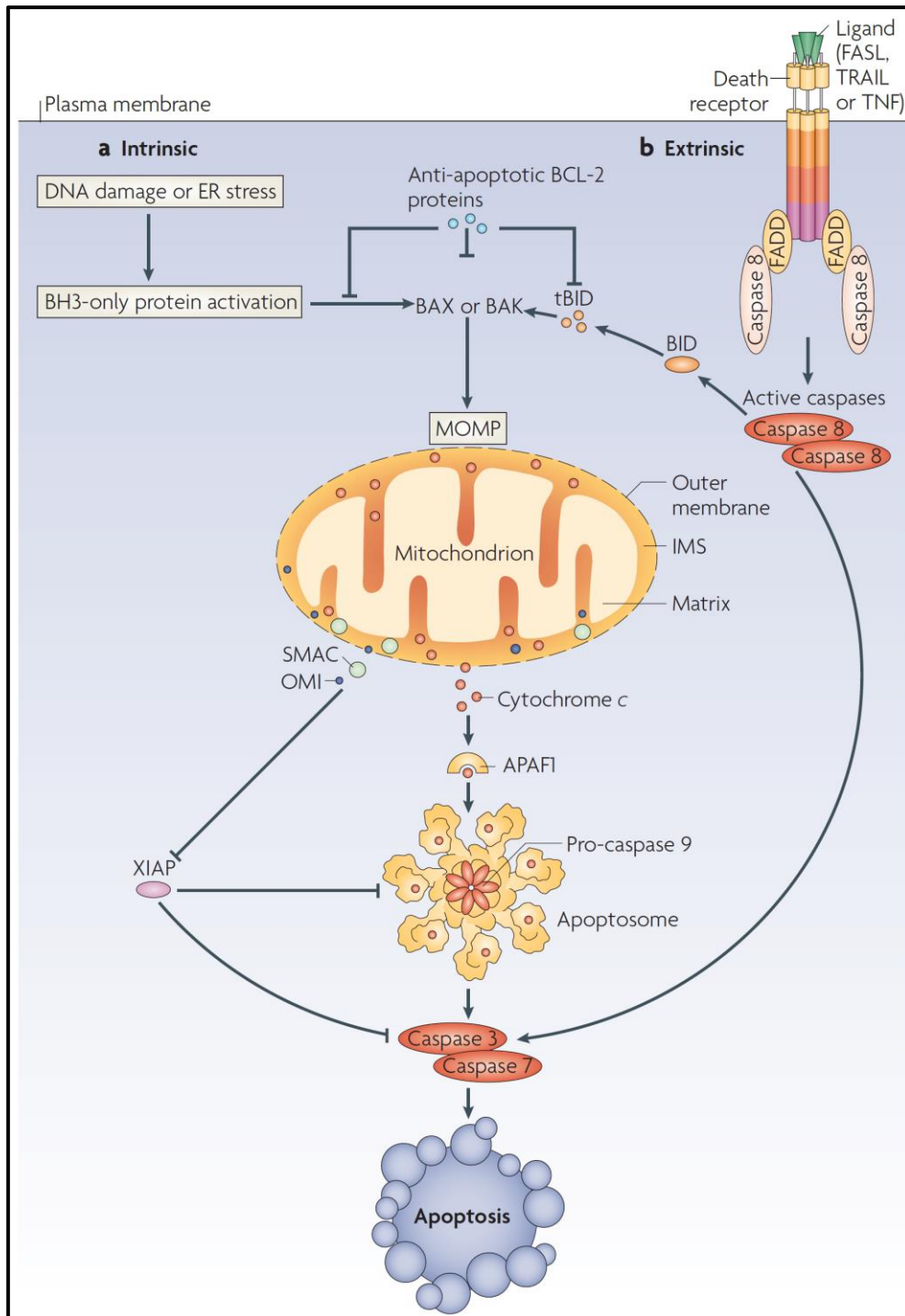


Figure 6: Apoptotic pathways

a) Intrinsic and b) extrinsic pathways. Explanations are given in the text. (Tait *et al.*, 2010)

1.6.2. Activation of the intrinsic pathway by nucleoside analogs

Activation of the intrinsic pathway is the main mechanism by which NAs induce apoptosis. This activation can be p53-dependent or independent.

p53-dependent mechanisms

As mentioned above, DNA damage induced by NAs is detected by the sensor kinases ATM, ATR and DNA-PK that, once activated, phosphorylate and stabilize the transcription factor p53. Activation of p53 can lead to cell cycle arrest, but also to initiation of the intrinsic apoptotic pathway. Indeed, p53 is able to induce MOMP through transcriptional regulation of apoptotic proteins. On the one hand, p53 increases the transcription of pro-apoptotic proteins, including BAX and BAK, and on the other hand, it decreases the transcription of anti-apoptotic proteins, such as Bcl-2. As explained above, these alterations result in the formation of pores in the outer membrane of mitochondria leading to the release of cytochrome c into the cytosol. Induction of MOMP due to p53 activation probably constitutes the main mechanism by which NAs activate the intrinsic pathway of apoptosis (Gartenhaus *et al.*, 1996; Biswal *et al.*, 2000; Genini *et al.*, 2000a; Marzo *et al.*, 2001; Pepper *et al.*, 2001; Galmarini *et al.*, 2003b).

p53-independent mechanisms

Beside p53 activation, several other mechanisms can be implicated in the activation of the intrinsic pathway by NAs.

Firstly, as described in section 1.4.1.3, some NAs, including F-Ara-A and gemcitabine, are able to inhibit RNA synthesis, which could consequently decrease the levels of anti-apoptotic proteins, such as Bcl-2 and Mcl-1 (myeloid cell leukemia 1), and favor the activation of the intrinsic pathway (Gottardi *et al.*, 1997; Nomura *et al.*, 2000; Sampath *et al.*, 2000; Marzo *et al.*, 2001; Chen *et al.*, 2005).

Secondly, perturbation of mitochondrial functions can result from the incorporation of NAs, especially CdA, into mitochondrial DNA after phosphorylation by mitochondrial kinases (Lim *et al.*, 2001; Lai *et al.*, 2004). This incorporation induces the same type of damage like in genomic DNA, which is potentiated in mitochondria by the lack of repair mechanisms (Parker *et al.*, 1988; Hentosh *et al.*, 1997). Damage in mitochondrial DNA results in a loss of metabolic functions through inhibition of the synthesis of the proteins involved in oxidative phosphorylations (Hentosh *et al.*, 1997). Moreover, it has been shown,

at least for the nucleotides of CdA and CAFdA, that they can bind directly to mitochondrial membrane proteins, such as F_1F_0 ATPase (mitochondrial adenosine triphosphate synthase), causing a decrease of the membrane potential and a loss of mitochondria integrity (Genini *et al.*, 2000a).

Finally, in addition to these mechanisms that affect directly mitochondria, a last mechanism not implicated in MOMP, but in caspase 9 activation has been reported. The triphosphate forms of CdA, CAFdA, F-Ara-A and Ara-C can replace dATP in the formation of the apoptosome and therefore promote the assembly of APAF-1 and the activation of caspase 9 (Leoni *et al.*, 1998; Genini *et al.*, 2000a; Genini *et al.*, 2000b).

1.7. Mechanisms of resistance

The effectiveness of NA treatment against cancer cells is principally limited by primary or acquired resistance. Several mechanisms of resistance have been described. They can be divided in three different groups (Jordheim *et al.*, 2005).

The first group contains alterations that result in insufficient conversion of NAs into their active triphosphate form. This can be due to impaired transport through the plasma membrane, decreased dCK activity, increased cytosolic 5'-NT activity as well as increased expression of ATP-dependent efflux pumps, such as MRPs 5 and 7 (Chen *et al.*, 2011). Insufficient activation of NAs is often associated with reduced activity of the activating enzyme dCK. Loss of dCK activity can result from mutations or deletions in the coding sequence of dCK, expression of alternatively spliced fragments coding for inactive protein, or decreased expression of dCK mRNA due to methylation of the promoter region of the gene (Kong *et al.*, 1991; Owens *et al.*, 1992; Veuger *et al.*, 2000). A complete loss of dCK has not been observed in clinical samples (Lotfi *et al.*, 2003; Mansson *et al.*, 2003), but a lack of dCK in NA-resistant tumor cell lines is frequent. In this case, sensitivity to NAs can be restored by reintroduction of dCK in dCK-deficient cells (Stegmann *et al.*, 1995; Hapke *et al.*, 1996). Accordingly, correlation between dCK activity and NA cytotoxicity has been reported, indicating that dCK activity could be important as a prognostic marker for response to therapy (Arner *et al.*, 1994; Kroep *et al.*, 2002). Due to this well demonstrated role of dCK in NA efficacy, several studies have been conducted to identify new mechanisms involved in the

regulation of dCK activity (see section 2.7.3.) or to elaborate strategies that could counteract dCK deficiency, like use of pro-nucleotides. This approach enables the NA monophosphate, conjugated to a lipophilic group, to enter the cells by passive diffusion (Wagner *et al.*, 2000; Bontemps *et al.*, 2006).

The second group comprises defects that make NAs unable to induce sufficient DNA damage. This can be caused by overexpression of RR, which leads to increase of dNTP pool and hence to decreased incorporation of NA triphosphate into DNA, or by mutations in RR that make it less sensitive to allosteric inhibition by NA triphosphates (Mansson *et al.*, 2003). Increased RR activity can also provoke an increase of the concentration of dCTP, which is a retro-inhibitor of dCK. Moreover, reduced ability to induce DNA damage can result from overexpression of DNA repair enzymes, like DNA-PK (Beck *et al.*, 2004; Van Den Neste *et al.*, 2005).

Finally, the third group includes defects in the DNA damage response and/or the apoptotic pathway, as p53 dysfunction or changes in the expression of apoptotic proteins (Feng *et al.*, 2000; Jordheim *et al.*, 2007). Defects in p53 prevent cell cycle arrest as well as induction of apoptosis. For that reason, p53 dysfunction is responsible for clinical resistance toward most chemotherapeutic agents, including NAs, and is a negative prognosis marker. Besides p53 defects, low sensitivity to NAs has also been related to increased level of anti-apoptotic proteins, such as overexpression of Bcl-2, or decreased levels of pro-apoptotic proteins, such as lack of BAX. Overexpression of inhibitors of caspases (survivin and XIAP), or a lack of expression of certain caspases also induce resistance to NAs (Mohammad *et al.*, 1998; Lotfi *et al.*, 2001; Sampath *et al.*, 2003).

2. Deoxycytidine kinase

2.1. Physiological role

Deoxycytidine kinase (dCK) belongs to the family of deoxynucleoside kinases (dNKs). These enzymes play a crucial role in the salvage pathway, one of the two pathways that lead to the synthesis of DNA precursors.

2.1.1. The two pathways of DNA precursor synthesis

Adequate maintenance of the intracellular deoxynucleoside triphosphate (dNTP) pool is essential for mammalian cells to replicate and repair its DNA. This is achieved by the *de novo* and the salvage pathways.

The *de novo* pathway is the main source of dNTPs for nuclear DNA replication in proliferating cells. This energy-consuming pathway, which includes multiple enzymatic steps, begins by the synthesis of ribonucleotides from small basic molecules (amino acids, carbohydrate, CO₂, tetrahydrofolate derivatives, ...). Ribonucleotides are then reduced at the diphosphate level to deoxynucleoside diphosphates (dNDPs) by ribonucleotide reductase. dNDPs are finally converted to dNTPs by NDPK (Reichard, 1988).

The salvage pathway represents a complementary route for supplying DNA precursors to both resting and dividing cells. As its name implies, this pathway recycles intra- or extracellular deoxyribonucleosides, which can originate from nutrients or degraded DNA. The principal salvage enzymes are the deoxyribonucleoside kinases (dNKs), including dCK, which phosphorylate deoxynucleosides to their corresponding deoxynucleoside monophosphates (dNMPs). These are then converted to dNTPs by the successive actions of NMPK and NDPK. Although the salvage pathway is not a prerequisite for cell survival in replicating mammalian cells since they can synthesize dNTPs *via* the *de novo* pathway, it is very important in some situations, especially in resting or in fully differentiated cells in which the *de novo* synthesis of dNTPs is assumed to be switched off due to the lack of ribonucleotide reductase activity (Reichard, 1988).

2.1.2. The deoxyribonucleoside kinases

The natural substrates for the dNKs are the four natural dNs: deoxycytidine (dCyd), thymidine (Thd), deoxyadenosine (dAdo) and deoxyguanosine (dGuo). In mammalian cells, four dNKs with overlapping specificity have been identified: two cytosolic enzymes, dCK and thymidine kinase 1 (TK1), and two mitochondrial enzymes, deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2).

Among the salvage kinases, **dCK** has the broadest specificity. It is indeed able to phosphorylate the pyrimidine nucleoside deoxycytidine and the purine nucleosides deoxyadenosine and deoxyguanosine, which can lead to the synthesis of dCTP, dATP and dGTP. In addition, dCK can supply cells with dTTP, since dCMP can be converted to thymidine monophosphate (dTMP) by the successive actions of dCMP deaminase and thymidylate synthase (Figure 7) (Arner *et al.*, 1995). Therefore, dCK is a very important enzyme of the salvage pathway since it is the only dNK that can supply cells with all four precursors of DNA.

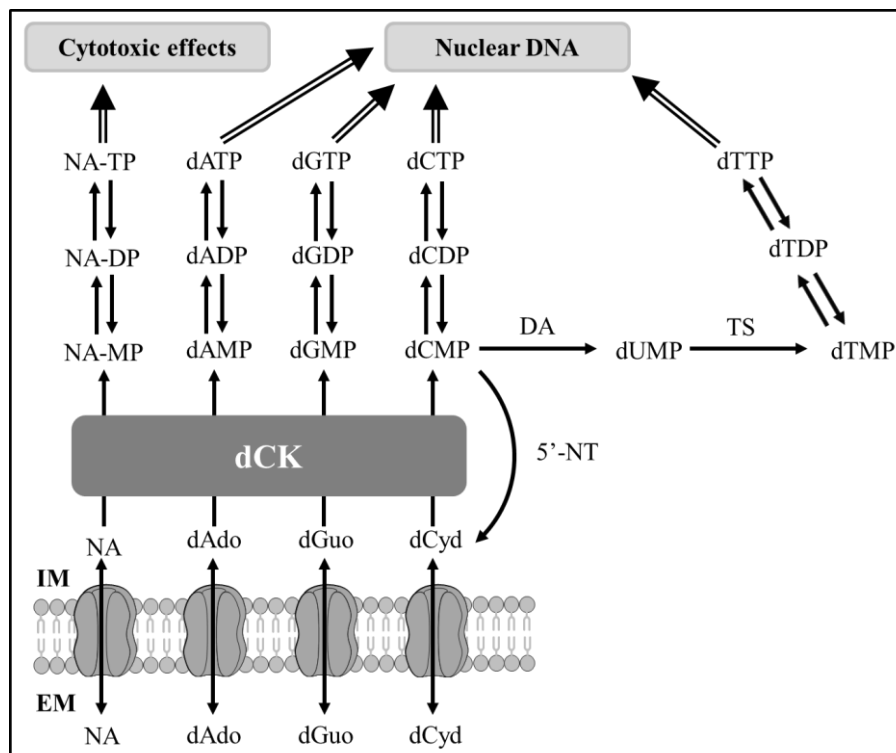


Figure 7: Role of dCK in dNTPs synthesis and nucleoside analog activation

Through the salvage pathway, dCK is able to supply cells with the four dNTPs required for nuclear DNA synthesis. dCK also activates numbers of NAs used in the treatment of cancer and viral infections (see section 1.1.). 5'-NT: 5'-nucleotidase; DA: dCMP deaminase; TS: thymidylate synthase; EM: extracellular medium; IM: intracellular medium (adapted from Arner *et al.*, 1995).

dCTP derived from the salvage pathway has been suggested to be utilized for DNA repair, whereas the *de novo* pathway would supply dCTP for DNA replication (Xu *et al.*, 1995). However, this functional compartmentalization of the dCTP pool has been disputed (Bianchi *et al.*, 1997). Besides DNA, dCK can also provide dCTP for the synthesis of the deoxyliponucleotides dCDP-choline, dCDP-ethanolamine (Spyrou *et al.*, 1989; Arner *et al.*, 1995) or dCDP-diacylglycerol (Spasokukotskaja *et al.*, 1991; Hrabak *et al.*, 1993). Moreover, dCK could play an important role in lymphocyte development, homeostasis and survival, at least in mice, and in hematopoiesis (Toy *et al.*, 2010; Austin *et al.*, 2012; Choi *et al.*, 2012), as well as in the migration and invasion of fibroblast-like synoviocytes from rheumatoid arthritis patients (Fan *et al.*, 2013). And last, but not least, dCK efficiently phosphorylates and activates a large number of therapeutic NAs, including the five anticancer NAs used in our study (see section 1.2.).

TK1, the cytosolic TK, has the most narrow substrate specificity of the four mammalian dNKs. TK1 phosphorylates only thymidine and deoxyuridine, but it has the highest catalytic efficiency with thymidine, its primary substrate. TK1 is also the only cell cycle-dependent dNK. Its activity is indeed low or absent in non-dividing cells, increased during late G1, maximal in S-phase and decreased in G2/M (Sherley *et al.*, 1988; Munch-Petersen *et al.*, 1991; He *et al.*, 1996; Munch-Petersen *et al.*, 2007). TK1 can also activate NAs, like Zidovudine (AZT), an antiviral NA used in human immunodeficiency virus (HIV) therapy, or floxuridine (5-fluoro-2'-deoxyuridine) approved for the treatment of advanced colon cancer, kidney cancer and stomach cancer (Jordheim *et al.*, 2013).

Unlike TK1, the mitochondrial **TK2** is constitutively expressed and shows broader substrate specificity, being able to phosphorylate the three pyrimidine nucleosides, thymidine, deoxyuridine and deoxycytidine with high efficiency. Together with the mitochondrial **dGK** that phosphorylates deoxyadenosine and deoxyguanosine, TK2 is essential for the synthesis of nucleotide precursors for mitochondrial DNA and for the maintenance of its integrity (Munch-Petersen *et al.*, 1991; Munch-Petersen *et al.*, 2007). Both TK2 and dGK can phosphorylate NAs, but often with lower efficiency than dCK. Their role in NA cytotoxicity is not yet very clear, though a role of dGK in apoptosis has been suggested (Jullig *et al.*, 2001).

2.2. Tissular and subcellular distribution

dCK level is highest in lymphoid tissues such as thymus, spleen, lymph nodes, stimulated peripheral blood mononuclear cells, bone marrow cells and in all malignancies related to these cells (Staub *et al.*, 1988; Eriksson *et al.*, 1994; Arner *et al.*, 1995). In both normal and leukemic lymphocytes, the amount of dCK is around 100 ng/mg of proteins, which corresponds to 0.01 % of total cellular proteins, with extreme values of 28 and 1500 ng/mg of protein (Kawasaki *et al.*, 1992; Eriksson *et al.*, 1994; Spasokoukotskaja *et al.*, 1995). In the same cells, dCK protein amount is usually well correlated to the catalytic activity of the enzyme (Kawasaki *et al.*, 1992; Kroep *et al.*, 2002; Hubeek *et al.*, 2005), which varies between 60 and 500 pmol/min/mg of protein (Arner ES, 1992; Spasokoukotskaja *et al.*, 1995; Jacobsson *et al.*, 2001). In proliferating epithelial cells (lung, colon, placenta) as well as in resting peripheral blood mononuclear cells, dCK levels are intermediate, whereas they are very low in differentiated tissues, like brain, kidney, liver and muscle (Arner *et al.*, 1995; Staub *et al.*, 2007). Several groups have shown that there was a correlation between neoplastic transformation and increased dCK protein levels and activities in solid cancer tissues compared to the corresponding normal tissues (Harkrader *et al.*, 1980; Singhal *et al.*, 1992; Ruiz van Haperen *et al.*, 1993; Weber *et al.*, 1993; Eriksson *et al.*, 1994; Spasokoukotskaja *et al.*, 1995; van der Wilt *et al.*, 2003; Karbownik *et al.*, 2005). In most carcinomas, dCK amount is about 3- to 7- fold higher than in normal tissues (Spasokoukotskaja *et al.*, 1995). The preferential expression of dCK in lymphoid and malignant cells explains the efficacy and the relative specificity of NAs in the treatment of hematological malignancies and some solid tumors.

The subcellular localization of dCK protein was a matter of discussion. Several studies showed that dCK was mainly cytosolic (Pegoraro *et al.*, 1971; Arner *et al.*, 1995), but overexpressed dCK was found to be located in the nucleus, independently on the absence or the presence of C-terminally fused GFP (Johansson *et al.*, 1997; Hatzis *et al.*, 1998). Moreover, a nuclear localization signal (NLS) sequence, enriched in basic amino acids (lysine, arginine), was identified in the N-terminal region of dCK (Figure 8). These discrepancies in the localization of dCK were resolved by Hatzis *et al.* (1998), who demonstrated that endogenous dCK is mostly cytosolic, but was mainly found in the nucleus when overexpressed. To explain this observation, the authors proposed that endogenous dCK could be retained in the cytosol by a putative binding protein, which would become saturated

when dCK is overexpressed, explaining its nuclear localization in this condition. In line with this, overexpression of a dCK lacking the NLS sequence resulted in cytosolic localization (McSorley *et al.*, 2008). The identity of this potential binding partner is still unknown and the role of the nuclear localization signal of dCK remains to be elucidated. Possibly, transport of dCK between subcellular compartments could play an important role in the regulation of its physiological function.

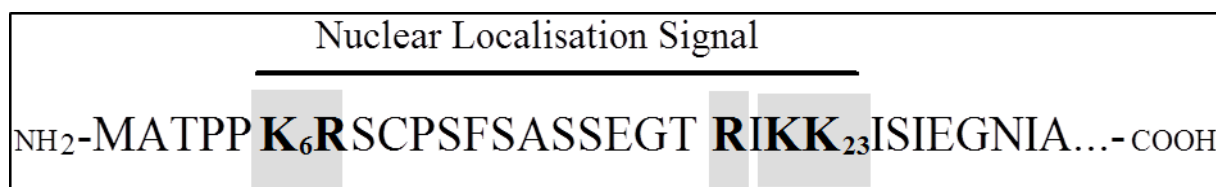


Figure 8: Nuclear localization signal (NLS) in human dCK.

2.3. Physicochemical properties

The first homogeneous purifications of human dCK were obtained from leukemic spleen (Bohman *et al.*, 1988) and T-lymphoblasts (Kim *et al.*, 1988; Datta *et al.*, 1989a; Datta *et al.*, 1989b) and performed by using affinity chromatography. Nevertheless, this purified dCK was unstable and several conditions were required to maintain its activity: a high dCK protein concentration, the presence of a reducing agent (2-25 μ M dithiothreitol), bovine serum albumin, 20 % glycerol and several protease inhibitors. The active human dCK appeared to be a dimer of 61 kDa, composed of two identical 30.5 kDa subunits (Bohman *et al.*, 1988; Datta *et al.*, 1989a; Chottiner *et al.*, 1991). After two-dimensional electrophoresis, pI (isoelectric point) values for polypeptides were determined as being between 5.5 and 6.1 depending on the source (Bohman *et al.*, 1988; Kim *et al.*, 1988). Regarding the optimum pH, it ranged from 5.5 to 10 (Durham *et al.*, 1970; Sarup *et al.*, 1987; Bohman *et al.*, 1988; Datta *et al.*, 1989b).

Due to the low amounts of dCK in tissues and its relative instability after purification, production of recombinant dCK was considered. After a failed attempt (Huang *et al.*, 1989), human dCK cDNA was successfully cloned and the recombinant protein dCK was produced in *E.coli* (Chottiner *et al.*, 1991). This recombinant dCK had characteristics similar to that of the enzyme purified from human tissues (Bohman *et al.*, 1988; Datta *et al.*, 1989a; Datta *et al.*, 1989b) and was, like native dCK, able to phosphorylate dCyd, dAdo and dGuo. Currently,

recombinant dCK is purified by metal affinity chromatography, based on the presence of an N-terminal histidine tag sequence (Johansson *et al.*, 1995).

Sequence of the dCK peptide is shown in Figure 9. The polypeptide chain contains 260 amino acids, corresponding to a 30.5 kDa subunit, in agreement with the molecular mass of 61 kDa determined by gel filtration for the active enzyme.

MATPPKRSCP	SFSASSEGR	IKKISIEGNI	AAGKSTFVNI	LKQLCEDWEV	VPEPVARWCN
VQSTQDEFEE	LTMSQKNGGN	VLQMMYEKPE	RWSFTFQTYA	CLSRIRAQLA	SLNGKLKDAE
KPVLFFERSV	YSDRYIFASN	LYESECNET	EWTIYQDWH	WMNNQFGQSL	ELDGIIYLQA
TPETCLHRIY	LRGRNEEQGI	PLEYLEKLHY	KHESWLLHRT	LKTNFDYLQE	VPILTLDVNE
DFKDKYESLV	EKVKEFLSTL				

Figure 9: Amino acid sequence of dCK (from Chottiner *et al.*, 1991).

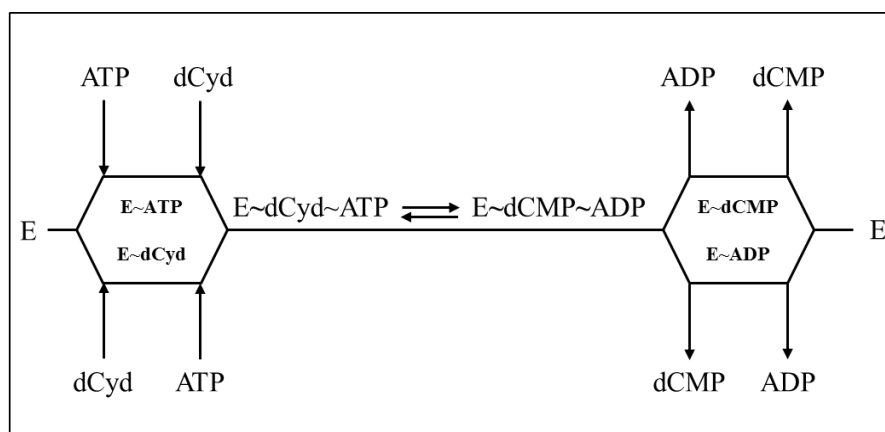
2.4. Kinetic properties

2.4.1. Kinetic mechanisms

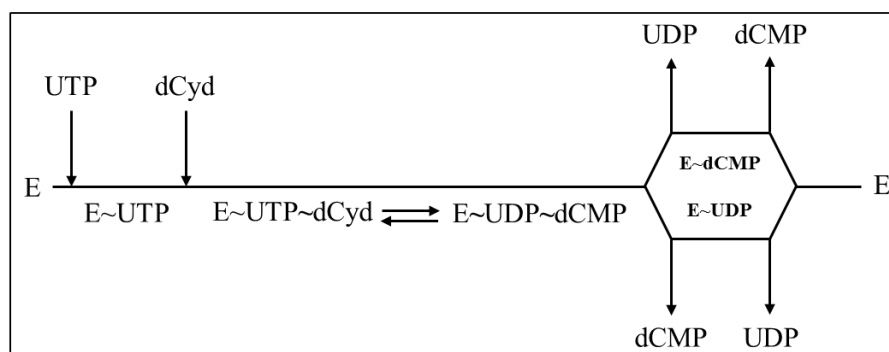
dCK (EC 2.7.1.74, deoxycytidine 5'-phosphotransferase) belongs to the group of phosphotransferases, which are enzymes that catalyze the transfer of a phosphoryl group from a nucleoside triphosphate to another substrate. On the basis of sequence and structure analysis, dCK has been classified in the group of Rossmann-like fold kinases, and more precisely in the family of the P-loop-containing kinases (Cheek *et al.*, 2002). Phosphoryl transfer reaction can occur by a ping-pong mechanism in which one of the products is released before the second substrate is bound or by a sequential mechanism, in which the two substrates are bound to the enzyme prior to phosphoryl transfer. Sequential mechanism can be “ordered” if the substrates combine with the enzyme and the products dissociate from them in an obligatory order or, “random” if there is no obligatory order of combination or release. The reaction catalyzed by dCK, which is named bi-bi because it involves two substrates and two products, occurs by a sequential mechanism. Both random bi-bi and ordered bi-bi mechanisms were proposed (Figure 10).

Steady-state substrate kinetic analyses and product inhibition studies with purified native or recombinant dCK have shown a random bi-bi mechanism when ATP was used as the phosphate donor (Datta *et al.*, 1989b) (Figure 10A), but an ordered pathway in the presence of UTP (the preferred phosphate donor as described in section 2.5.3.), with UTP

binding before the nucleoside substrate and a random release of the products (Figure 10B). In other words, the kinetic data indicate that, although dCK can follow a random bi-bi reaction sequence, binding to UTP prior to dCyd is preferred when UTP is the phosphate donor (Hughes *et al.*, 1997). Results obtained from circular dichroism and fluorescence spectroscopy studies as well as from crystallography (Kierdaszuk *et al.*, 1993; Mani *et al.*, 2003; Godsey *et al.*, 2006) demonstrated that dCK assumes different conformational states in the presence of ATP or UTP, which may account for the distinct kinetic behaviors of dCK in the presence of the two nucleotides (see section 2.5.2.).



A. Random bi-bi mechanism (Datta *et al.*, 1989b).



B. Ordered and random bi-bi mechanism (Hughes *et al.*, 1997).

Figure 10: The two kinetic mechanisms described for dCK.

Explanations are given in the text. E: enzyme.

2.4.2. Kinetic characteristics

As mentioned previously, human dCK is characterized by very broad substrate specificity. dCK is indeed able to catalyze the phosphorylation of three natural

deoxyribonucleosides, dCyd, dAdo, and dGuo, but also of numerous anticancer and antiviral NAs. In addition, dCK can use several nucleotides as phosphoryl donor (see section 2.5.3), indicating substrate promiscuity at both the nucleoside acceptor and nucleotide donor sites. It is important to note that the nucleotide donors can be used as substrates only in the presence of a divalent cation, Mg^{2+} being the preferred (Momparker *et al.*, 1968; Sarup *et al.*, 1987), which forms a complex with the nucleotide. The true substrate of the enzyme is in fact the complex Mg^{2+} -nucleotide, like Mg^{2+} -ATP or Mg^{2+} -UTP (Datta *et al.*, 1989b; Shewach *et al.*, 1992).

The kinetic behavior of dCK is relatively complex. Indeed, although dCK presents an hyperbolic saturation curve with a variety of substrates, negative cooperativity, characterized by a Hill coefficient < 1 , has been described, resulting in bimodal kinetics with two sets of K_m and V_{max} values (Ives *et al.*, 1969; Sarup *et al.*, 1987; Kierdaszuk *et al.*, 1990; White *et al.*, 1991; Usova *et al.*, 1997). Negative cooperativity generally indicates that the binding of a ligand to one enzyme subunit decreases the affinity for the ligand of the neighboring subunit, because of a conformational modification (Conway *et al.*, 1968). The negative cooperativity observed with dCyd was found to be abolished by 0.4 M NaCl (Usova *et al.*, 1997), probably due to alterations in the conformation of the active site. Discrepancies in the literature concerning the kinetic properties of dCK could be explained in part by this salt effect.

According to the source of the enzyme, its method of purification, its relative instability, and the method used to measure its activity, dCK from human tissues displayed K_m values for dCyd, with ATP as phosphoryl donor, which ranged between 0.3 (Sarup *et al.*, 1987) and 700 μM (Singhal *et al.*, 1992). By contrast, when human recombinant dCK, overexpressed in *E. coli*, was used, K_m values for dCyd in the presence of ATP varied between 0.16 (Johansson *et al.*, 1995) and 9 μM (Shaffiee *et al.*, 1998), as shown in Table 1 that also reports kinetic parameters determined for dAdo and dGuo in several studies. Obviously, dCK has higher affinity for dCyd than for dAdo and dGuo, whereas maximal activities (V_{max} or K_{cat} values) are several-fold lower for dCyd as compared with purine nucleosides. However, the resulting catalytic efficiency (V_{max}/K_m or K_{cat}/K_m) is higher for dCyd, which is thus the preferred substrate of dCK. It is also clear from Table 1 that affinity of dCK for its nucleoside substrate is greatly increased when UTP is used as phosphoryl donor instead of ATP.

Phosphoryl acceptor	Phosphoryl donor	K_m (μM)	$V_{\max}$ (nmol/min/mg) or K_{cat} (s^{-1})	References
dCyd	5 mM ATP	0.16	33* (0.02)	(Johansson <i>et al.</i> , 1995)
	5 mM UTP	0.05	16* (0.01)	
	5 mM ATP	1.3	69* (0.04)	(Usova <i>et al.</i> , 1997)
	5 mM ATP	9	240* (0.14)	(Shafiee <i>et al.</i> , 1998)
	5 mM ATP	1.5	68* (0.04)	(Usova <i>et al.</i> , 2002)
	5 mM UTP	0.3	27* (0.02)	
	1 mM ATP	6.2	0.03	(Sabini <i>et al.</i> , 2003)
	1 mM ATP	<1 μM	0.033	(Godsey <i>et al.</i> , 2006)
	1 mM UTP	<1 μM	0.044	
	1 mM ATP	<3 μM	0.033	(Sabini <i>et al.</i> , 2007)
	1 mM UTP	<3 μM	0.049	
dAdo	2 mM ATP	1.7	0.04	(McSorley <i>et al.</i> , 2008)
	1 mM ATP	1	0.064	(Iyidogan <i>et al.</i> , 2008)
	1 mM ATP	<3 μM	0.04	(Hazra <i>et al.</i> , 2009)
	5 mM ATP	480	1500* (0.85)	(Johansson <i>et al.</i> , 1995)
	5 mM UTP	19	90* (0.05)	
	5 mM ATP	160	1280* (0.73)	(Usova <i>et al.</i> , 2002)
	5 mM UTP	41	410* (0.23)	
	1 mM ATP	100	1.7	(Godsey <i>et al.</i> , 2006)
dGuo	1 mM UTP	13	0.33	
	2 mM ATP	117	0.77	(McSorley <i>et al.</i> , 2008)
	1 mM ATP	81	3.1	(Iyidogan <i>et al.</i> , 2008)
	1 mM ATP	115	2.1	(Sabini <i>et al.</i> , 2008a)
	1 mM UTP	8.5	0.37	
	1 mM ATP	115	2.1	(Hazra <i>et al.</i> , 2009)
	5 mM ATP	130	81* (0.05)	(Usova <i>et al.</i> , 1997)
	5 mM ATP	120	360* (0.2)	(Usova <i>et al.</i> , 2002)
dGuo	5 mM UTP	44	160* (0.09)	
	1 mM ATP	231	2.6	(Godsey <i>et al.</i> , 2006)
	1 mM UTP	21	0.33	
	2 mM ATP	246	0.73	(McSorley <i>et al.</i> , 2008)
	1 mM ATP	154	3.3	(Iyidogan <i>et al.</i> , 2008)
	1 mM ATP	181	2.5	(Sabini <i>et al.</i> , 2008a)
	1 mM UTP	41	0.53	
	1 mM ATP	231	2.6	(Hazra <i>et al.</i> , 2009)

Table 1: Kinetic parameters of recombinant dCK determined for its natural substrates

Maximal dCK activity was expressed in $V_{\max}$ (nmol/min/mg) (*), or in K_{cat} (s^{-1}) (not marked). Values shown are means of several observations. SEM are not indicated, but can be found in the corresponding references.

Regarding NAs (Table 2), they appear also to be good substrates of dCK. Interestingly, CdA seems to be a better substrate of dCK than its natural counterpart dAdo. Finally, affinity of dCK for NAs is also highly increased when UTP is used instead of ATP.

Phosphoryl acceptor	Phosphoryl donor	K_m (μ M)	V_{max} (nmol/min/mg) or K_{cat} (s^{-1})	References
Ara-C	1 mM ATP	15.5	0.26	(Sabini <i>et al.</i> , 2003)
	1 mM ATP	6.9	0.30	(Godsey <i>et al.</i> , 2006)
	1 mM UTP	2.3	0.17	
	2 mM ATP	5.9	0.32	(McSorley <i>et al.</i> , 2008)
	1 mM ATP	13.1	0.34	(Hazra <i>et al.</i> , 2009)
dFdC	1 mM ATP	22	0.37	(Sabini <i>et al.</i> , 2003)
	2 mM ATP	6.2	0.44	(McSorley <i>et al.</i> , 2008)
	1 mM ATP	16.1	0.39	(Hazra <i>et al.</i> , 2009)
CdA	5 mM ATP	24	760* (0.43)	(Johansson <i>et al.</i> , 1995)
	5 mM UTP	0.99	120* (0.068)	
	1 mM ATP	78	0.6	(Sabini <i>et al.</i> , 2008a)
	1 mM UTP	5.1	0.37	

Table 2: Kinetic parameters of recombinant dCK determined for NAs

Maximal dCK activity was expressed in V_{max} (nmol/min/mg) (*), or in K_{cat} (s^{-1}) (not marked). Values shown are means of several observations. SEM are not indicated, but can be found in the corresponding references.

2.4.3. The physiological phosphate donor

Even though several nucleoside triphosphates like ATP, UTP, GTP, CTP, dGTP, dTTP can be used by dCK as phosphoryl donor *in vitro*, ATP and UTP are considered as the most probable candidates for a physiological role. Indeed, with an intracellular concentration of 2-4 mM, ATP is not limiting for the phosphorylation of deoxynucleosides, since K_m values reported for ATP range between 7 and 54 μ M (Godsey *et al.*, 2006). However, UTP, with intracellular concentration around 1 mM, could also be implicated. Indeed, dCK has higher affinity for UTP than ATP with K_m values close to 1 μ M (Coleman *et al.*, 1975; Sarup *et al.*, 1987; White *et al.*, 1987; Datta *et al.*, 1989b; Sarup *et al.*, 1989; White *et al.*, 1991; Shewach *et al.*, 1992; Johansson *et al.*, 1995; Krawiec *et al.*, 1995; Hughes *et al.*, 1997; Godsey *et al.*, 2006) and displays also higher affinity for most of its nucleoside substrates (see Tables 1 and 2) with UTP instead of ATP as phosphate donor. In addition, when a nucleotide mixture is used to mimic the intracellular medium, the rate of phosphorylation of nucleosides is clearly decreased after UTP removal whereas there is no change when another nucleotide is removed

(White *et al.*, 1991; Shewach *et al.*, 1992). Finally, UTP is also the preferred phosphate donor for dCK when equimolar or physiological concentrations of ATP and UTP were used in phosphate transfer experiments (Krawiec *et al.*, 1995). So, even if its intracellular concentration is 2- to 4-fold lower than that of ATP, UTP seems to be the preferred phosphoryl donor for dCK *in vivo* (Hughes *et al.*, 1997; Godsey *et al.*, 2006).

2.5. Three-dimensional structure

2.5.1. Overall structure

In 2003, Sabini *et al.* succeeded to solve the structure of human dCK in complex with its physiological substrate dCyd or the NAs Ara-C or dFdC at the acceptor site, ADP at the phosphoryl donor site, and the essential divalent cation Mg^{2+} , to a resolution of 1.6 Å. These crystal structures, with a nucleoside and $ADP-Mg^{2+}$, represent a dead-end complex composed of a mixture of substrates and products, which nonetheless allowed the authors to observe a closed conformation of the enzyme similar to the one expected in the presence of ATP. As illustrated in Figure 11A and B, human dCK is a homodimeric globular protein. Each monomer consists of a five-stranded, parallel β -sheet core surrounded by ten α -helices. The dimer interface is composed of a four-helix bundles corresponding to helices $\alpha 4$ and $\alpha 7$ of each monomer. Human dCK contains a 15-residue insert (Ser63-Asn77) between helix $\alpha 2$ and helix $\alpha 3$, which could be modeled in only one of the monomers. The dCK N-terminus (residues 1-19) was flexible and could not be modeled.

Three sequence motifs are important for dCK function (Figure 11C). The first sequence, which is highly conserved among deoxynucleoside kinases, is called the **P-loop** motif. It is located at residues Gly28-Ser35 and is implicated in the binding and positioning of the α - and β -phosphoryl groups of the phosphoryl donor. The second is called the **ERS** motif as it contains a glutamic acid (Glu-127 or E127), an arginine (Arg-128 or R128) and a serine (Ser-129 or S129). E127 is able to interact directly with Mg^{2+} . R128 is implicated in the substrate binding and facilitates the catalysis by bringing the 5'-OH group of dCyd in close proximity to the γ -phosphate of the phosphoryl donor allowing Glu-53, which acts as a base, to deprotonate the 5'-OH (Figure 12). The resulting activated 5'-O⁻ is responsible for the

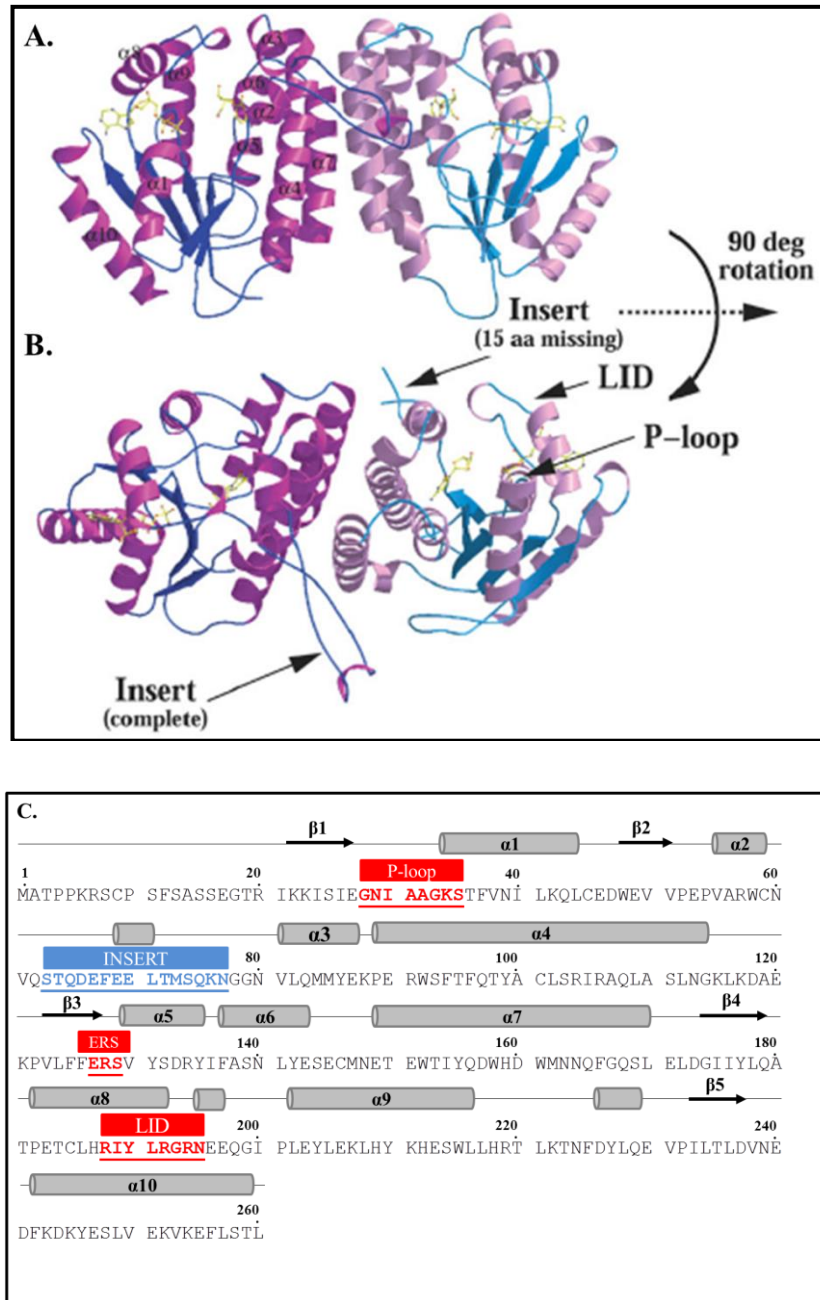


Figure 11: Overall fold of dCK.

A. Ribbon diagram of the dCK dimer in the presence of dCyd and ADP. **B.** View of "A" rotated 90°. **C.** Amino acid sequence of dCK with indication of the position of the β -sheets (1-5) and the α -helices (1-10). The insert region is in blue, whereas P-loop, ERS motif and LID region are in red (adapted from Sabini *et al.* 2003).

nucleophilic attack of the γ -phosphate of the phosphoryl donor. The last residue, S129, has a structural role. The third motif is the **LID** region. This loop, located at residues 188-195 (RIYLRGRN), is a flexible segment containing three conserved arginine residues. The LID

region encloses the phosphoryl donor when it binds and supplies critical residues involved in ATP binding and catalysis.

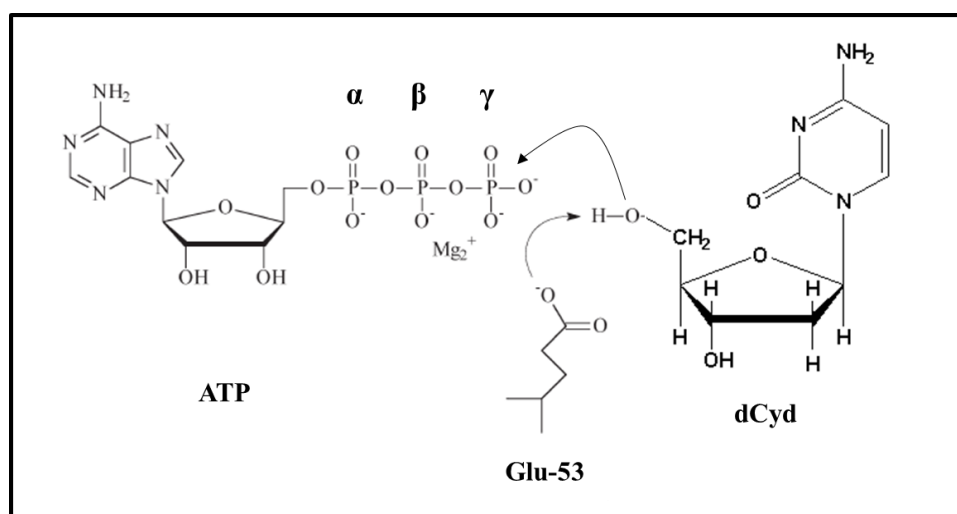


Figure 12: Mechanism of catalysis of dCK.

Glu-53 allows the deprotonation of the 5'-OH of dCyd into 5'-O⁻ that can then make a nucleophilic attack on the γ -phosphate of ATP (adapted from (Eriksson *et al.*, 2002)).

2.5.2. Influence of the nature of the phosphoryl acceptor and/or phosphoryl donor on dCK structure

Although it has been suggested that dCK could have different conformation states with pyrimidine and purine nucleosides (Bohman *et al.*, 1988; Kierdaszuk *et al.*, 1993; Johnsamuel *et al.*, 2005; Mani *et al.*, 2006), Zhang *et al.* (2006), but also Sabini *et al.* (2008b) demonstrated that dCK takes the same conformation when it is bound to a purine or a pyrimidine nucleoside and that the active site of dCK in the presence of a purine nucleoside is very similar to that obtained with a pyrimidine nucleoside in ADP-bound dCK. However, according to the size, shape and hydrogen binding capabilities of the substrate, several active residues move slightly to maintain the substrate-enzyme interactions. For example (Figure 13), when dCyd is replaced by dAdo, Asp-133 get closer to the NH₂ group of the adenine ring (2.5 Å) compared to the NH₂ group of the cytosine ring (2.9 Å). In the same time, Arg-104 moves subtly to make a tighter interaction with Asp-133 (Sabini *et al.*, 2008b).

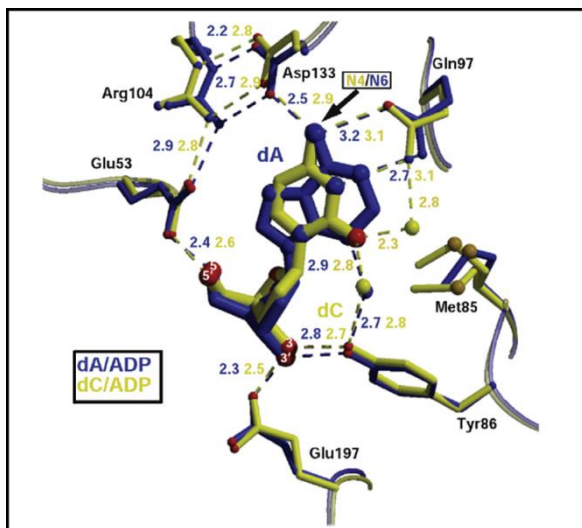


Figure 13: Binding of dAdo relative to dCyd requires only a slight adjustment of active sites residues. A stereoview of the overlay of the dAdo(dA)/ADP complex (blue) on the dCyd(dC)/ADP complex (green). Explanations are given in the text. (Sabini *et al.* 2008b).

In contrast to the preserved enzyme conformation between dCK-dCyd/ADP and dCK-dAdo/ADP complexes, the presence of UDP instead of ADP at the phosphoryl donor site with dAdo at the nucleoside site (dCK-dAdo/UDP *versus* dCK-dAdo/ADP) results in changes in the overall-fold structure of the protein (Sabini *et al.*, 2008b). The main conformational differences between the two complexes are limited to three distinct regions (labeled A, B and C in Figure 14A). These regions are discontinuous in their sequence, but together build a continuous surface. It is proposed that they communicate between each other upon binding of either ADP or UDP, which ultimately affects the conformation of the protein. The **region A** (residues 240-254), which is involved in the binding of the base moiety of the nucleotides, senses the differences between ADP and UDP and adopts a main-chain conformation that varies according to the nature of the nucleotide, resulting in different enzyme-nucleotide interactions. Compared with ADP, the presence of UDP induces inward shift of the base-sensing loop to make closer contact with the smaller uracil base. By reorganizing Phe-242 (F242), the carbonyl group of Glu-240 (E240) moves away from the uracil ring, while the side chain of Asp-241 (D241) is getting closer to the uracil base (Figure 14B). The second region influenced by the phosphoryl donor is the **region B** (residues 179-207 encompassing the LID motif). It is not yet clear how this region is connected to region A, but the difference in its conformation would occur mainly through Arg-188 (R188) that belongs to the LID motif and senses directly the nature of the donor (Figure 14C). In fact, position of the side chain of Arg-188 adjusts to the adenine or uracil base of the nucleotides and this adjustment propagates to the entire region. The third region that differs significantly in conformation between the ADP and UDP structures is the **region C** (residues 54-90). This region contains the flexible insert sequence (residues 63-77), which was found to adopt in the presence of UDP a very different

conformation from the one modelled in the presence of ADP (Sabini *et al.*, 2008b). This conformational change would involve mainly the displacement of three residues located just before and just after the insert region: Trp-58 (W58), Asn-80 (N80) and Tyr-86 (Y86) (Figure 14D). Importantly and in brief, dCK is able to sense the nature of the nucleotide at the donor site and adopts a more open conformation in regions A, B and C, when it is bound to UDP instead of ADP.

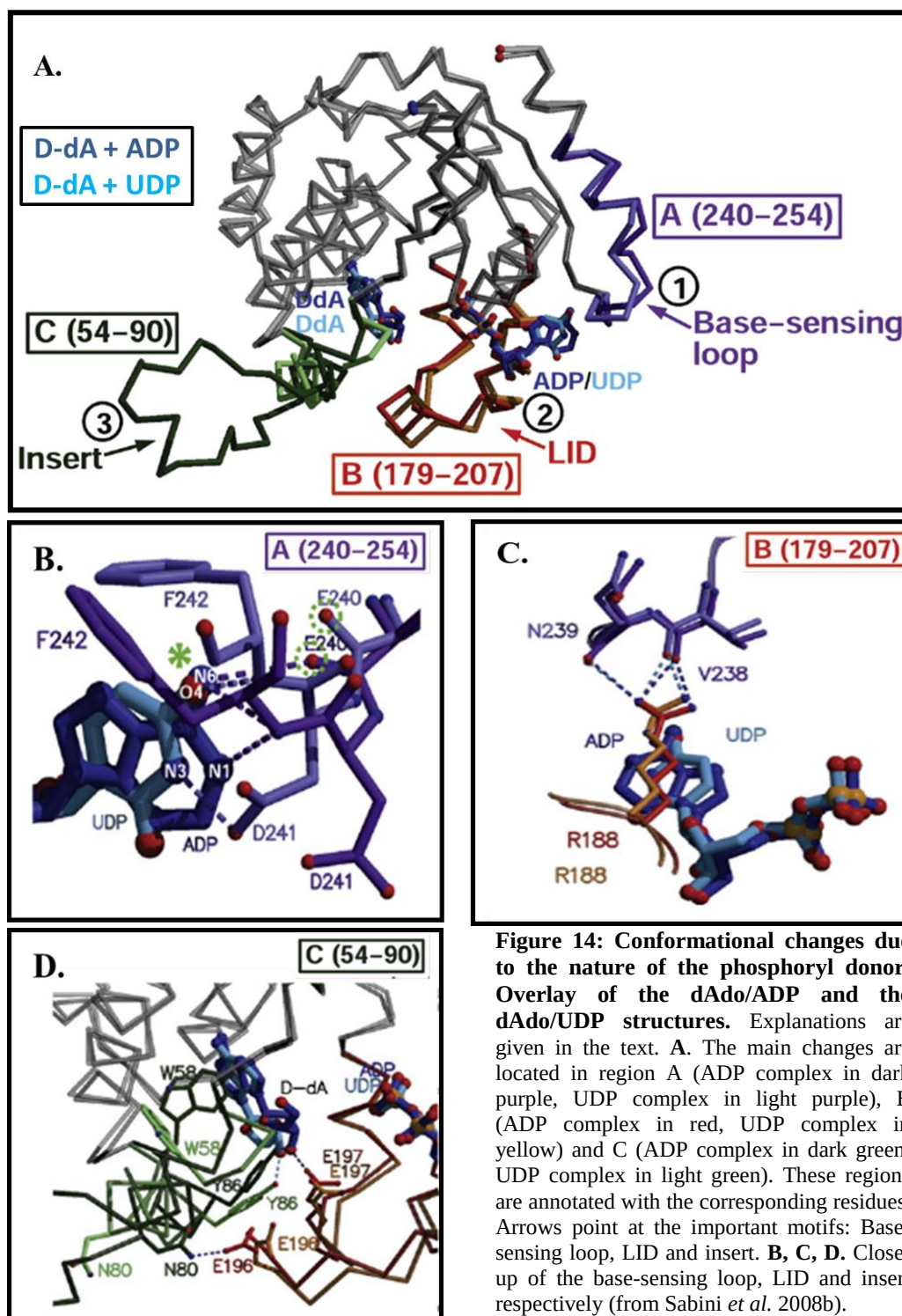


Figure 14: Conformational changes due to the nature of the phosphoryl donor. Overlay of the dAdo/ADP and the dAdo/UDP structures. Explanations are given in the text. **A.** The main changes are located in region A (ADP complex in dark purple, UDP complex in light purple), **B** (ADP complex in red, UDP complex in yellow) and **C** (ADP complex in dark green, UDP complex in light green). These regions are annotated with the corresponding residues. Arrows point at the important motifs: Base-sensing loop, LID and insert. **B**, **C**, **D.** Close-up of the base-sensing loop, LID and insert respectively (from Sabini *et al.* 2008b).

In addition to the conformational modifications in these three regions, superposition of dCK structures solved with ADP or UDP highlights an unexpected difference (Figure 15) in the conformation of the nucleoside dAdo (Sabini *et al.*, 2008b). In the ADP complex, dAdo binds in the *anti* conformation, whereas a *syn* conformation is observed in the UDP complex, which means a 180° rotation for the base moiety of dAdo. Nevertheless, the adenine ring conserves identical hydrogen-bonding pattern with dCK and occupies the same binding site regardless of being in *syn* or *anti* conformation. In contrast, the sugar moieties of the nucleoside occupy totally different positions. The distance between the 5'-OH group of the sugar and the catalytic Glu-53 residue is almost doubled in the *syn* conformation relative to the *anti* conformation, which suggests that the dAdo/UDP structure would be catalytically inactive. However, kinetic data prove that dCK is active with UTP as phosphoryl donor, which indicates that this inactive enzyme conformation would represent an intermediate state in the catalytic cycle. Finally, an important conclusion of the study performed by Sabini *et al.* (2008b) is that all these conformational differences are consistent with the different kinetic parameters measured in the presence of ATP or UTP (see section 2.4.2.).

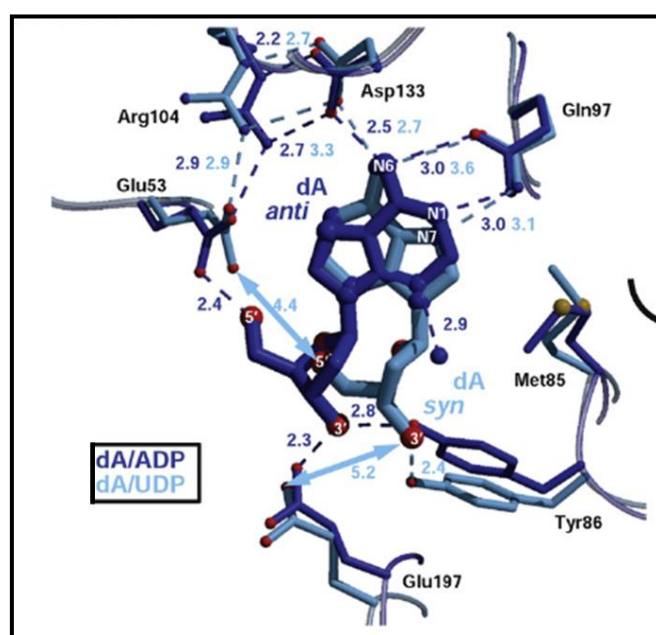


Figure 15: The nature of the phosphoryl donor influences the conformation of the phosphoryl acceptor. Overlay of the dAdo/ADP and the dAdo/UDP structures. dAdo binds in *anti* (dark blue) or *syn* (light blue) conformation in presence of ADP or UDP respectively (from Sabini *et al.* 2008b).

2.6. Regulation of dCK activity

Enzyme activity can be controlled by several mechanisms, including feedback inhibition, change in the rate of synthesis or degradation of the protein and covalent modification. The first of these mechanisms is rapid and reversible, whereas the two others induce a stable change of the enzyme activity.

2.6.1. Feedback regulation

Deoxynucleoside kinases are regulated by a feedback mechanism through the products of the salvage pathway. The best inhibitor of a dNK is usually the triphosphate form of its preferred substrate. Therefore, dCK is negatively regulated by dCTP for which a K_i of 0.7-6 μM has been determined in the presence of ATP (Kessel, 1968; Ives *et al.*, 1970; Bohman *et al.*, 1988; Datta *et al.*, 1989b; Kim *et al.*, 1989). Since the intracellular concentration of dCTP is around 10-20 μM (Datta *et al.*, 1989b; Sarup *et al.*, 1989), it might be expected that dCK could be inhibited in physiological conditions. However, dCTP was found to be much less inhibitory, with a K_i of 57.2 μM , when dCK activity was analyzed in the presence of UTP, suggesting that the enzyme should not be significantly inhibited in the cellular environment (Shewach *et al.*, 1992). Concerning the mechanism by which dCTP inhibits dCK, it was proposed that dCTP acts as a bisubstrate analog, binding and bridging both substrates sites on the enzyme (Jansson *et al.*, 1990). Other nucleotides considered as potential inhibitors of dCK, like dADP, dCDP, dGMP, would not play a role in the regulation of dCK activity, due to low intracellular concentrations (Datta *et al.*, 1989b).

2.6.2. Regulation at the protein level

The human dCK gene (HGNC:2704) is localized on chromosome 4 band q13.3-q21.1 and is a single-copy gene of 34 kb with a coding region composed of seven exons (from 90 to 1544 base pairs (bp)) (Song *et al.*, 1993; Stegmann *et al.*, 1993). The dCK promoter activity was localized to a 697-bp upstream fragment. This highly GC-rich (71%) transcription regulatory region lacks a TATA-box, but contains several binding sites for transcription factors, such as Sp1 (specificity protein 1) and USF (upstream stimulatory factor). This initiation region also contains an imperfect (half) E2F (eukaryotic transcription factor 2)

binding site (Johansson *et al.*, 2000; Ge *et al.*, 2003; Kim *et al.*, 2008). Although the transcriptional importance of these binding sites has been evidenced by site-directed mutagenesis, the detailed mechanisms implicated in the regulation of dCK gene expression are still unclear (Ge *et al.*, 2003).

Though not yet explained, several data are in favor of the existence of regulatory mechanisms for dCK gene transcription. Indeed, it has been observed that 5-azacytidine, a DNA methyltransferase inhibitor, could induce re-expression of the dCK gene, leading to increase of dCK protein and activity in leukemic blasts or resistant cell lines. These results suggest that expression of the dCK gene could be down-regulated by methylation (Avramis *et al.*, 1989; Kong *et al.*, 1991). These results were confirmed by Hodzic *et al.* (2011) who demonstrated the role of promoter methylation in the regulation of dCK gene expression in different cancer cell lines. Moreover, the same authors highlight the existence, in various colon and lung cancer cell lines, of a negative correlation between micro-RNA 330 expression and dCK mRNA expression, suggesting a role of this micro-RNA in the post-transcriptional regulation of dCK (Hodzic *et al.*, 2011).

In addition, increased mRNA and/or dCK protein levels have been observed in rat liver after administration of dCyd or steroids over 2-3 days (Weber *et al.*, 1993), in leukemic cells after interleukin-3 treatment (Cao *et al.*, 1996), in colon cancer cell lines and in various murine and human cell lines after a 24 h-exposure to γ -irradiation (Wei *et al.*, 1999; Gregoire *et al.*, 2002), in pancreatic tumor cells after administration of fluvastatin (Bocci *et al.*, 2005) or in neuroblastoma after treatment with cyclopentenyl cytosine, a CTP synthetase inhibitor (Bierau *et al.*, 2006). More recently, hypoxia was identified as a novel regulator of dCK expression, at both mRNA and protein dCK level, in chronic lung disease (Weng *et al.*, 2013).

Although it is well-admitted that dCK is preferentially expressed in lymphoid tissues and is up-regulated in several solid tumors, the molecular mechanisms leading to tissue-specific and proliferation-dependent transcription of dCK are not clearly understood (Arner *et al.*, 1995). In 1993, Song *et al.* proposed that the signals responsible for the tissue-specific expression levels of dCK in lymphocytes could be carried by the second intron of the dCK gene. Later, it was proposed that a direct interaction between Sp1 and USF proteins could, depending on the relative levels and tissue distributions of these proteins, regulate the transcription of the human dCK gene (Ge *et al.*, 2003). Despite these hypotheses, the true

reason for which dCK is predominantly expressed in lymphoid tissues and is almost absent in tissues such as resting or terminally differentiated cells remain to be elucidated.

Concerning cell cycle regulation of dCK, discrepancies exist in the literature. Different groups reported an increase of dCK activity in S-phase (Pegoraro *et al.*, 1971; Persohn *et al.*, 1976; Wan *et al.*, 1978), whereas others showed that dCK activity remained constant through the cell cycle (Howard *et al.*, 1974; Arner *et al.*, 1988). Subsequent studies demonstrated that cell cycle variation in dCK activity was in fact dependent on the cell type. In contrast, in all these studies, dCK mRNA level was found to be constant at all growth stages (Hengstschlager *et al.*, 1993). Moreover, contrary to TK1 mRNA, dCK mRNA was not increased by mitogens, like phorbol ester or phytohemagglutinin (Mitchell *et al.*, 1993; Spasokoukotskaja *et al.*, 1995), confirming that dCK expression is not dependent on the cell cycle phases. The fact that there is only an half E2F site in the dCK promotor region might explain why the dCK gene is not cell cycle-regulated, like TK1 (Arner *et al.*, 1995).

Finally, to the best of our knowledge, the regulation of dCK degradation has not yet been carefully investigated. Preliminary data obtained in our laboratory indicate that dCK half-life is high, being around 17 h (personal communication from Eliza Starczewska).

2.6.3. Post-translational regulation

2.6.3.1. Evidences for regulation by reversible phosphorylation

The hypothesis of a regulation of dCK activity by post-transcriptional or post-translational mechanisms was initially proposed by Hengstschläger *et al.* (1993) following the observation that dCK activity varied during the cell cycle of some cell lines without any changes in dCK mRNA level. Afterwards, it was reported that human dCK from leukemic blasts could be phosphorylated *in vitro* by protein kinase $C\alpha$ (PKC α), a kinase implicated in various cellular processes including proliferation, apoptosis and differentiation (Wang *et al.*, 1994). Phosphorylation of dCK by this PK induced increase of the V_{max} , but also of the K_m , so that the catalytic efficiency of dCK was finally reduced. Another study reported that recombinant dCK was a poor substrate of PKC, whereas PKA was able to phosphorylate it efficiently, but without changing its kinetic parameters (Spasokoukotskaja *et al.*, 2000).

On the other hand, in the late 1990s and the early 2000s, several studies showed that dCK activity could be increased, up to 3-fold, upon short-term treatments (0.5 to 2h) by genotoxic agents, including therapeutic NAs (CdA, Ara-C, F-ara-A and CAFdA), aphidicolin (a DNA polymerase inhibitor), etoposide (a topoisomerase II inhibitor), hydrogen peroxide, and UV- or γ -irradiation in human normal or leukemic lymphocytes (Sasvari-Szekely *et al.*, 1998; Spasokoukotskaja *et al.*, 1998; Spasokoukotskaja *et al.*, 1999; Cardoen *et al.*, 2001; Csapo *et al.*, 2001; Csapo *et al.*, 2003; Van Den Neste *et al.*, 2003). Activation of dCK by these different agents could be explained neither by an allosteric effect nor by an increase in dCK protein level, suggesting that dCK might be regulated by a post-translational covalent modification, such as phosphorylation. In addition, dCK activity was found to be increased by protein phosphatase inhibitors, such as NaF or calyculin A, in intact cells (Csapo *et al.*, 2001; Csapo *et al.*, 2003) and decreased by sorbitol-induced osmotic stress, known to induce important changes in the activation state of various protein kinases and phosphatases (Csapo *et al.*, 2003). Also, it was found in our lab (Smal *et al.*, 2004) that dCK activity was enhanced in different types of leukemic cells by several cell-permeable protein kinase inhibitors, like genistein (a protein tyrosine kinase inhibitor), AG-490 (a JAK2 and JAK3 inhibitor) and inhibitors of the MAPK/ERK pathway, as well as by the protein phosphatase inhibitor okadaic acid. Finally, it was shown that partially purified dCK or dCK from crude lysates could be inactivated by the recombinant λ protein phosphatase (λ -PP), a PP with Ser/Thr/Tyr dephosphorylating activity (Csapo *et al.*, 2001; Smal *et al.*, 2004).

2.6.3.2. Evidences for regulation by Ser-74 phosphorylation

The direct demonstration that dCK was regulated *in vivo* by reversible phosphorylation was provided in 2006 by our group (Smal *et al.*, 2006). It was indeed shown that dCK overexpressed in HEK293T cells could be labeled during incubation with [32 P]orthophosphate and that this labeling was reduced by osmotic stress known to reduce dCK activity. After purification of 32 P-labeled dCK, digestion by trypsin, and analysis of radioactive peptides by tandem mass spectrometry, four *in vivo* phosphorylation sites were identified: Thr-3, Ser-11, Ser-15 and Ser-74, the latter being the major phosphorylated residue. This residue, located in the insert region, is approximately 30 Å from the active site (Soriano *et al.*, 2007) (Figure 16) and is well conserved among vertebrates (Figure 17). Site-directed mutagenesis experiments in HEK293T cells demonstrated that phosphorylation of the three N-terminus sites did not influence dCK activity, whereas Ser-74 phosphorylation was

crucial (Smal *et al.*, 2006; Smal *et al.*, 2010a). In addition, use of a specific anti-phospho-Ser-74 antibody allowed for detection of Ser-74 phosphorylation on endogenous dCK in leukemic CCRF-CEM cells and increase of this phosphorylation after treatment of cells by agents that enhance dCK activity (Smal *et al.*, 2006). Further studies performed in lymphocytes isolated from CLL patients showed that interindividual variability in dCK activity could be related to variation in the level of dCK phosphorylation on Ser-74 (Smal *et al.*, 2007b). All these data gave direct evidences that phosphorylation of Ser-74 plays an essential role in the control of dCK activity in intact cells.

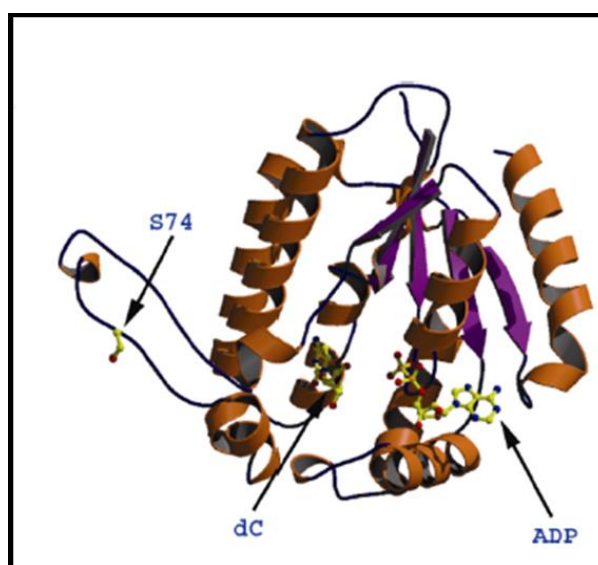


Figure 16: Ribbon diagram of a dCK monomer highlighting the Ser-74 phosphorylation site in the insert region (from McSorley *et al.*, 2008).

	S74
Human dCK	WCNVQS-TQDEF E ELTM S QKNGGNVLQMMY
<i>M.musculus</i> dCK	WCNVQS-TQEEF E ELTT S QKSGGNVLQMMY
<i>X.laevis</i>	WCNIQS-CKDEF E ELTT S QKSGGNLLQMMY
<i>G.gallus</i>	WCNVQQNSEED C EELTT S QKSGGNVLQMMY
<i>D.rerio</i>	WCNVQT-QHDDH E ELTT S QKSGGNVLQMMY
<i>T.nigroviridis</i>	WCNVQT-QGDDFQ E ELTI S QKSGGNVLKMMY

Figure 17: Conservation of Ser-74 phosphorylation site of dCK in vertebrate species

The dCK or homologous protein amino acid sequences were obtained from NCBI: human (accession number A38585); mouse (accession number NP_31858.1); frog (*Xenopus laevis*, accession number AAH84070.1); chicken (*Gallus gallus*, accession number NP_001006451.1); fish (*Danio rerio*, accession number AAH83277.1; *Tetraodon nigroviridis*, accession number CAF97102.1). The sequences were aligned by ClustalW (www.ebi.ac.uk/clustalw/). Conserved residues are in boldface; phosphorylated residues are in red; acidic residues are in green. (Smal *et al.*, 2006)

2.6.3.3. Influence of Ser-74 phosphorylation on dCK kinetics and structure

In all the experiments reported above, dCK activity was assayed in the presence of 10 μ M dCyd, which is the concentration regularly used in dCK activity assays. An important question was to know whether phosphorylation of dCK on Ser-74 also increases dCK activity toward its numerous other substrates. This problem was investigated by McSorley *et al.* (2008) who mimicked Ser-74 phosphorylation by introducing a Ser-74-Glu (S74E) mutation in bacterially produced dCK. By comparing the enzyme kinetics of this mutated dCK to that of wild-type dCK, they found that S74E mutation increased the K_{cat} values ~11-fold for dCyd, ~3-fold for the pyrimidine NAs dFdC and Ara-C, but decreased it for dAdo and dGuo. On the other hand, it was observed that the S74E mutation systematically increased the K_m values. Therefore, the catalytic efficiency, as given by the K_{cat}/K_m ratio, was increased about 2 fold for dCyd, remains unchanged for dFdC and Ara-C, and was decreased about 5-fold in the case of dAdo and dGuo. These results showed that the influence of Ser-74 phosphorylation on dCK activity varies depending on the substrate. The first aim of this thesis was to deal with this question more in depth and particularly to investigate whether an increase of Ser-74 phosphorylation could favor intracellular activation of NAs (Chapter II).

Insight into the mechanism by which phosphorylation of Ser-74, a residue far from the active site, modifies dCK activity has been provided by Hazra *et al.* (2011). The authors of this study, who succeeded in crystallizing dCK containing the S74E mutation, propose a model in which Glu-74 stabilizes the open conformation of dCK by direct interaction with Trp-58 (Figure 18). As this conformation is compatible with binding of the nucleoside and release of the product, but not with phosphoryl transfer (Sabini *et al.*, 2008b), the S74E mutation increases the energy barrier to the formation of the catalytically competent closed state, explaining the higher K_m of the S74E dCK for its substrates. The increased K_{cat} of this variant, as observed for dCyd, would result from a more rapid transition to the open state after phosphoryl transfer, allowing for nucleoside monophosphate release (Hazra *et al.*, 2011). However, the reason why the S74E dCK displays a decreased activity toward the purines dAdo and dGuo (McSorley *et al.*, 2008) remains unclear.

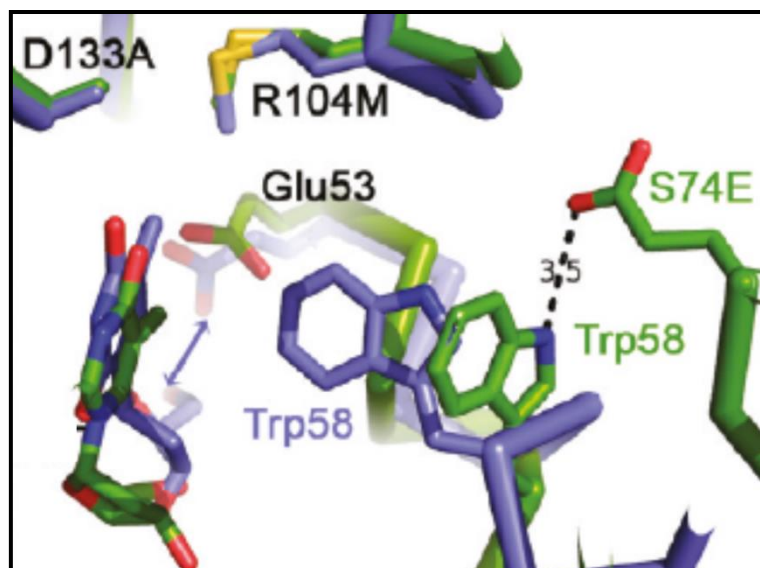


Figure 18: The S74E mutation, done to mimic S74 phosphorylation, stabilizes the open dCK conformation.

Explanations are given in the text. The presence of S74E mutation (green) stabilizes the open dCK conformation mainly through direct interaction with Trp-58 (Hazra *et al.*, 2011).

2.6.3.4. Signaling pathway(s) involved in the phosphorylation of Ser-74

As Ser-74 phosphorylation plays a key role in the regulation of dCK activity, it is important to identify the protein kinase(s) and phosphatase(s) that directly phosphorylate(s) or dephosphorylate(s) this residue. Their identification constitutes one of the main objectives of our group. Our investigations (see Annex Section) have shown that casein kinase 1 δ was able to phosphorylate dCK on Ser-74 *in vitro*, but would not be responsible for its *in vivo* phosphorylation (Smal *et al.*, 2010b). By contrast, the DNA-damage-response kinase ATM was found to phosphorylate Ser-74 *in vitro* and seems also to phosphorylate it *in vivo*, at least after γ -irradiation, since increase in Ser-74 phosphorylation induced by this treatment could not be detected in ATM-deficient cells or in ATM shRNA transfected cells (Yang *et al.*, 2012). These results were in accordance with a previous large-scale proteomic study identifying Ser-74-dCK as one of the 700 proteins phosphorylated by ATM (Matsuoka *et al.*, 2007). Moreover, ATM-mediated dCK phosphorylation could contribute to the activation of the G2/M checkpoint. Indeed, when dCK is phosphorylated on Ser-74, it could interact with and inhibit Cdk1 (cyclin-dependent kinase 1), a kinase essential for G2/M phase transition (Yang *et al.*, 2012). Finally, work in progress in our lab suggests that another kinase than ATM could also phosphorylate Ser-74 and regulate dCK activity (personal communication from Maxime Beyaert).

Concerning the protein phosphatase (PP) that dephosphorylates Ser-74 in intact cells, its responsiveness to okadaic acid (OA) has been well established. Indeed, this PP inhibitor increased dCK activity (Smal *et al.*, 2004) and Ser-74 phosphorylation (Smal *et al.*, 2010b) in cultured cells. These results constituted the starting point of the study presented in Chapter III, which aimed to identify the OA-sensitive PP implicated in Ser-74 dephosphorylation.

3. Phosphoprotein phosphatases

3.1. Introduction to reversible protein phosphorylation

Protein phosphorylation is a major control mechanism of a wide range of cellular processes, including cell division, growth and development, metabolism, survival, proliferation and apoptosis (Hunter, 1995; Novak *et al.*, 2010; Zhang *et al.*, 2013). This control is possible through a precise modulation of the phosphorylation level of proteins by both phosphorylation and dephosphorylation reactions, catalyzed by protein kinases and phosphatases, respectively. Aberrant protein phosphorylation, which can be due to imbalance of their activities, has been linked to a variety of diseases, such as cancer, cardiac hypertrophy, neurodegeneration and diabetes, illustrating the importance of this regulatory mechanism (De Munter *et al.*, 2013; Zhang *et al.*, 2013).

Phosphorylation of proteins is catalyzed by a superfamily of enzymes called protein kinases (Brautigan, 2013). These enzymes catalyze the transfer of the γ -phosphoryl group of ATP onto the side chains of acceptor proteins. In eukaryotic cells, phosphorylation mainly occurs on three amino acid residues containing a hydroxyl group: tyrosine (Tyr), threonine (Thr) and serine (Ser), the latter being by far the most phosphorylated residue. Indeed, proteomic analysis of 6600 phosphorylation sites on 2244 human proteins showed that phosphotyrosine (pTyr), phosphothreonine (pThr) and phosphoserine (pSer) accounted for 1.8, 11.8 and 86.4% respectively, of the phosphorylated residues (Olsen *et al.*, 2006). Human genome contains 518 putative protein kinases that can be classified into two families: 90 protein tyrosine kinases (PTKs) and 428 protein Ser/Thr kinases (PSKs) (Lander *et al.*, 2001; Venter *et al.*, 2001; Johnson *et al.*, 2005). All these protein kinases share a similar structure, operate with the same mechanism and select their protein substrates through the recognition of consensus amino acid sequences surrounding the target phosphorylation sites (Peti *et al.*, 2013). The biochemistry of the kinase superfamily is thus relatively simple.

In contrast to protein kinases, protein phosphatases developed a diverse biochemistry, with separate superfamilies of enzymes that have different 3D structures, and different active sites with different mechanisms of hydrolysis. There are 107 putative protein Tyr phosphatases (PTPs), which roughly match with the number of PTKs (Alonso *et al.*, 2004).

However, there are only 43 protein Ser/Thr phosphatases (PSPs), which represents barely 10% of the number of the corresponding PSKs (Zhang *et al.*, 2013). Therefore, PSPs have evolved diversified strategies to act upon a greater variety of substrates. PSPs and PTPs have distinct structural characteristics, which give rise to very different catalytic and regulatory mechanisms. PTPs, which were *a priori* not involved in Ser-74 dephosphorylation, will not be considered in this section.

3.2. Classification and mechanisms of catalysis of PSPs

PSPs can be classified into three major families: PPPs (phosphoprotein phosphatases), which represent the largest family, PPMs (metal-dependent protein phosphatases) and aspartate-based phosphatases (Shi, 2009) (Figure 19).

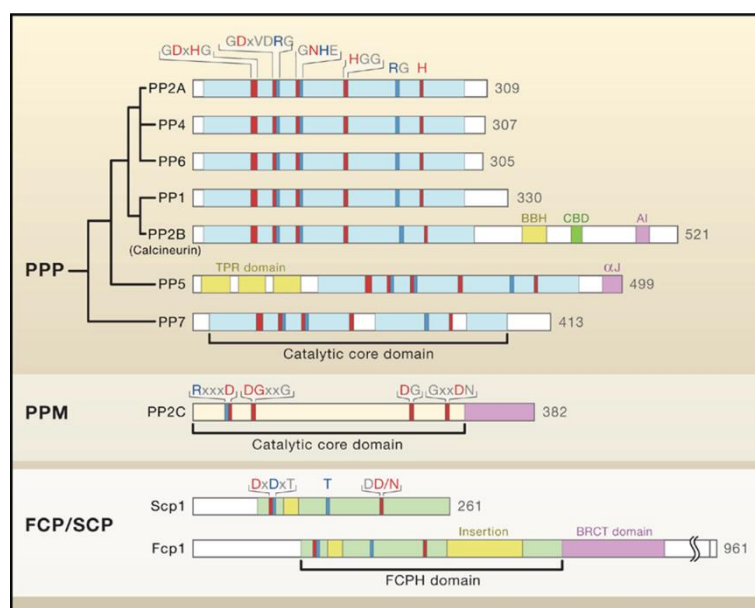


Figure 19: The three families of Ser/Thr protein phosphatases.

The PSPs can be categorized into three families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases such as FCP (TFIIF-associated component of RNA polymerase II CTD phosphatase) and SCP (small CTD phosphatase). Representative members of each family are presented. The catalytic core domains of each protein are indicated below the diagram. (Reviewed in more details in Shi (2009)).

PPP family, which plays the major role for dephosphorylation of Ser/Thr residues in proteins, comprises several sub-families: PP1, PP2A and PP2B (known also as calcineurin) as well as the more recently identified PP4, PP5, PP6 and PP7, which all share conserved active site residues (Figure 20A) (Shi, 2009). Also, except PP5, they are multimeric. The PPM family phosphatases are Mg^{2+}/Mn^{2+} -dependent enzymes that are mostly monomeric proteins, such as PP2C and pyruvate dehydrogenase phosphatase. The third family, the aspartate-based phosphatases, represented by FCP (TFIIF-associating component of RNA polymerase II CTD phosphatase) and SCP (small CTD phosphatase), are also single subunit proteins that require the presence of a metal ion (Mg^{2+}) in the active site for the catalysis. For both PPPs and PPMs, the presence of two metal ions (Mg^{2+} , Mn^{2+} , Fe^{2+} , Zn^{2+}) is essential for catalysis (Figure 20B) (Zhang *et al.*, 2013). These two metal ions are coordinated at the active site to mediate the phosphate transfer without forming an intermediate. A hydroxyl ion bridging the two metal ions takes part in nucleophilic attack on the phosphate group of the substrate. In contrast, for the aspartate-based phosphatases, the catalysis mechanism is different. The phosphate transfer requires the Dx Dx(T/V) (D: aspartate; T: threonine; V: valine) motif and the presence of a Mg^{2+} ion in the active site (Figure 20C). In the first step, the N-terminal aspartate of the Dx Dx(T/V) motif makes a nucleophilic attack on the phosphate group of the substrate to form a phospho-aspartyl intermediate, which is then hydrolyzed in the second step (Zhang *et al.*, 2013).

The vast majority of Ser and Thr dephosphorylations in eukaryotic cells are catalyzed by members of the PPP and PPM families, while the only known substrate for FCP/SCP is the serine-rich C-terminal domain (CTD) of RNA polymerase II (Shi, 2009). Although both PPPs or PPMs could theoretically be involved in the regulation of dCK activity *via* Ser-74 dephosphorylation, the previous observation that Ser-74 dephosphorylation was responsive to okadaic acid (OA) (Smal *et al.*, 2010b) indicated that the PP responsible for this dephosphorylation could belong to the PPP family. Indeed, most of the PPP members are sensitive to OA, whereas PPMs cannot be inhibited by this toxin (Swingle *et al.*, 2007). Therefore, we will only focus on the different members of the PPP family in the next part of this section.

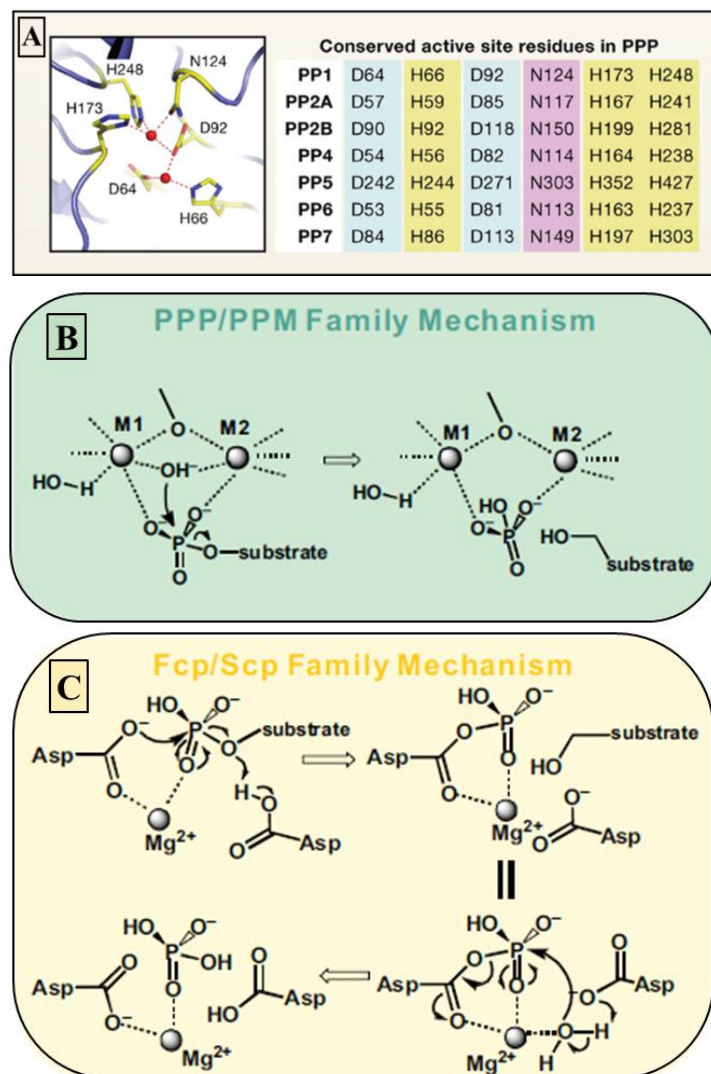


Figure 20: Catalytic mechanisms of Ser/Thr protein phosphatases.

(A) Conserved coordination of the binuclear metal center in the members of the PPP family (Shi, 2009). Catalytic mechanisms of (B) PPP/PPM and (C) FCP/SCP families. Explanations are given in the text (Zhang *et al.*, 2013).

3.3. Highlighting individual PPP

3.3.1. Protein phosphatase 1

Protein phosphatase 1 (PP1) is a major protein Ser/Thr phosphatase ubiquitously expressed in all eukaryotic cells. PP1 has been highly investigated and found to dephosphorylate a multitude of substrates and to regulate a variety of cellular processes, including meiosis, cell division, apoptosis, metabolism, protein synthesis, cytoskeletal reorganization, and regulation of membrane receptors and channels (Cohen, 2002; Ceulemans *et al.*, 2004; Shi, 2009).

Each functional PP1 enzyme is composed of two subunits, a catalytic subunit and a regulatory subunit (R subunit). The catalytic subunit of PP1 is highly conserved in eukaryotes, with protein sequence identity exceeding 70% in any pairwise alignment, and shares a conserved fold and a similarly positioned active site with all members of the PPP family (Figure 20A). Human genome contains three highly related genes that encode four distinct catalytic subunits of PP1: PP1 α , PP1 β/δ and the splice variants PP1 γ 1 and PP1 γ 2 (Cohen, 1988; Dombradi *et al.*, 1990; Sasaki *et al.*, 1990; Cohen, 2002). These isoforms are highly homologous and only differ in the C-terminal domain. Except PP1 γ 2, which is found exclusively in the testis, all the isoforms are ubiquitously expressed. Although the catalytic PP1 subunits appear to have little intrinsic specificity, they typically acquire their specificity by forming dimers with regulatory (R) proteins. These R subunits may target the PP1 catalytic subunits to specific cellular compartment, modulate substrate specificity, or serve as substrates themselves. Until now, more than 200 putative R subunits have been identified (Bollen *et al.*, 2010; Brautigan, 2013; Peti *et al.*, 2013), representing a large number of PP1-binding possibilities. Most PP1 regulatory proteins and some PP1 substrates interact with PP1 via a small PP1 binding motif, the RVxF motif. This motif generally conforms to the consensus sequence [K/R][K/R][V/I][x][F/W] (K: Lys, R: Arg, V: Val, I: Ile, F: Phe, W: Trp), where x is any residue other than Phe, Ile, Met, Tyr, Asp or Pro (Wakula *et al.*, 2003; Meiselbach *et al.*, 2006; Hendrickx *et al.*, 2009; Peti *et al.*, 2013). While the RVxF interaction is required for anchoring the regulatory subunit to the catalytic PP1 subunit, it does not induce conformational changes of PP1 and does not influence PP1 activity (Egloff *et al.*, 1997; Terrak *et al.*, 2004; Hurley *et al.*, 2007; Bollen *et al.*, 2010). However, this binding event is essential to allow the R subunit to direct PP1 specificity. Critically, the RVxF binding site comprises residues that are either invariant or highly conserved among all PP1 isoforms from yeast to humans, but are not conserved in PP2A or PP2B, which explains why PP1 regulatory proteins with RVxF motifs do not bind other PPPs (Watanabe *et al.*, 2001).

In addition to targeting subunits, PP1 is regulated by several inhibitory proteins such as inhibitor-1 (I-1), inhibitor-2 (I-2), and DARPP-32. Specific inhibition of PP1 by I-1 and DARPP-32 requires phosphorylation of a conserved threonine at the N-termini of the inhibitory proteins, which is thought to bind the active site of PP1 (Desdouits *et al.*, 1995; Shi, 2009; Peti *et al.*, 2013). PP2A and PP2B are not sensitive to inhibition by I-1 or I-2, which was the basis for classification of type 1 (PP1) versus type 2 phosphatases (PP2) (Ingebritsen *et al.*, 1983).

3.3.2. Protein phosphatase 2A

PP2A is ubiquitously expressed and one of the most abundant enzymes, accounting for more than 1% of total cellular proteins in some tissues (Shi, 2009; Peti *et al.*, 2013). Together with PP1, PP2A constitutes the majority of Ser/Thr phosphatase activity in a given tissue or cell (Lambrecht *et al.*, 2013). PP2A is thus implicated in many cellular events, including development, cell proliferation, apoptosis, cell motility, cell-cycle regulation and many other signaling pathways (Shi, 2009). It also controls cell metabolism by regulating the activity of enzymes involved in glycolysis, lipid metabolism and catecholamine synthesis (Tung *et al.*, 1985). To be functional, PP2A requires at least two distinct components: a catalytic subunit (C subunit) and a structural (or scaffold) subunit (also known as the A or PR65 subunit), which together form the core of the enzyme. This heterodimer (PP2A_D) can also interact with a variety of regulatory B-type subunits, leading to the formation of a wide range of heterotrimeric holoenzymes (PP2A_T) with diverse specificities (Eichhorn *et al.*, 2009; Lambrecht *et al.*, 2013). It is to note that the dimeric complexes (PP2A_D) constitute about one-third of PP2A complexes, whereas the majority of them are heterotrimers, consisting of one catalytic C subunit, one structural A subunit and one variable regulatory B-subunit (Figure 21) (Janssens *et al.*, 2001; Lambrecht *et al.*, 2013).

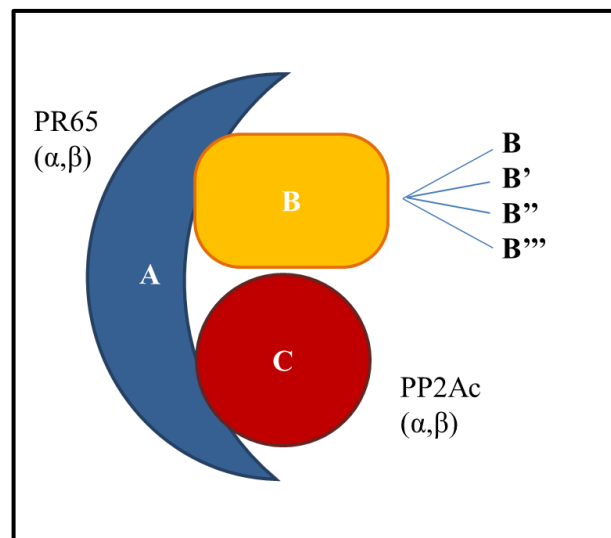


Figure 21: Schematic representation of the structural diversity of PP2A holoenzyme complexes.

PP2A holoenzymes consist of a core dimer composed of a structural A subunit (PR65, existing as α and β isoform) and a catalytic C subunit (PP2Ac, existing as α and β isoform), which is generally associated with one of the regulatory B-type subunits (divided in four different sub-families: B, B', B'', B''') to form an heterotrimer. Adapted from Lambrecht *et al.* (2013).

3.3.2.1. The core enzyme

The catalytic subunit of PP2A is encoded by two distinct ubiquitously expressed genes, *PPP2CA* and *PPP2CB*. The resulting α and β isoforms of 35 kDa in size are very similar as they share 97% sequence identity. However, due to a weaker promoter, the expression of the β isoform is tenfold lower than that of the α isoform (Janssens *et al.*, 2001; Eichhorn *et al.*, 2009). Once expressed, the catalytic C subunit requires interaction with the structural A subunit to be active. The latter also exists as a α (PR65/A α) and a β (PR65/A β) isoform encoded by two different genes, *PPP2R1A* and *PPP2R1B*. The PR65/A α isoform is expressed at much higher level than PR65/A β and is found in 90% of PP2A_D complexes. Both isoforms are very similar as they contain 15 tandem HEAT (Huntingtin/elongation/A-subunit/TOR) repeats (Hemmings *et al.*, 1990) essential for the binding of B-type and C subunits (Lambrecht *et al.*, 2013). Canonical HEAT motif consists of a helical hairpin made by two helices. To form the core enzyme, the C subunit binds to the structural A subunit via the five C-terminal (11-15) HEAT repeats. This interaction induces a conformational change of the structural A subunit, which folds in on itself forming a horseshoe shape-like structure (Xu *et al.*, 2006; Cho *et al.*, 2007).

Although other members of the PPP family show important similarity with the C subunit of PP2A, they do not associate with the structural A subunit. Indeed, the amino acid residues that allow the interaction of the C subunit with the A subunit are mostly replaced by non-conserved amino acids in PP1, PP2B, PP5 and PP7. However, PP4 and PP6 represent an exception as they contain the majority, but not all, of these interface residues (Shi, 2009). Table 3 summarizes the main characteristics of structural and catalytic PP2A subunits.

Subunit	Gene	Isoform	Other name	Chromosome number	Normal tissue distribution	Subcellular distribution	Aberrant tissue distribution
Structural (A)	<i>PPP2R1A</i>	α	PR65 α , PP2A-A α	19q13.33	Ubiquitously expressed in all the tissues (Hemmings <i>et al.</i> , 1990)	Cytosol	Lung, breast, melanoma, endometrial and ovarian cancer (Calin <i>et al.</i> , 2000)
	<i>PPP2R1B</i>	β	PR65 β , PP2A-A β	11q23.2	Ubiquitously expressed and highly expressed in ovary (oogenesis) (Hemmings <i>et al.</i> , 1990)	Cytosol	Lung, breast, colon, ovarian, B-CLL (Calin <i>et al.</i> , 2000)
Catalytic (C)	<i>PPP2CA</i>	α	PP2A α	5q31.1	Brain and heart (HE) (Arino <i>et al.</i> , 1988; Khew-Goodall <i>et al.</i> , 1988)	Cytoplasm and nucleus	Prostate (Bhardwaj <i>et al.</i> , 2011)
	<i>PPP2CB</i>	β	PP2A β	8p12	Brain and heart (HE) (Arino <i>et al.</i> , 1988; Khew-Goodall <i>et al.</i> , 1988)	Cytoplasm and nucleus	–

Table 3: The various isoforms of PP2A A and C subunits, their nomenclature, chromosomal location and normal/aberrant expression in various cancer and normal tissues.

HE: highly expressed (From Seshacharyulu *et al.*, 2013).

3.3.2.2. The regulatory B subunits

By interacting with the core enzyme, the regulatory B-type subunits are responsible for the localization and the specificity of the PP2A holoenzymes. By contrast with A and C subunits, their expression differs in a tissue-specific manner. In human genome, 15 genes have been identified that encode at least 24 different regulatory B-type subunits. They are divided into four families: B (also known as PR55 or B55), B' (PR61 or B56), B'' (PR72) and B''' (PR93/PR110), the different numbers corresponding to the approximate molecular weight of each peptide in kDa. Each family contains between three and eight isoforms, which can be, in some cases, the result of alternative splicing. Despite their large diversity and total lack of sequence similarity between the different families, B-type subunits always bind to the 1-10 HEAT repeat region of the structural A subunit (Janssens *et al.*, 2001). Main characteristics of regulatory B-type subunits are given in Table 4.

PP2A-B. Encoded by four distinct genes (*PPP2R2A*, *PPP2R2B*, *PPP2R2C* and *PPP2R2D*), the 55kDa B subunits exist in five isoforms (α , β_1 , β_2 , γ , δ). Both α and δ isoforms are widely distributed, whereas β and γ isoforms are particularly expressed in brain. The B subunits play important roles in many cellular processes and signaling pathways, encompassing mitosis (Barr *et al.*, 2011; Wurzenberger *et al.*, 2011), apoptosis (Janssens *et al.*, 2012), tau dephosphorylation (Torrent *et al.*, 2012), ERK (extracellular signal-regulated kinase) pathway (Eichhorn *et al.*, 2009) and DNA damage response (Kalev *et al.*, 2012). Furthermore, PP2A-B has been associated with cytoskeletal dynamics and nuclear translocation. Indeed, specific depletion of PP2A-B protein in fibroblasts induces hyperphosphorylation of the intermediate filament vimentin, leading to disassembly of the vimentin and to alterations in the interphase dynamics (Turowski *et al.*, 1999; Seshacharyulu *et al.*, 2013).

PP2A-B'. With at least eight isoforms (α , β_1 , β_2 , γ_1 , γ_2 , γ_3 , δ and ϵ) encoded by five different genes (*PPP2R5A*, *PPP2R5B*, *PPP2R5C*, *PPP2R5D* and *PPP2R5E*), the B' subunits represent the largest B-type subunit family (Janssens *et al.*, 2001). Among them, B' γ_1 is the only isoform that is not a phosphoprotein (McCright *et al.*, 1996). All B' isoforms contain a highly conserved (80% identical) central region implicated in the interaction with the A and possibly the C subunit. By contrast, both C- and N-termini are significantly distinct, suggesting that these regions could play an important role in substrate specificity and subcellular targeting. Intracellular localization of the PP2A-B' isoforms varies: α , β and ϵ isoforms are cytoplasmic, whereas B' γ_1 - γ_3 are concentrated in the nucleus and B' δ is found in both compartment (Janssens *et al.*, 2001). These isoforms also do not share the same tissue distribution: B' α and B' γ_1 - γ_3 are widely expressed, though predominantly in heart and skeletal muscles, whereas B' β , B' δ and B' ϵ are mainly expressed in brain (McCright *et al.*, 1996). PP2A holoenzymes comprising B' family members are implicated in mitosis/meiosis (Barr *et al.*, 2011; Wurzenberger *et al.*, 2011), development (Yang *et al.*, 2010), apoptosis (Janssens *et al.*, 2012) and dopaminergic signaling (Walaas *et al.*, 2011). Furthermore, due to their key role in the regulation of p53, Akt, Wnt and c-Myc signaling, they are considered as the major tumor suppressive PP2A subunits (Janssens *et al.*, 2001; Janssens *et al.*, 2005; Arnold *et al.*, 2008; Eichhorn *et al.*, 2009).

PP2A-B''. Encoded by three different genes, *PPP2R3A*, *PPP2R3B* and *PPP2R3C*, the human B'' subunits exist in five isoforms (α_1 , α_2 , β_1 , β_2 , γ). B'' α_2 and B'' β_2 are mainly found in heart and skeletal muscle, while the other isoforms are ubiquitously expressed (Yan *et al.*, 2000; Stevens *et al.*, 2003). The B'' subunits contain two EF-hand domains that, by binding calcium, induce conformational change in protein structure (Janssens *et al.*, 2003). One of these domains (EFX1) is essential for binding to the structural A subunit (Eichhorn *et al.*, 2009). The B'' subunits take part in noncanonical Wnt signaling (Eichhorn *et al.*, 2009), in EGF (epidermal growth factor) signaling (Zwaenepoel *et al.*, 2010), in DNA replication (Davis *et al.*, 2008) and in cell cycle regulation through the modulation of retinoblastoma protein activity (Kolupaeva *et al.*, 2013).

PP2A-B'''. Encoded by three different genes (*STRN*, *STRN3* and *STRN4*), the B''' subunits (striatin, SG2NA and zinedin) are highly expressed in brain. They are calmodulin-binding proteins and interact with the core enzyme to enhance calcium-dependent signaling. These proteins also require ATP and Mg^{2+} to stimulate their own activity (Seshacharyulu *et al.*, 2013). Roles of these subunits in Hippo signaling, Golgi polarization and cytokinesis have been reported (Lambrecht *et al.*, 2013).

In conclusion, although there are only two catalytic C and two structural A subunits, the large number of B-type subunits allows the formation of more than 90 different PP2A complexes, each being potentially able to achieve a particular physiological function. Presently, these functions are not yet completely unraveled. They have mostly been investigated in cellular models, whereas only few studies have been performed in animal models. In the future, use of knockout models will certainly provide more reliable knowledge about the function of each B-type isoform. For instance, it has been recently evidenced that knockout in mice of a specific B-type subunit, namely the subunit B' δ , induced neurological defects, indicating non-redundant functions for the different isoforms of this B-type subunit family (Louis *et al.*, 2011; Lambrecht *et al.*, 2013; Sents *et al.*, 2013).

Subunit	Gene	Isoform	Other name	Chromosome number	Normal tissue distribution	Subcellular distribution	Aberrant tissue distribution
Regulatory (B)	<i>PPP2R2A</i>	α	PR55 α , PP2A-B α	8p21.1	Widely distributed in all tissues (Mayer-Jaekel <i>et al.</i> , 1994)	Membranes, cytoplasm, microtubules nucleus.	Golgi complex, endoplasmic reticulum and neurofilaments AML, prostate P (Cheng <i>et al.</i> , 2011; Ruvolo <i>et al.</i> , 2011)
	<i>PPP2R2B</i>	β 1-2	PR55 β , PP2A-B β	5q31–5q32	Brain and testis (HE) (Mayer-Jaekel <i>et al.</i> , 1994)	Cytosol	–
	<i>PPP2R2C</i>	γ	PR55 γ , PP2AB γ	4p16.1	Brain (SE) (Zolnierowicz <i>et al.</i> , 1994)	Mainly in cytoskeletal fraction	Lung cancer, melanoma (Banerjee <i>et al.</i> , 2007)
	<i>PPP2R2D</i>	δ	PR55 δ , PP2AB δ	10q26.3	Wide spread distribution in tissues, testis (HE) (Strack <i>et al.</i> , 1999)	Cytosol	–
Regulatory (B')	<i>PPP2R5A</i>	α	PR56/61 α , PP2AB' α	1q32.2–q32.3	Cardiac tissues and skeletal muscles (HE) (McCright <i>et al.</i> , 1996)	Cytoplasm	–
	<i>PPP2R5B</i>	β	PR56/61 β , PP2AB' β	11q12–q13	Brain (HE) (McCright <i>et al.</i> , 1996)	Cytoplasm	–
	<i>PPP2R5C</i>	γ 1, 2, 3	PR56/61 γ , PP2AB' γ	14q32	Cardiac tissues and skeletal muscles (HE) (McCright <i>et al.</i> , 1996)	Cytoplasm and nucleus	Lung cancer (HE) reduced level in melanoma cells (Francia <i>et al.</i> , 1999; Shouse <i>et al.</i> , 2010)
	<i>PPP2R5D</i>	δ	PR56/61 δ , PP2AB' δ	6p21.1	Primarily exist in brain (McCright <i>et al.</i> , 1996)	Cytoplasm, nucleus, mitochondria, microsomes	–
	<i>PPP2R5E</i>	ϵ	PR56/61 ϵ , PP2AB' ϵ	14q23.1	Primarily exist in brain (McCright <i>et al.</i> , 1996)	Cytoplasm	Breast cancer, soft tissue sarcoma (Dupont <i>et al.</i> , 2010)
Regulatory (B'')	<i>PPP2R3A</i>	α 1	PR130, B'' α 1	3q22.1	Brain (HE), heart, lung, kidney and muscle (Mayer-Jaekel <i>et al.</i> , 1994)	Centrosome and Golgi complex	–
	<i>PPP2R3A</i>	α 2	PR72, B'' α 2	3q22.1	Heart (HE) and skeletal muscle (Mayer-Jaekel <i>et al.</i> , 1994)	Cytosol and nucleus	–
	<i>PPP2R3B</i>	β	PR70, PR48, B'' β	Xp22.33, Y11.3	Placenta (Yan <i>et al.</i> , 2000)	Nucleus	–
	<i>PPP2R3C</i>	γ	G5PR, G4-1	14q13.2	During developmental process expressed in fetal brain (Kamnasaran <i>et al.</i> , 2005)	Nucleus	–
	<i>PPP2R3D</i>	δ	PR59, B'' δ		Cardiac tissue, kidney and lungs (Voorhoeve <i>et al.</i> , 1999)	Nucleus	–
Regulatory (B''')	<i>STRN</i>		Striatin, PR110	2p22.2	Brain (Moreno <i>et al.</i> , 2000)	Membrane and cytoplasm	–
	<i>STRN3</i>		SG2NA	14q13–q21	Neurons (Moreno <i>et al.</i> , 2000)	Nucleus	–
	<i>STRN4</i>		zinedin		Brain (HE)	Golgi, caveolae	–

Table 4: The various isoforms of B-type subunits, their nomenclature, chromosomal location and normal/aberrant expression in various cancer and normal tissues.

HE : highly expressed ; SE : specific expression (Lambrech *et al.*, 2013; Seshacharyulu *et al.*, 2013).

3.3.2.3. Regulation by inhibitory proteins

In addition to the important regulation by B-type subunits, the catalytic activity of PP2A can be also directly inhibited by endogenous specific PP2A inhibitory proteins that may either target all or very specific PP2A holoenzymes. Among them are ANP32a (Acidic Nuclear Phosphoprotein 32a), SET (Su(var) 3-9/Enhancer of zeste/Trithorax) and CIP2A (Cancerous Inhibitor of PP2A). An aberrant expression of these inhibitors has been related to numerous cancers and constitutes a major mechanism by which the tumor suppressive function of PP2A can be impaired in tumors (Haesen *et al.*, 2012).

3.3.2.4. Regulation by post-translational modifications

PP2A catalytic efficiency can also be modulated by post-translational modifications, namely phosphorylation (Brautigan, 1995) and methylation (Tolstykh *et al.*, 2000). These modifications, which occurs in a conserved sequence (³⁰⁴TPDYFL³⁰⁹) of C-terminus PP2A C subunit (Janssens *et al.*, 2001), can provide additional interactions with specific B-type subunits and hence influence positively or negatively the holoenzyme formation (Janssens *et al.*, 2008). For instance, phosphorylation of Thr-304 (Schmitz *et al.*, 2010; Sents *et al.*, 2013) and/or Tyr-307 (Chen *et al.*, 1992), by kinases not yet identified, is associated with inactivation of specific PP2A holoenzymes. Possible explanations are that Thr-304 phosphorylation might affect B (PR55) subunit binding, whereas Tyr-307 phosphorylation might inhibit the recruitment of B' (PR61) subunits (Janssens *et al.*, 2008). On the other hand, methylation of the C subunit at Leu-309, which is catalyzed by the leucine carboxyl methyltransferase 1 (LCMT1) (De Baere *et al.*, 1999) and reversed by the methylesterase PME-1 (Ogris *et al.*, 1999), modulates the recruitment of regulatory subunits and, in the case of B (PR55) subunits, is absolutely required for the holoenzyme assembly (Janssens *et al.*, 2008).

The C subunit of PP2A is not the only post-translationally regulated subunit. Indeed, B-type subunits, and particularly B' subunits, can also be phosphorylated by different protein kinases. For instance, phosphorylation of B' α by PKR (protein kinase RNA-activated) (Xu *et al.*, 2000), or of B' γ by ATM (Shouse *et al.*, 2011; Sents *et al.*, 2013) or of B' δ by PKA increases the activity of each specific PP2A heterotrimer. By contrast, phosphorylation of B' γ

on Ser-337 by ERK induces the dissociation from the core enzyme and thus the inactivation of PP2A (Letourneux *et al.*, 2006).

3.3.3. Protein phosphatase 2B (PP2B)

PP2B (also known as calcineurin or PP3) is implicated in numerous calcium-dependent biological processes, including immune response, cardiac hypertrophy, neurodevelopment and memory, muscle development and signal transduction (Rusnak *et al.*, 2000). PP2B consists of a catalytic A subunit (calcineurin A or CNA) and a regulatory B subunit (calcineurin B or CNB) related to calmodulin (Moorhead *et al.*, 2007; Shi, 2009). As shown in Figure 19A, the catalytic A subunit of PP2B contains in addition to the N-terminal phosphatase domain, a CNB-binding helical domain (BBH), a calcium-calmodulin-binding motif (CBD), and an autoinhibitory sequence (AI). The structure of the phosphatase domain of CNA is very similar to that of the catalytic subunit of PP1 and presents the same pattern of metal ion coordination (Griffith *et al.*, 1995; Kissinger *et al.*, 1995). The association of CNA with calcium-calmodulin (Ca^{2+} -CaM) is essential for the phosphatase activity of PP2B. The tridimensional structure of PP2B also revealed a mechanism of autoinhibition, in which the autoinhibitory sequence forms a α helix able to interact with the phosphatase domain, preventing thus the access to the catalytic center (Kissinger *et al.*, 1995).

3.3.4. Protein phosphatase 4 (PP4)

In human, the catalytic subunit of PP4 (PP4c) is encoded by the *PPP4C* gene, located on chromosome 16p11-p12 (Bastians *et al.*, 1997). Although closely related to the catalytic subunit of PP2A, PP4c does not interact with the structural A subunits of PP2A, and possess its own binding partners, namely R1 and R2 (Cohen *et al.*, 2005; Brautigan, 2013). Localized in both nucleus and cytoplasm, PP4 plays an important role in numerous cellular events, including centrosome duplication (Sumiyoshi *et al.*, 2002), cortical development (Xie *et al.*, 2013), DNA replication and DNA damage response (Chowdhury *et al.*, 2008a; Nakada *et al.*, 2008; Lee *et al.*, 2010). Moreover, it has been shown that PP4 knockout in mice is embryonic lethal and that PP4 null thymocytes display decreased proliferation and enhanced apoptosis (Shui *et al.*, 2007), indicating that PP4 has essential roles separate from other PPPs in cell signaling.

3.3.5. Protein phosphatase 5 (PP5)

In contrast with other PPPs, PP5 contains a catalytic and a regulatory domain in the same polypeptide. PP5 is expressed in all types of mammalian tissues, but particularly in the brain (Shi, 2009). Though it presents a C-terminal nuclear localization sequence, it is localized in both nucleus and cytoplasm (Borthwick *et al.*, 2001). PP5 has been implicated in hormone and stress-induced signaling, such as in glucocorticoid receptor signaling, but it is also implicated in cellular proliferation, differentiation, migration, survival and death, and DNA damage repair *via* the ATM/ATR and p53 pathways. Given all these effects, the development of viable PP5-deficient mice was unexpected and suggested that PP5 could exert a modulatory, rather than essential, role in cell signaling (Hinds *et al.*, 2008).

Structural analysis of PP5 showed two particular regions: three tetratricopeptide repeats (TPRs), known to mediate protein-protein interactions, in the N-terminal regulatory domain and a helix αJ in the C-terminal catalytic domain. Similar to PP2B, free PP5 is maintained in an inactive conformation and has low basal activity. Indeed, in the absence of interacting proteins, the N-terminal domain folds over the catalytic phosphatase domain, blocking access to substrates. This association between the TPR domain and the phosphatase domain is reinforced by direct binding of the C-terminal helix αJ to a surface groove on the TPR domain. Release of PP5 auto-inhibition and hence PP5 activation can be triggered *in vitro* by binding of the TPR domain to ligands, such as Hsp90 (heat shock protein 90) or long-chain fatty acyl-CoA esters, like arachidonyl CoA (Moorhead *et al.*, 2007; Hinds *et al.*, 2008; Shi, 2009). Nevertheless, if these molecules are really endogenous activators of PP5 remains unclear.

3.3.6. Protein phosphatase 6 (PP6)

In human genome, the catalytic subunit of PP6 (PP6c) is encoded by the gene *PPP6C*, located on chromosome 9q33.3. As PP4c, PP6c is closely related to the C subunit of PP2A. PP6c (or Sit4 in yeast) has several related binding partners referred to as Sit4-associated proteins (SAPs) (Moorhead *et al.*, 2007). In analogy to PP2A, PP6c is thought to form a heterotrimeric holoenzyme, with a structural SAP domain-containing subunit and a regulatory subunit containing ankyrin repeats (Stefansson *et al.*, 2007). Mainly implicated in cell cycle progression, PP6 also plays a role in DNA repair following ionizing radiation (Mi *et al.*, 2009; Douglas *et al.*, 2010; Zhong *et al.*, 2011).

3.3.7. PP7 or PPEF protein phosphatase

PP7 is the most recently identified PPP. PP7 was a term used at the same time to designate a plant phosphatase (Andreeva *et al.*, 1998) and a mammal PPP phosphatase (Huang *et al.*, 1998), the latter also assigned as PPEF (protein phosphatase with EF-hand) by other groups (Montini *et al.*, 1997; Sherman *et al.*, 1997). Although designation of the animal and plant phosphatases as PP7 was fortuitous, phylogenic analysis of their catalytic domains suggested that they form in fact a separate clade. Therefore, the designation “PP7” was conserved for the plant phosphatase that does not contain EF-hand domains, whereas the mammalian PP7 was designated as PPEF/PP7 or more simply PPEF (Andreeva *et al.*, 2009). PPEF phosphatase was first detected especially in sensory neurons, suggesting a specific role in sensory signaling, but was found thereafter in numerous tissues, such as testis, uterus, bone marrow, B lymphocytes, cardiomyocytes, etc. Like PP2B, PPEF is stimulated by Ca^{2+} (Moorhead 2007). Among possible functions of PPEF, roles in apoptotic and oxidative stress responses, cell proliferation, oncogenesis and differentiation have been suggested (Andreeva *et al.*, 2009).

3.4. The PPPs in DNA damage response

As already mentioned above several times, PPPs are involved in the regulation of a multitude of cellular processes, including metabolism, cell growth and proliferation, DNA repair, apoptosis, and still several others. As an example, we will illustrate some functions of PPPs in the DNA damage response (DDR), one of the major signaling pathways activated by NAs. As explained before (section 1.5.), DNA damage can be detected by three sensor protein kinases, ATM, ATR and DNA-PK, which trigger multiple signaling cascades through phosphorylation of a variety of substrates (Achanta *et al.*, 2001; Shiloh, 2003). ATR and ATM phosphorylate and activate the transducer kinases, Chk1 (Checkpoint kinase-1) and Chk2 (Checkpoint kinase-2), respectively, which convey signals to numerous downstream effectors, such as p53 and pRb (retinoblastoma tumor suppressor protein). These events initiate various biological responses, including transient cell cycle arrest mediated by checkpoints, DNA repair, and apoptosis. In addition to the evident role of protein kinases in the DDR, it has become increasingly clear that phosphatases, particularly PPPs, also play an important role, not only by counteracting the function of kinases, but also through the initiation of specific steps during the DDR (Shimada *et al.*, 2013). Several studies have now

shown that PPPs control DDR process by dephosphorylating sensor kinases, transducer kinases, histones and effector molecules, as summarized below from a recent review (Shimada *et al.*, 2013).

Sensor kinases: ATM, ATR and DNA-PK. It has been shown that PP1 and PP2A operate as regulators of ATM, by preventing its activation. In the absence of DNA damage, they associate with and directly dephosphorylate ATM at Ser-1981. Upon DNA damage, dissociation of PP1 and PP2A from ATM occurs, allowing increase in Ser-1981 phosphorylation, and ATM recruitment to damaged sites. By contrast, PP5 associates with ATM upon DNA damage and most likely is involved in its activation. Indeed, expression of a catalytically inactive PP5 mutant inhibits ATM activation. Similarly, PP5 is involved in ATR activation and appears to be required for adequate phosphorylation of ATR targets, especially Chk1. Although association of PP5 with ATR is DNA damage-dependent, the precise mechanism of its action remains unknown. Particularly, whether ATR is a substrate of PP5 has not been demonstrated. Finally, DSBs induce interaction of PP2A with the DNA-PK subunits Ku70 and Ku80 whose dephosphorylation by PP2A enhances the formation of a functional DNA-PK, leading to DSBs repair. PP5 also interacts with DNA-PK and dephosphorylates most probably the autophosphorylated sites Ser-2056 and Ser-2609, since their phosphorylation is reduced in cells overexpressing PP5 (Shimada *et al.*, 2013).

Transducer kinases: Chk1 and Chk2. In unperturbed cells, PP2A prevents phosphorylation of Chk1 at Ser-317 and/or Ser-345, two phosphorylations that promote Chk1 release from chromatin and increase its kinase activity (Leung-Pineda *et al.*, 2006). PP2A also maintains Chk2 in an inactive state by dephosphorylating Thr-68 under normal conditions. Under DNA damage, PP2A dissociates from Chk2 and permits Chk2 phosphorylation by ATM. After completion of DNA repair, PP2A is also able to reduce the DDR partly through dephosphorylation of Chk2 at Thr-68 (Shimada *et al.*, 2013).

Histones. In response to DSBs, phosphorylation of the histone H2AX at Ser-139 (γ -H2AX) by ATM is required for the recruitment, to damaged sites, of different DDR proteins (Burma *et al.*, 2001). Phosphorylation level of this DNA damage marker is controlled by PP2A, PP4 and PP6 (Shimada *et al.*, 2013). On the other hand, PP1 prevents the phosphorylation of histone H3 by Chk1 at Thr-11 following DNA damage, leading to

transcriptional repression of cell cycle-regulated genes such as *CDK1* and *CYCLIN B* (Shimada *et al.*, 2008).

Effector molecules: pRB and p53. In its hypophosphorylated state, pRb inhibits cell cycle progression by binding and inhibiting transcription factors of the E2F family. This inhibitory state is maintained by PP2A and PP1 that are responsible for the dephosphorylation of pRb at multiple sites. Regarding p53, upon DNA damage, it is submitted to several phosphorylations that contribute to its stabilization and activation. One of these sites is Ser-15 that is dephosphorylated by PP1. But binding of PP1 to p53 is inhibited by DNA damage, which prevents dephosphorylation of p53 at Ser-15. Another site is Ser-37 whose phosphorylation is required for p53 transcriptional activity (Dohoney *et al.*, 2004) and both PP1 and PP2A have been shown to dephosphorylate it (Shimada *et al.*, 2013).

All these examples demonstrate the involvement of protein phosphatases in addition to kinases in the DDR. However, several questions remain to be investigated, such as the mechanism(s) by which DNA damage regulates the activity of each phosphatase to induce dephosphorylation events.

3.5. Small Molecule Inhibitors of PhosphoProtein Phosphatases

Study of the role of Ser/Thr phosphatases in cellular processes has been greatly facilitated by use of cell-permeable inhibitors. Indeed, their sensitivity to various inhibitors can be very different and the analysis of this sensitivity can constitute an interesting step in the identification of a specific protein phosphatase in a target pathway. Several PPP inhibitors have been identified, which all are natural compounds. The relative sensitivity of PP1-PP7/PPEF to commercially available inhibitors is shown in Table 5. Examination of this table indicates that microcystin-LR, nodularin, calyculin A and tautomycin are all very potent inhibitors of PP1, PP2A, PP4 and PP5, with limited activity against PP2B or PP7. In addition, they have virtually no effect on PPM phosphatases and phosphotyrosine phosphatases. However, microcystin-LR and nodularin are weakly cell-permeable and are most useful as *in vitro* inhibitors in cell homogenates. Regarding calyculin A and tautomycin, they cannot be used to distinguish the actions of sensitive PP from each other. The most potent and selective inhibitors discovered to date are okadaic acid and fostriecin, on which we will focus below.

Compound	PP1	PP2A	PP2B	PP4	PP5	PP7
Okadaic acid	15-50	0.1-0.3	~4,000	0.1	3.5	>1,000
Microcystin-LR	0.3-1	<0.1-1	~1,000	0.15	1.0	>1,000
Nodularin	2.4	0.3	>1,000	ND	~4	>1,000
Calyculin A	0.4	0.25	>1,000	0.4	3	>1,000
Tautomycin	0.23-22	0.94-32	>1,000	0.2	10	ND
Cantharidin	1,100	194	>10,000	50	600	ND
Fostriecin	45,000-58,000	1.5-5.5	>100,000	3.0	50,000-70,000	ND

Table 5 : Inhibition of PPP catalytic subunits by some natural compounds.

The IC₅₀ values provided are in nM and represent the amount of inhibitor needed to inhibit 50% of the activity in an assay. The different values shown likely reflect variations in the assay conditions used by the laboratory reporting these values. ND, not determined. This table, issued from Swingle *et al.* (2007), had been adapted from Honkanen and Golden (2002).

Okadaic acid (OA, C₄₄H₆₈O₁₃; Figure 22), a complex fatty acid derivative containing many cyclic polyether linkages, was the first potent and selective inhibitor of the PPP family to be described. This toxin, synthesized by marine dinoflagellates, accumulates in marine organisms such as sponges and shellfish, which feed on them. In this way, it enters the food chain, and at high concentration level causes diarrhetic seafood poisoning. Study of its effects revealed that OA induced contraction of isolated smooth muscle, which was known to result from phosphorylation of one of the myosin light chains. This led to the finding that OA was a potent cell-permeable PP1 and PP2A inhibitor (Shibata *et al.*, 1982; Bialojan *et al.*, 1988). Although OA inhibits both PP1 and PP2A, it shows substantial preference *in vitro* for PP2A (IC₅₀ = 0.1-0.3 nM) over PP1 (IC₅₀ = 15-50 nM) (Swingle *et al.*, 2007). The selectivity of OA for PP2A has also been demonstrated *in vivo* in intact cells in which 100 nM OA has been shown to inhibit PP2A without affecting PP1 activity (Favre *et al.*, 1997). Structural comparison of OA binding by PP1 and PP2A showed that the catalytic subunit of PP2A, but not of PP1, contained a hydrophobic cage that was able to accommodate the hydrophobic end of OA, providing a likely explanation for the observed difference in IC₅₀ (Xing *et al.*, 2006). Besides PP1 and PP2A, OA also potently inhibits PP4, PP5 and likely PP6. However, OA weakly inhibits PP2B and PPEF/PP7 and does not inhibit PP2C and protein tyrosine phosphatases.

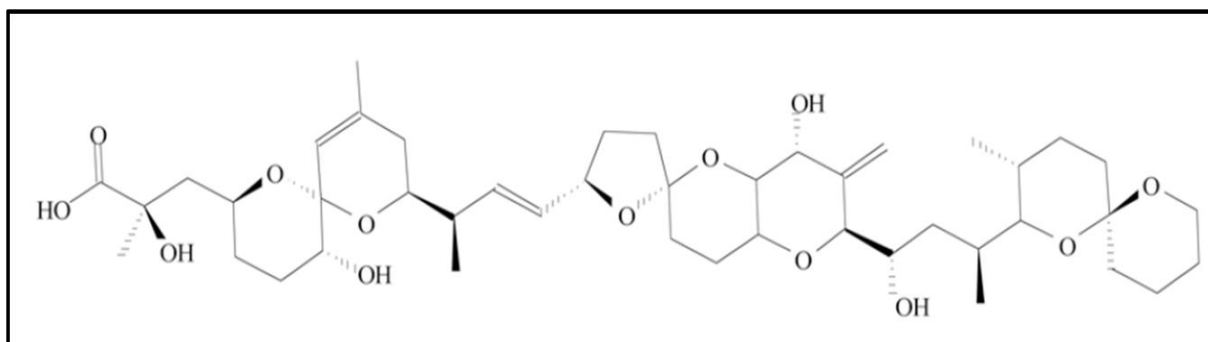


Figure 22: Structure of okadaic acid

Fostriecin ($C_{19}H_{26}O_9P$; Figure 23) is an antitumor antibiotic isolated from the fermentation beer of *Streptomyces pulveraceus*. This phosphate ester demonstrated marked cytotoxicity against many cancer cell lines and potent antitumor activity in animals. This antitumor activity was first ascribed to inhibition of topoisomerase II (Boritzki *et al.*, 1988), but was subsequently attributed to inhibition of the mitotic entry checkpoint due to potent and selective inhibition of PP2A (Cheng *et al.*, 1998). Indeed, fostriecin is a strong inhibitor of PP2A and PP4 (see Table 5) and very interestingly, inhibition of PP1 by this compound requires >10,000-fold higher concentrations. Fostriecin has no apparent effect on the activity of PP2B. Fostriecin enters cells via a carrier mediated transporter, likely the reduced folate carrier system (Fry *et al.*, 1984). The mechanism of inhibition of PP2A has been shown to involve the covalent binding of fostriecin to the Cys-269 residue of PP2Ac *in vitro* (Takeuchi *et al.*, 2009). This finding may provide valuable information for the development of new biological tools and hopefully novel drugs for the treatment or prevention of cancer.

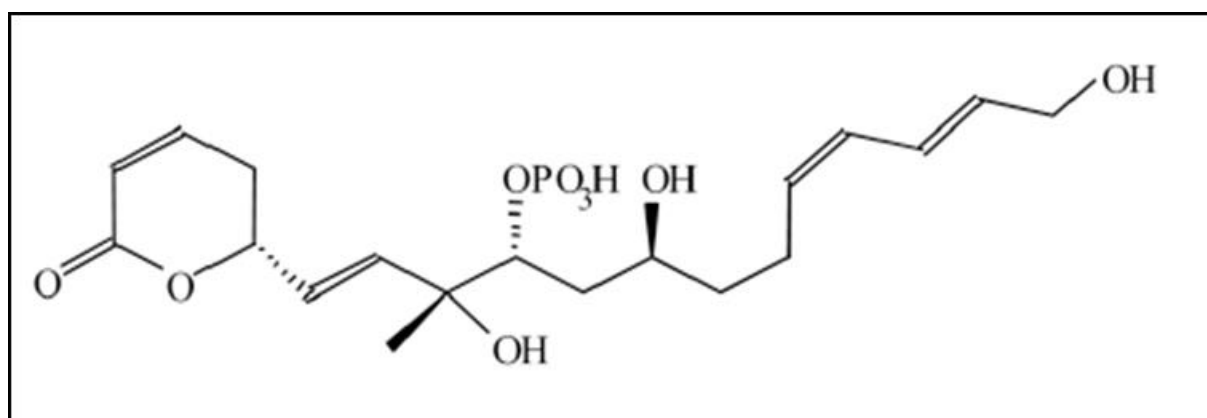


Figure 23: Structure of fostriecin

4. Objectives of the thesis

Due to its essential role in salvage of deoxynucleosides and in activation of chemotherapeutic nucleoside analogs, dCK is an enzyme that interests both basic scientists and clinical researchers. Better knowledge of the mechanisms that regulate dCK activity could indeed contribute to improvement of anticancer and antiviral chemotherapy. Prior to the start of my thesis project, researches conducted in our laboratory had demonstrated that dCK activity is regulated by post-translational modification, and more precisely that its activity can be increased *via* Ser-74 phosphorylation. These findings raised a series of questions, some of which were at the origin of my PhD work.

Activation of dCK via Ser-74 phosphorylation was detected in assays using dCyd as substrate, as usually performed. An important question was to know whether phosphorylation of dCK on Ser-74 increases dCK activity toward all its substrates, including nucleoside analogs. This matter was firstly investigated, but only partially, by McSorley *et al.* (2008) who mimicked Ser-74 phosphorylation by introducing a Ser-74-Glu (S74E) mutation in bacterially produced dCK. By comparing the kinetic properties of this mutated dCK to those of wild-type dCK, the authors showed that Ser-74 phosphorylation increased dCK activity toward dCyd and pyrimidine analogs, but not toward natural purine deoxynucleosides. **The first objective** of my thesis was to analyze this question more in depth with other dCK substrates, and in addition to investigate whether an increase of dCK phosphorylation at Ser-74 could enhance activation of nucleoside analogs in cancer cells and hence their cytotoxicity. The results of this study were published in *Biochemical Pharmacology* (Chapter II).

Other questions concerned the identity of the protein kinase(s) and phosphatase(s) that control Ser-74 phosphorylation level and thereby dCK activity. While identification of Ser-74-dCK kinase was already under investigation in the lab, **my second objective** was to identify the protein phosphatase responsible for Ser-74 dephosphorylation. Preliminary results indicated that the protein phosphatase implicated in this process was sensitive to okadaic acid (Smal et al., 2004; Smal et al., 2010b), and may thus belong to the PPP family. The results of this study were published in *FEBS Letters* (Chapter III).

Finally, I also contributed to a study conducted by Dr C. Smal to identify the protein kinase that phosphorylates Ser-74. We demonstrated that casein kinase 1 δ was able to phosphorylate dCK at Ser-74 *in vitro*, but not in cells. This work was published in *Archives of Biochemistry and Biophysics* (Annex section).

CHAPTER II:
Phosphorylation of deoxycytidine kinase on Ser-74:
Impact on kinetic properties and nucleoside analog
activation in cancer cells

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Phosphorylation of deoxycytidine kinase on Ser-74: Impact on kinetic properties and nucleoside analog activation in cancer cells

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ABSTRACT

Deoxycytidine kinase (dCK) (EC 2.7.1.74) is a key enzyme in the activation of several therapeutic nucleoside analogs (NA). Its activity can be increased in vivo by Ser-74 phosphorylation, a property that could be used for enhancing NA activation and clinical efficacy. In line with this, studies with recombinant dCK showed that mimicking Ser-74 phosphorylation by a S74E mutation increases its activity toward pyrimidine analogs. However, purine analogs had not been investigated. Here, we show that the S74E mutation increased the k_{cat} for cladribine (CdA) by 8- or 3-fold, depending on whether the phosphoryl donor was ATP or UTP, for clofarabine (CAFdA) by about 2-fold with both ATP and UTP, and for fludarabine (F-Ara-A) by 2-fold, but only with UTP. However, the catalytic efficiencies (k_{cat}/K_m) were not, or slightly, increased. The S74E mutation also sensitized dCK to feed-back inhibition by dCTP, regardless of the phosphoryl donor. Importantly, we did not observe an increase of endogenous dCK activity toward purine analogs after in vivo-induced increase of Ser-74 phosphorylation. Accordingly, treatment of CLL cells with aphidicolin, which enhances dCK activity through Ser-74 phosphorylation, did not modify the conversion of CdA or F-Ara-A into their active triphosphate form. Nevertheless, the same treatment enhanced activation of gemcitabine (dFdC) into dFdCTP in CLL as well as in HCT-116 cells and produced synergistic cytotoxicity. We conclude that increasing phosphorylation of dCK on Ser-74 might constitute a valuable strategy to enhance the clinical efficacy of some NA, like dFdC, but not of CdA or F-Ara-A.

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1. Introduction

Deoxycytidine kinase (dCK) (EC 2.7.1.74) is a cytosolic enzyme with broad substrate specificity, catalyzing the phosphorylation of deoxycytidine (dCyd), deoxyadenosine (dAdo) and deoxyguanosine (dGuo) with ATP or UTP as phosphoryl donor. This reaction constitutes the first and rate-limiting step of the deoxynucleoside salvage pathway, which provides cells with deoxynucleotides for DNA synthesis as an alternative to the de novo synthesis [1]. In addition, dCK phosphorylates a number of therapeutic nucleoside analogs including anticancer drugs such as fludarabine (F-Ara-A), cladribine (CdA), clofarabine (CAFdA), gemcitabine (dFdC), cytarabine (Ara-C), and antiviral compounds, such as zalcitabine (ddC) and lamivudine (3TC) [2,3]. Phosphorylation of these compounds by dCK is most often the rate-limiting step in the pathway that

converts them to their active triphosphate form. Given its key role in the activation and therapeutic efficacy of nucleoside analogs [4], dCK has been the subject of many functional and structural investigations in the last years.

The finding that several genotoxic agents, including aphidicolin, etoposide (VP-16), nucleoside analogs, UV-C- and γ -radiation, were able to increase dCK activity without change in dCK protein level led to the hypothesis of a regulation of dCK through post-translational modification [5–10]. Previous work of our group revealed that dCK is a phosphoprotein containing at least four phosphorylation sites: Thr-3, Ser-11, Ser-15 and Ser-74, the latter being the major phosphorylated residue. Site-directed mutagenesis demonstrated that phosphorylation of the three first sites does not influence dCK activity, whereas phosphorylation of Ser-74 increases its activity [11,12]. Moreover, use of a specific anti-phospho-Ser-74 antibody showed that activation of dCK by genotoxic agents in leukemic cells is related to an increase of Ser-74 phosphorylation [11,13]. In all these experiments, dCK activity was measured using 10 μ M dCyd as substrate, as usually performed [14]. Unexpectedly, further investigations showed

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that mimicking phosphorylation of Ser-74 by a Ser-74-Glu mutation increases dCK activity for dCyd and dCyd analogs, but decreases it for dAdo and dGuo, suggesting that Ser-74 phosphorylation could positively influence dCK activity toward pyrimidine, but not purine nucleosides [15]. Nevertheless, the influence of Ser-74 phosphorylation on dCK activity toward purine analogs, which are widely used in the treatment of hematological malignancies and particularly chronic lymphocytic leukemia (CLL) [16], remained to be analyzed. Also, implication of Ser-74 phosphorylation in the activation of nucleoside analogs, either purine or pyrimidine, inside cancer cells had not been investigated yet.

In the present study, we further analyzed the effects of mimicking Ser-74 phosphorylation on the kinetic properties of recombinant dCK, particularly toward purine analogs. We also investigated the effect of an increase of the phosphorylation of endogenous dCK on Ser-74 on its activity toward various substrates and on the intracellular conversion of nucleoside analogs into their active triphosphate form. The results show that increasing dCK activity via Ser-74 phosphorylation can enhance the intracellular activation of some nucleoside analogs, like gemcitabine, but not of CdA or F-Ara-A.

2. Materials and methods

2.1. Materials

Ficoll-Paque Plus (density: 1.077) was from GE Healthcare (Buckinghamshire, UK). Dulbecco's Modified Eagle Medium (DMEM), ultraglutamine, FCS and penicillin–streptomycin were purchased from Lonza (Basel, Switzerland). RPMI-1640 was from Gibco/Invitrogen (Carlsbad, CA, USA). [5-³H]-dCyd (30 Ci/mmol), [5-³H]-dFdC (21.9 Ci/mmol), [2,8-³H]-dAdo (10.4 Ci/mmol), [8-³H]-CdA (7 Ci/mmol), [8-³H]-CAFdA (2.4 μ Ci/mmol), [8-³H]-F-Ara-A (4.4 Ci/mmol) and [5-³H]-Ara-C (20.6 Ci/mmol) were from Moravek Biochemicals (Brea, CA, USA). dCyd, dAdo, F-Ara-A, Ara-C, etoposide (VP-16), genistein (GNT), aphidicolin (APC) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St Louis, MO, USA). CdA was synthesized and supplied by Prof. J. Marchand (Laboratory of Organic Chemistry, Université catholique de Louvain, Louvain-la Neuve). dFdC from Eli Lilly (Indianapolis, USA) was kindly provided by V. Gregoire (Centre for Molecular Imaging and Experimental Radiotherapy, Université catholique de Louvain, Brussels). Ac-DEVd-AMC (Ac-Asp-Glu-Val-Asp-AMC) and AMC (7-amino-4-methyl coumarin) were purchased from Alexis Biochemicals (San Diego, CA, USA). Other chemicals, materials and reagents were from Sigma–Aldrich (St Louis, MO, USA), Merck Biosciences (Gibbstown, NJ, USA) or Bio-Rad (Hercules, CA, USA) Laboratories.

2.2. Cell culture

Peripheral blood from CLL patients was obtained after written informed consent in accordance with the hospital ethics committee. All patients had an established diagnosis of CLL, were free of any anticancer treatment for at least 3 months, and had lymphocytes $>30 \times 10^9/L$. Mononuclear cells ($\geq 95\%$ CLL cells) were isolated by Ficoll-Paque sedimentation, washed in PBS, suspended in RPMI-1640 supplemented with 10% heat-inactivated FCS and 1% penicillin–streptomycin, and cultured at a concentration of 10×10^6 cells/ml. EHEB cells, a B-cell line established from CLL lymphocyte [17], purchased from DSMZ-German Collection of Microorganisms and Cell Culture (Braunschweig, Germany), were cultured in RPMI-1640, supplemented with 10% heat-inactivated FCS at 37 °C in an atmosphere of 5% CO₂ in air. Before experiments,

cells were counted and suspended at a concentration of 0.5×10^6 cells/ml. Hydrophobic activators of dCK (aphidicolin, etoposide and genistein) were dissolved in DMSO and equal amounts of DMSO were added in control cells. In each independent experiment, control condition was performed in duplicate.

HCT-116 and HT-29 colon cancer cell lines, kindly provided by G. Bommer (de Duve Institute, Brussels) were maintained in DMEM supplemented with 10% heat-inactivated FCS, 1% penicillin–streptomycin, and 2 mM ultraglutamine in an atmosphere of 5% CO₂ in air.

2.3. Kinetic studies of recombinant dCK

Human wild-type (WT) dCK or the mutant S74E were expressed in *Escherichia coli* and purified as previously described [18]. The purified proteins were stored at –80 °C in the presence of 20% (w/v) glycerol. dCK activities were determined using a radiochemical method [14]. All measurements were done at 37 °C in a buffer containing 50 mM Tris–HCl, pH 7.6, 2 mM dithiothreitol, 100 mM KCl, 5 mM MgCl₂, 2 mM ATP or UTP and labeled nucleosides, at concentrations that varied according to the nucleoside. The enzyme concentration varied between 5 and 20 nM. The K_m and k_{cat} values were calculated using non-linear regression with the GraphPad Prism 5.0 software. To investigate inhibition by dCTP, we measured dCK activity at variable concentrations of labeled dCyd or CdA in the presence of different fixed dCTP concentrations (ranging from 2 to 200 μ M). K_i values were calculated with the GraphPad Prism 5.0 software from secondary plots of inhibitor concentration versus intercept or slope of the double reciprocal plots using linear least-squares regression analysis to determine the best-fit line describing the data points.

2.4. dCK activity in cell lysates

Activity of dCK in cell lysates was measured as reported previously [10], using 20–80 μ g of cell extract protein depending on the cell line, with 10 μ M [5-³H]-dCyd (~900 cpm/pmol), 50 μ M [5-³H]-dFdC (~300 cpm/pmol), 50 μ M [2,8-³H]-2'-dAdo (~200 cpm/pmol), 50 μ M [8-³H]-CdA (~200 cpm/pmol) or 50 μ M [8-³H]-F-Ara-A (~200 cpm/pmol) as substrates. When dAdo was used, 10 μ M erythro-9-(2-hydroxy-3-nonyl)adenine was included in the assay to prevent its deamination by adenosine deaminase. In some experiments, dCyd and CdA were used at several concentrations as indicated. The protein content of samples was determined by the method of Bradford by using bovine serum albumin as standard [19].

2.5. Analysis of deoxycytidine and nucleoside analog metabolism in intact cells

To investigate the influence of the phosphorylation state of Ser-74 on the metabolism of nucleoside (analog) in CLL cells, samples containing 30×10^6 cells were preincubated without or with 3 μ M aphidicolin for 30 min and incubated for additional 2 h with labeled nucleosides (~2 μ Ci/ml) at the indicated concentrations. For analysis of dFdC activation in HCT-116 cells, cells were plated at a concentration of 0.5×10^6 cells/well in 6-well plates and incubated without or with 3 μ M aphidicolin for 24 h. Incubation medium was then replaced by fresh medium to remove aphidicolin and cells were incubated for additional 24 h in the presence of [5-³H]-dFdC at the indicated concentrations. Preparation of perchloric cell extracts and separation of labeled nucleotides by HPLC were performed as described previously [20]. The amounts of nucleotides synthesized were calculated from the specific radioactivity of the precursor. To analyze the incorporation of labeled nucleosides into nucleic acids, radioactivity was measured in the

acid-insoluble fraction of the cellular extract after two washings with 1 M HClO₄ and solubilization with 0.5 M NaOH.

2.6. Cytotoxicity and drug interaction analysis

To analyze whether aphidicolin enhances dFdC-mediated cytotoxicity, CLL cells (2×10^6 cells/well) were incubated in the absence or presence of 3 μ M aphidicolin and without or with dFdC at the indicated concentrations in 96-well plates for 96 h. When HCT-116 cells were used, they were incubated in 96-well plates (2.5×10^3 cells/well) without or with 3 μ M aphidicolin for 24 h, cleared from aphidicolin and then incubated for additional 72 h without or with various concentrations of dFdC. Cell viability was measured using the MTT assay as previously [21]. Each condition was done in triplicate in the same experiment. The absorbance of each well was measured at 570 nm and 655 nm with a Multiwell Scanning Spectrophotometer (Bio-Rad, Hercules, CA, USA). Cell survival, expressed as a percentage, was calculated by the equation: (mean absorbance of dFdC-treated cells/mean absorbance of control cells) $\times$ 100. The IC₅₀ value was determined graphically.

Interaction between aphidicolin and dFdC, i.e. synergism, additivity or antagonism, was defined according to the multiplicative model as previously reported [22]. In this model, the expected effect of a drug combination is the product of the effect of each single drug. The interaction is synergic when the observed cell survival (drug A + drug B) < expected cell survival (drug A $\times$ drug B); additive if expected cell survival (drug A $\times$ drug B) < observed cell survival (drug A + drug B) < cell survival of the most active single drug; antagonist if observed cell survival (drug A + drug B) > the most active single drug.

2.7. Assay for caspase-3 activity

HCT-116 cells (1×10^6 cells/6-cm dishes) were incubated without or with 3 μ M aphidicolin for 24 h. After changing the medium to remove aphidicolin, dFdC was added at the indicated concentrations. After 24 h of incubation, cells were lysed and caspase-3 activity was determined by fluorometric assay using Ac-DEVD-AMC as fluorogenic substrate, as previously described [23]. Release of AMC was measured using a spectrofluorometer (Bio-Rad) with a pair of excitation/emission wavelength of 360/460 nm. Caspase-3 activity was calculated by converting fluorescence units into micromoles of AMC released per min per mg of protein using a standard curve obtained from free AMC.

2.8. Statistical analysis

All the results are the means $\pm$ SEM of at least three independent experiments. Significance was analyzed by paired Student's *t*-tests. Changes were considered significantly different when *P* < 0.05.

3. Results

3.1. Influence of S74E mutation on dCK activity toward dCyd and purine analogs

The effect of Ser-74 phosphorylation was analyzed by comparing the kinetic properties of WT dCK, which is unphosphorylated when produced in bacteria, and of its variant S74E dCK, in which Glu mimics Ser-74 phosphorylation [11,15,18]. The Michaelis–Menten kinetic constants of both dCKs (Table 1) were then determined with ATP or UTP as phosphoryl donor, the latter being considered as the physiological phosphoryl donor by several authors [24–26]. Results obtained with ATP confirmed that mimicking Ser-74 phosphorylation increased both the *K_m* and *k_{cat}* of dCK for dCyd [15], by about 4- and 12-fold, respectively, resulting in an almost 3-fold increase in the dCK specificity constant (*k_{cat}*/*K_m*). With UTP as phosphoryl donor, the increases in *K_m* and *k_{cat}* induced by the S74E mutation were approx. 2-fold less than with ATP, but the dCK specificity constant was similarly increased.

Previous studies had shown that the S74E dCK, versus the WT dCK, displayed an up to 5-fold decrease of the specificity constant for the natural purines dAdo and dGuo [15]. Therefore, it was important to know whether Ser-74 phosphorylation similarly affects dCK activity toward antileukemic purine analogs. Contrary to what was observed for natural purines, we found that the S74E mutation increased the *k_{cat}* values for CdA and CAFdA, by ~8- and ~2-fold respectively, with ATP as the phosphoryl donor, resulting in a slight increase of the specificity constant despite increase in the *K_m* values (Table 1). With UTP, changes induced in the *K_m* and *k_{cat}* by the S74E mutation were less pronounced than with ATP, particularly for CdA, with no change in the specificity constant values. Regarding F-Ara-A, the S74E mutation slightly decreased the *k_{cat}* when ATP was the phosphoryl donor, but increased it by 2-fold with UTP. However, due to increase in *K_m*, the specificity constant of S74E dCK for F-Ara-A was reduced, to about half, with both ATP and UTP.

Altogether, these results indicate that the effect of phosphorylation of Ser-74 on the kinetic parameters of dCK varies from nucleoside to nucleoside, and does not depend on the purine or

Table 1

Kinetic constants of WT and S74E dCK for dCyd or purine analogs. dCK activity was measured with increasing concentrations of the indicated nucleosides and 2 mM ATP or UTP as phosphoryl donor. Kinetic constants (*K_m* and *k_{cat}*) were calculated using non-linear regression with the GraphPad Prism 5.0 software. Values shown are the means $\pm$ SEM of at least three experiments. *K_m* are in μ M, *k_{cat}* are in 10^{-3} s⁻¹, and *k_{cat}*/*K_m* are in 10^3 M⁻¹ s⁻¹.

Nucleoside		With 2 mM ATP as phosphoryl donor			With 2 mM UTP as phosphoryl donor		
		WT dCK	S74E dCK	Ratio S74E/WT	WT dCK	S74E dCK	Ratio S74E/WT
dCyd	<i>K_m</i>	0.07 $\pm$ 0.01	0.27 $\pm$ 0.04	3.9	0.09 $\pm$ 0.02	0.18 $\pm$ 0.05	2.0
	<i>k_{cat}</i>	15 $\pm$ 0.2	183 $\pm$ 25	12.4	21 $\pm$ 2	123 $\pm$ 13	5.7
	<i>k_{cat}</i> / <i>K_m</i>	236 $\pm$ 32	676 $\pm$ 44	2.9	257 $\pm$ 35	745 $\pm$ 150	2.9
CdA	<i>K_m</i>	2.4 $\pm$ 0.2	15.5 $\pm$ 1.3	6.5	0.65 $\pm$ 0.09	1.6 $\pm$ 0.14	2.5
	<i>k_{cat}</i>	172 $\pm$ 14	1386 $\pm$ 81	8.1	80.4 $\pm$ 3.6	211 $\pm$ 1.4	2.6
	<i>k_{cat}</i> / <i>K_m</i>	74.3 $\pm$ 8.1	90.2 $\pm$ 5.4	1.2	130 $\pm$ 23	136 $\pm$ 12	1.0
CAFdA	<i>K_m</i>	22.0 $\pm$ 1.3	32.5 $\pm$ 4.7	1.5	8.7 $\pm$ 0.6	15.5 $\pm$ 1.3	1.8
	<i>k_{cat}</i>	1110 $\pm$ 125	2450 $\pm$ 188	2.2	486 $\pm$ 31	880 $\pm$ 84	1.8
	<i>k_{cat}</i> / <i>K_m</i>	50.1 $\pm$ 4.0	76.8 $\pm$ 4.8	1.5	56.3 $\pm$ 7.1	57.6 $\pm$ 5.9	1.0
F-Ara-A	<i>K_m</i>	450 $\pm$ 38	551 $\pm$ 27	1.2	28 $\pm$ 1.8	142 $\pm$ 28	5.1
	<i>k_{cat}</i>	1334 $\pm$ 161	1005 $\pm$ 46	0.8	177 $\pm$ 8	363 $\pm$ 50	2.0
	<i>k_{cat}</i> / <i>K_m</i>	3 $\pm$ 0.45	1.8 $\pm$ 0	0.6	6.4 $\pm$ 0.6	2.6 $\pm$ 0.15	0.4

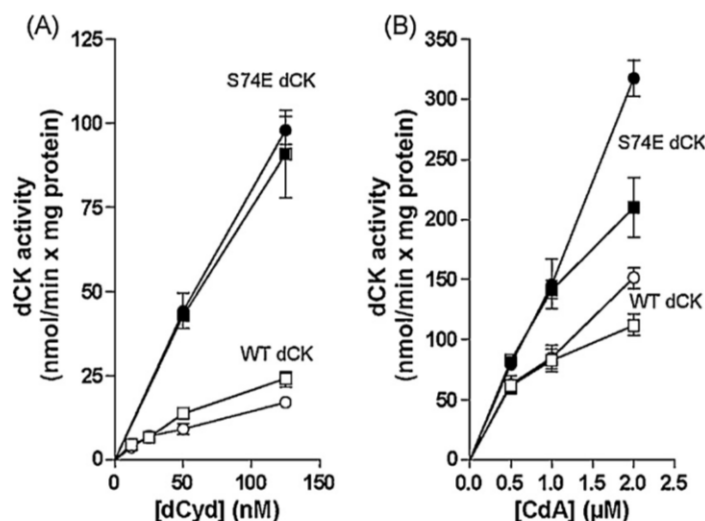


Fig. 1. Activity of WT and S74E dCK at physiological concentrations of dCyd or therapeutic concentrations of CdA. WT (open symbols) or S74E (closed symbols) dCK activity was measured with dCyd (A) or CdA (B) at the indicated concentrations in the presence of ATP (circles) or UTP (squares) as phosphoryl donor. Data derive from the kinetic studies shown in Table 1.

pyrimidine nature of the base moiety. Also, the effect of Ser-74 phosphorylation on dCK activity toward a particular nucleoside will depend on the concentration of the latter, since Ser-74 phosphorylation as a rule decreases the affinity of dCK for its substrates. At substrate concentrations $\leq K_m$, the effect of Ser-74 phosphorylation will strongly depend on the k_{cat}/K_m ratio, whereas at higher concentrations the effect observed at the k_{cat} level will prevail. So, if we consider dCK activity at physiological concentrations of dCyd ($\sim 0.05 \mu\text{M}$ [27]) (Fig. 1A) or at cytotoxic concentrations of CdA (around $0.5 \mu\text{M}$ [20]) (Fig. 1B), the kinetic advantage due to Ser-74 phosphorylation is clearly more noticeable for dCyd than for CdA, although k_{cat} values are markedly increased for both substrates.

3.2. Influence of S74E mutation on dCK inhibition by dCTP

While dCTP is a potent inhibitor of dCK when ATP is the phosphoryl donor, it is a relatively weak inhibitor when dCK activity is measured with UTP as the phosphoryl donor, so that the enzyme should not be significantly inhibited at physiological levels of dCTP ($10\text{--}20 \mu\text{M}$) [28,29]. However, it was not known whether this feed-back inhibition was modulated by Ser-74 phosphorylation. Therefore, we analyzed the effect of dCTP on the activity of WT and S74E dCK with respect to dCyd or CdA and at saturating concentrations of ATP or UTP (Table 2). All inhibition studies yielded linear kinetics on the double reciprocal plots and showed changes both in slope and intercept when ATP was the phosphoryl

donor, suggestive of mixed inhibition, and only in intercept with UTP, which was consistent with uncompetitive inhibition (not shown). The inhibition pattern of dCK by dCTP was similar with dCyd and CdA, although the mixed inhibition seemed to be more competitive than uncompetitive with respect to dCyd ($K_{is} < K_{ii}$) and rather non-competitive with respect to CdA ($K_i \sim K_{is}$). Mimicking Ser-74 phosphorylation did not change the pattern of inhibition, but sensitized dCK to inhibition by dCTP. The results also confirmed that dCK was more sensitive to inhibition by dCTP when ATP was the phosphoryl donor, with both dCyd or CdA as the substrate, and showed that inhibition was higher with respect to CdA than dCyd.

Finally, we compared the effect of dCTP, in a physiological range of concentrations, on the activity of both forms of dCK at a physiological concentration of dCyd (Fig. 2A) or a pharmacological concentration of CdA (Fig. 2B), with ATP at 3 mM and UTP at 1 mM , mimicking in vivo conditions [28]. With dCyd as substrate, dCK activity was barely inhibited by dCTP. With CdA, inhibition was more pronounced, reaching about 40% at $50 \mu\text{M}$ dCTP for the S74E dCK. With both substrates, the S74E dCK was slightly more inhibited by dCTP than the WT dCK.

3.3. Influence of Ser-74 phosphorylation on the activity of endogenous dCK toward various nucleosides

To verify whether an increase of phosphorylation of Ser-74, as may occur in intact cells after treatment with the DNA polymerase

Table 2

Kinetic constants for inhibition of WT and S74E dCK by dCTP. dCK activity was measured with respect to dCyd and CdA in the presence of 2 mM ATP or UTP. K_{is} and K_{ii} are inhibition constants for slope and intercept effects, respectively. Each value represents the average of two separate experiments giving similar results: variation was less than 20%.

Variable substrate	Saturating substrate	dCK	Pattern of inhibition	K_{is} (μM)	K_{ii} (μM)
dCyd	ATP	WT	Mixed	30	132
		S74E	Mixed	2.9	46
	UTP	WT	Uncompetitive	–	398
		S74E	Uncompetitive	–	54
CdA	ATP	WT	Mixed	5.8	7.0
		S74E	Mixed	1.5	1.8
	UTP	WT	Uncompetitive	–	50
		S74E	Uncompetitive	–	27

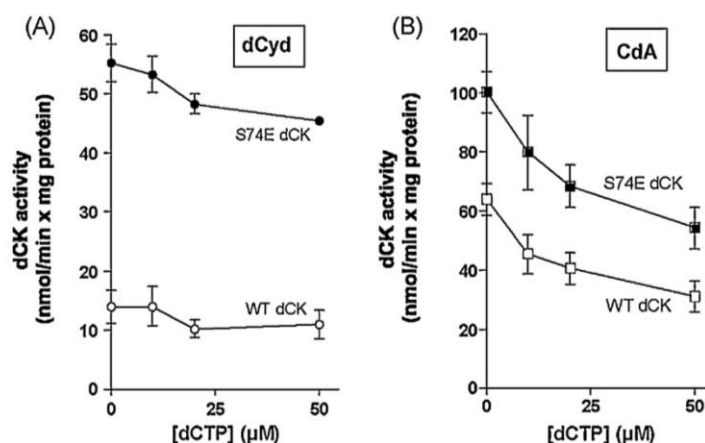


Fig. 2. Effect of dCTP on WT and S74E dCK activity. WT (open symbols) or S74E (closed symbols) dCK activity was measured with 0.05 μM dCyd (A) or 0.5 μM CdA (B) in the presence of 3 mM ATP and 1 mM UTP and the indicated concentrations of dCTP.

inhibitor aphidicolin [13], could result in an increase of dCK activity toward substrates other than dCyd, we treated leukemic EHEB cells for 60 min without or with this compound. As expected, when dCK activity was assayed in cell lysates with dCyd as substrate, activation by aphidicolin was observed at all the tested concentrations (Fig. 3A). On the contrary, when dCK activity was measured in the same lysates with CdA, activation by aphidicolin was not detected, or very weakly at high CdA concentrations (Fig. 3B). We next investigated the effect of other agents reported to enhance phosphorylation of dCK on Ser-74 [13] and measured dCK activity with various pyrimidine or purine nucleosides as substrates (Fig. 4). Increase in dCK activity induced by aphidicolin, but also etoposide, UV-C light and genistein was similar when dCyd or dFdC was used as the substrate, all the treatments producing a 2- to 3-fold rise in enzyme activity. In contrast, with dAdo, CdA or F-Ara-A as substrates, activation of dCK could not be detected. Similar results were obtained in CLL lymphocytes (not shown). These results indicate that phosphorylation of dCK on Ser-74 in intact cells induces an increase of dCK activity only toward pyrimidine nucleosides.

3.4. Influence of aphidicolin on nucleoside analog activation in CLL cells

Our finding that dCK activity could be enhanced in leukemic cells by increasing its phosphorylation on Ser-74 [13] and the observation that mimicking Ser-74 phosphorylation increased catalytic activity of recombinant dCK for anti-cancer pyrimidine analogs [15] provided a rationale for chemotherapy combining nucleoside analogs with drugs that activate dCK through Ser-74 phosphorylation. However, this strategy might not be useful for purine analogs, as indicated by the results obtained above with endogenous dCK. To analyze the influence of an increased phosphorylation of dCK on Ser-74 on the conversion of various nucleoside analogs into their active triphosphate forms, we treated primary CLL lymphocytes with aphidicolin, which increases dCK activity by 2- to 3-fold in this cell model [13]. Practically, CLL cells were incubated during 30 min without or with 3 μM aphidicolin before the addition of labeled nucleoside analogs at concentrations around their IC₅₀ in these cells [30–32]. The intracellular accumulation of nucleotides was measured after 2 hours of

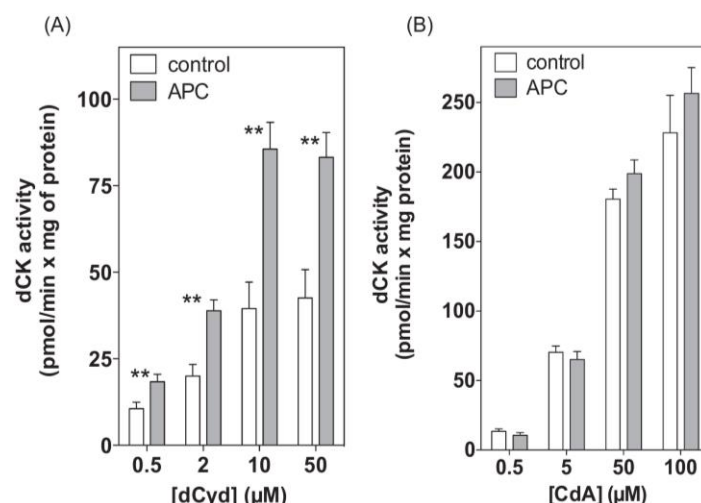


Fig. 3. Effect of aphidicolin treatment on dCK activity in leukemic cells. EHEB cells were incubated without (control) or with 3 μM aphidicolin (APC) for 1 h prior to determination of dCK activity with variable concentrations of dCyd (A) or CdA (B). Results are means ± SEM of 4 independent experiments. Significance relative to the absence of APC: ***P* < 0.01.

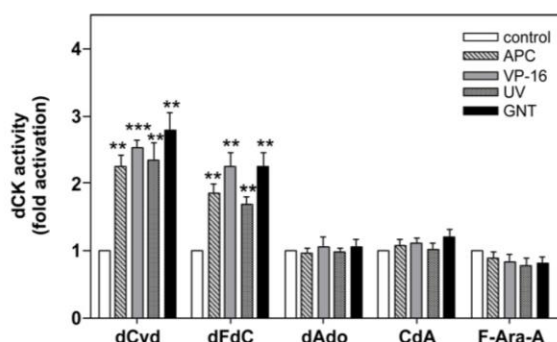


Fig. 4. Effect of several treatments that increase phosphorylation of dCK on Ser-74 on its activity toward various nucleosides. EHEB cells were incubated for 90 min without (control) or with 3 μ M aphidicolin (APC), 100 μ M etoposide (VP-16), 100 μ M genistein (GNT) or were UV-C-irradiated (30 J/m²) before the 90-min incubation. Activity of dCK was measured in cell lysates with the indicated substrates, at a concentration of 10 μ M for dCyd and 50 μ M for the other nucleosides, as explained in Section 2.4. Control dCK activities with dCyd, dFdC, dAdo, CdA and F-Ara-A were 23 ± 3 , 30 ± 8 , 22 ± 4 , 101 ± 9 , 9 ± 2 pmol/min $\times$ mg protein, respectively. Results are means $\pm$ SEM of 3–5 independent experiments. Significance relative to the absence of treatment: ** $P < 0.01$; *** $P < 0.001$.

incubation (Table 3), which was the time required for nucleoside analog triphosphates to reach a plateau in CLL cells [20]. We observed that the conversion of F-Ara-A and CdA into F-Ara-ATP and CdATP, respectively, was not influenced by aphidicolin. In contrast, activation of Ara-C into Ara-CTP tended to be enhanced ($P < 0.13$), while that of dFdC into dFdCTP was significantly increased, up to 1.7-fold, by aphidicolin ($P < 0.01$). Similarly, we noted a significant increase of the conversion of dCyd into dCTP upon incubation with aphidicolin. The mono- and diphosphate forms of all the nucleosides accumulated in parallel with the nucleoside triphosphates (not shown). Incorporation of labeled nucleosides into DNA (and possibly RNA for some of the nucleoside analogs) was very low, accounting for less than 1% (for nucleoside analogs) and 5% (for dCyd) of the radioactivity measured in the nucleotides after a 2 h-incubation, and was not modified noticeably by aphidicolin.

Since enhanced conversion of nucleoside analogs into their triphosphate form is expected to result in higher cytotoxicity, we investigated the effect of 3 μ M aphidicolin on the sensitivity of CLL cells to dFdC using the MTT assay. We found that aphidicolin itself reduced only slightly CLL cell viability (from 0 to 18%) depending on the CLL sample, but clearly potentiated the cytotoxicity of dFdC, decreasing by 5.8-fold the IC₅₀ value: from 40.3 ± 20.3 to

7 ± 3.2 μ M (Fig. 5A). To characterize the in vitro drug interaction and to differentiate between synergism, additivity and antagonism, CLL cell survival was analyzed according to the multiplicative model, as detailed in Section 2.6. Analysis of the three separate experiments shown in Fig. 5A at five different concentrations of dFdC and one concentration of aphidicolin, resulting in 15 individual comparisons between observed and expected CLL survival, showed synergistic interaction in almost all combinations (Fig. 5B).

3.5. Influence of aphidicolin on dFdC cytotoxicity in colon cancer cell lines

As dFdC is not given to treat CLL, but solid tumors [33], we also analyzed the influence of aphidicolin on dFdC cytotoxicity in the human colon adenocarcinoma-derived cell line HCT-116. However, contrary to CLL cells that are resting cells, HCT-116 cells are dividing cells in which aphidicolin will inhibit DNA replication and hence their proliferation, which can complicate the interpretation of the MTT assays. Therefore, we first investigated whether dCK activation induced by aphidicolin could be maintained after removal of the drug. Indeed, we found that dCK activity, which was increased up to 4-fold after a 24 h-incubation in the presence of 3 μ M aphidicolin, was still 2- to 3-fold increased 24 h after its removal (not shown). Thus, HCT-116 cells were incubated for 24 h without or with 3 μ M aphidicolin, washed with fresh medium and then incubated again for 72 h with increasing concentrations of dFdC before the MTT assay (Fig. 6A). Results showed that the IC₅₀ of dFdC, which was very low in HCT-116 cells compared to CLL cells, was further reduced, from 6 ± 1 to 3 ± 1 nM ($P < 0.05$), when cells were preincubated with aphidicolin. Moreover, the residual cell survival, which was about 20% in the control condition, decreased to about 4% in cells that were preincubated with aphidicolin. To characterize the in vitro drug interaction, HCT-116 cell survival measured in experiments shown in Fig. 6A was analyzed according to the multiplicative model, as described above for CLL cells. Analysis of three separate experiments resulting in 17 individual comparisons between observed and expected HCT-116 cell survival (Fig. 6B) showed synergistic interaction in all combinations ($P < 0.001$).

Cytotoxicity of dFdC in HCT-116 cells has been associated with activation of caspase-3, a critical effector of apoptosis [34]. We found that caspase-3 activity was not yet augmented after a 24 h-incubation with dFdC alone, but was increased up to 4- and 9-fold at 0.03 and 0.1 μ M dFdC, respectively, in cells that were preincubated with aphidicolin (Fig. 6C).

As expected, analysis of dFdCTP by HPLC showed that aphidicolin treatment strongly enhanced the conversion of dFdC

Table 3

Influence of aphidicolin on the conversion of various nucleoside analogs and dCyd into triphosphate compounds in CLL cells. CLL cells were preincubated for 30 min without or with 3 μ M aphidicolin (APC) before addition of the labeled nucleoside analog at the indicated concentration. Nucleotides were determined by HPLC after 2 h of incubation. Results are means $\pm$ SEM of separate experiments, (n) indicating the number of experiments. Significance relative to the absence of APC: * $P < 0.05$; ** $P < 0.01$.

Nucleoside	(μ M)	(n)	Nucleoside triphosphates (pmol/mg protein)		Ratio +APC/–APC
			–APC	+APC	
F-Ara-A	0.3	(3)	31.9 ± 16	31.5 ± 14.5	0.99
	5.0	(3)	307 ± 58	301 ± 68	0.98
CdA	0.1	(3)	6.4 ± 3.0	5.6 ± 2.4	0.88
	0.5	(8)	19.6 ± 3.0	19.2 ± 2.6	0.98
Ara-C	10	(5)	1013 ± 209	1104 ± 273	1.1
	30	(3)	1105 ± 433	1750 ± 768	1.6
dFdC	10	(4)	1314 ± 327	$1664 \pm 450^*$	1.3
	50	(7)	1840 ± 328	$3182 \pm 619^{**}$	1.7
dCyd	10	(6)	24.2 ± 1.1	$38.2 \pm 5.1^{**}$	1.6

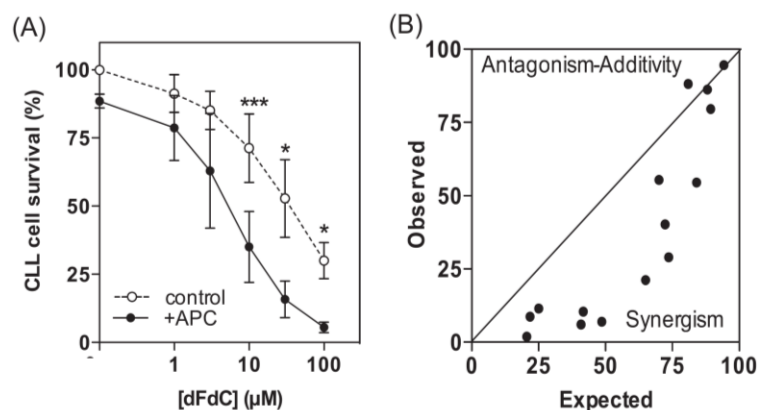


Fig. 5. Effect of aphidicolin on cell death induced by dFdC in CLL cells. (A) CLL cells were incubated without (○) or with 3 μM aphidicolin (APC) (●) in the presence of increasing concentrations of dFdC. CLL cell survival was measured by the MTT assay after a 96 h-incubation. (B) Comparisons ($n = 15$) between observed and expected cell survival for the combination dFdC + APC performed according to the multiplicative model as explained in Section 2.6. The continuous line is $x = y$.

into dFdCTP, by 9- and 4-fold at 0.03 and 0.1 μM dFdC, respectively (Fig. 6D). A 1.7-fold-increase of dFdC incorporation into DNA was also recorded at 0.03 μM dFdC, from 0.66 ± 0.28 pmol/mg of protein to 1.15 ± 0.33 pmol/mg of protein ($P < 0.05$), whereas no change could be detected at 0.1 μM dFdC. Similar results were observed in HT-29 cells, another colon cancer cell line (not shown).

4. Discussion

Low dCK activity resulting in poor accumulation of the active triphosphate form of nucleoside analogs can contribute to resistance to these drugs [4]. Our finding that dCK activity can be increased by Ser-74 phosphorylation in intact cells, including

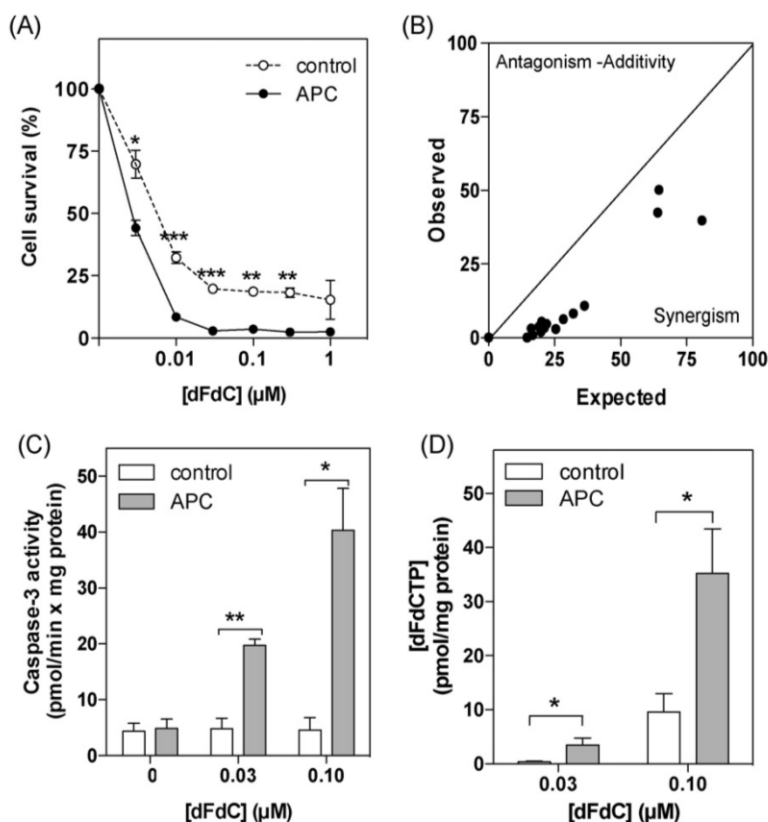


Fig. 6. Effect of aphidicolin on dFdC-induced cell death, caspase-3 activity and dFdC activation in HCT-116 cells. (A) HCT-116 cells were preincubated without (○) or with (●) 3 μM aphidicolin (APC) for 24 h. After removal of APC, cells were incubated in the presence of the indicated concentrations of dFdC and cell survival was measured by the MTT assay after a 72 h-incubation. Values obtained in the absence of dFdC for untreated and APC-treated cells were set at 100%. (B) Comparisons ($n = 18$) between observed and expected cell survival done according to the multiplicative model as explained in Section 2.6. The continuous line is $x = y$. (C) Caspase-3 activity and (D) dFdCTP concentration were measured after a 24 h-incubation in the presence of the indicated concentrations of dFdC in cells preincubated in the absence (open bars) or the presence (gray bars) of APC, as described in (A). Results are means $\pm$ SEM of 3 or 4 separate experiments. Significance relative to the absence of APC: * $P < 0.05$; ** $P < 0.01$.

CLL lymphocytes [13], led to the attractive hypothesis that combining nucleoside analogs with agents that increase phosphorylation of dCK on Ser-74 could improve their efficacy. However, although it was definitely demonstrated that Ser-74 phosphorylation increases dCK activity for dCyd [11,15] and dCyd analogs [15], whereas it decreases it for dAdo and dGuo [15], nothing was known about purine nucleoside analogs. We show here that mimicking Ser-74 phosphorylation increased the k_{cat} of dCK for purine analogs, and particularly CdA, in contrast with what was observed for natural purines. However, since the S74E mutation also increased the K_m for purine analogs as previously observed for other nucleosides, the catalytic efficiency (k_{cat}/K_m) was not radically modified, being slightly increased for CdA and CAFdA and decreased for F-Ara-A. Taken together, these data indicate that the effect of Ser-74 phosphorylation on dCK activity varies from nucleoside to nucleoside and should be analyzed for each analog in particular.

Insight into the mechanism by which phosphorylation of Ser-74, a residue far from the active site, modifies dCK activity has recently been provided [35]. The authors of this study, who succeeded in crystallizing dCK containing the S74E mutation, propose a model in which Glu-74 stabilizes the open conformation of dCK by directly interacting with Trp-58. As this conformation is compatible with binding of the nucleoside and release of the product, but not with phosphoryl transfer [36], the S74E mutation increases the energy barrier to the formation of the catalytically competent closed state, explaining the higher K_m of the S74E dCK for its substrates. The increased k_{cat} of this variant, as observed for dCyd, would result from a more rapid transition to the open state after phosphoryl transfer, allowing for nucleoside monophosphate release [35]. However, it remains unclear why the S74E dCK displays a decreased activity toward the purines dAdo and dGuo [15]. Also, we cannot explain why Glu-74 increases the catalytic rate of dCK toward CdA (Table 1), whereas it decreases it for its natural counterpart dAdo [15]. Since it has been established that the Trp58-Glu74 interaction plays a key role in modifying the activity of dCK, we can hypothesize that the degree of this interaction, and therefore the compactness of the nucleoside-binding pocket, will depend on the nature of the nucleoside. Indeed, it is well known that dCK can adopt several distinct conformations, depending on the nature of the nucleoside bound, very small changes in its structure being sufficient to select the open or the closed conformation [36–39]. As an example, CdA and dAdo that differ only by a chlorine atom at carbon 2 of the purine ring, have been shown to determine different dCK conformations [36].

The conformation of dCK at the acceptor site is determined not only by the nucleoside substrate, but also by the phosphoryl donor, UTP being more favorable to the open state than ATP [36]. Therefore, the effect of the S74E mutation on dCK activity could be different if UTP is used as the phosphoryl donor instead of ATP. Comparison of the results obtained with ATP and UTP (Table 1) confirmed that UTP enhances the affinity of dCK for purine nucleosides [39], and showed that the S74E mutation similarly influences the k_{cat}/K_m ratio with both phosphoryl donors, regardless the nucleoside examined. However, individual changes induced by Glu-74 in K_m and k_{cat} varied according to the nucleoside, being lower with UTP than with ATP for dCyd and CdA, similar for CAFdA and higher for F-Ara-A. Most probably, these different effects of the S74E mutation on K_m and k_{cat} in the presence of UTP versus ATP have to be related to different conformational states of dCK, which could be unraveled only by additional structural studies.

Inhibition of dCK by its distal end product dCTP has generally been investigated using dCK purified from human tissues, implying that this dCK might be, at least partially, phosphorylated on Ser-74.

As indicated by results shown in Table 2, this post-translational modification does not modify the inhibition pattern of dCK by dCTP, which was found to be mixed in the presence of ATP and uncompetitive in the presence of UTP, as already reported by others [25,40,41]. Nevertheless, Ser-74 phosphorylation could sensitize dCK to inhibition by dCTP, especially when dCyd is the variable substrate (Table 2). To explain inhibition without a separate allosteric site, it has been proposed that dCTP functions as a multi-substrate analogue, binding and bridging both the nucleoside and the nucleotide site of dCK [42]. Since Glu-74 favors the open dCK conformation and therefore the binding of nucleoside to dCK, we can hypothesize that it also favors the binding of the nucleoside moiety of dCTP, and hence the inhibition of dCK. However, in conditions simulating the cellular environment where both ATP and UTP are present, dCTP clearly does not prevent the increase in dCK activity induced by the S74E mutation (Fig. 2). Also, the physiological impact of dCTP inhibition appears to be weak toward dCyd, even for the S74E variant. In contrast, inhibition by dCTP is more pronounced when CdA was the dCK substrate, as it was previously observed for dFdC [43], reinforcing the hypothesis that reduction in cellular dCTP level could lead to enhanced nucleoside analog activation.

Although the S74E mutation increased the activity of recombinant dCK for CdA (Table 1 and Figs. 1 and 2), treatment of leukemic cells with aphidicolin, which leads to Ser-74 phosphorylation, did not enhance the activity of endogenous dCK toward CdA (Fig. 3b). However, the same treatment induced increase of dCK activity toward dCyd (Fig. 3a), though to a less extent than Glu-74 in recombinant dCK (Figs. 1 and 2). One explanation for the discrepancies observed between the recombinant and endogenous dCK might be that the true phosphorylation of Ser-74 does not induce exactly the same changes in dCK structure as Glu-74. Also, the recombinant WT dCK corresponds to a completely unphosphorylated enzyme and S74E dCK to a fully phosphorylated form, whereas endogenous dCK in leukemic cells is already partly phosphorylated on Ser-74 in basal conditions. Hence, increase of phosphorylation of endogenous dCK on Ser-74, such as induced by genotoxic agents, will not provoke a change as large as that induced by the S74E mutation. Results similar to those obtained with aphidicolin were found with other compounds that increase Ser-74 phosphorylation (Fig. 4), showing in addition that these treatments did not augment dCK activity toward dAdo and F-Ara-A, but well toward dFdC.

Finally, a key point was to analyze in intact cells whether activation of dCK through Ser-74 phosphorylation could enhance the conversion of therapeutic NA into their active triphosphate form. We clearly demonstrated that aphidicolin, which augments phosphorylation of dCK on Ser-74 in CLL cells [13], does not enhance the accumulation of the triphosphate form of CdA or F-Ara-A in this cell model (Table 3) in line with the results shown in Figs. 3 and 4. These results indicate that combination of CdA or fludarabine with a compound that increases phosphorylation of dCK on Ser-74 would not translate into improved clinical efficacy. On the opposite, aphidicolin significantly increases the intracellular accumulation of dFdCTP from dFdC (Table 3 and Fig. 6D) and amplifies dFdC-induced cytotoxicity in both CLL and HCT-116 cells (Figs. 5 and 6A and B), which could be associated with enhanced apoptosis, as shown by increase in caspase-3 activity (Fig. 6C). Therefore, increase of dCK activity induced by Ser-74 phosphorylation could enhance the in vivo activation of dFdC and hence its clinical efficacy.

In conclusion, effects of Ser-74 phosphorylation on dCK activity are not easily predictable. Indeed, the S74E mutation increases or decreases dCK activity depending on the nucleoside substrate. Moreover, increase in dCK activity induced by the S74E mutation in recombinant dCK appears to be higher than changes induced in the

activity of endogenous dCK by in vivo-induced Ser-74 phosphorylation. Therefore, assessment of the physiological or pharmacological interest of increasing Ser-74 phosphorylation will only be possible by measuring its effect on the conversion of the nucleoside (analog) of interest into nucleotides in intact cells. As reported here, this approach allowed demonstration that the strategy combining a nucleoside analog, like dFdC, with a compound that induces Ser-74 phosphorylation is promising and may constitute a new option in specific cancer therapies.

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CHAPTER III:
**Protein phosphatase 2A regulates deoxycytidine
kinase activity via Ser-74 dephosphorylation**

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Protein phosphatase 2A regulates deoxycytidine kinase activity via Ser-74 dephosphorylation

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ABSTRACT

Deoxycytidine kinase (dCK) is a critical enzyme for activation of anticancer nucleoside analogs. Its activity is controlled via Ser-74 phosphorylation. Here, we investigated which Ser/Thr phosphatase dephosphorylates Ser-74. In cells, the PP1/PP2A inhibitor okadaic acid increased both dCK activity and Ser-74 phosphorylation at concentrations reported to specifically target PP2A. In line with this, purified PP2A, but not PP1, dephosphorylated recombinant pSer-74-dCK. In cell lysates, the Ser-74-dCK phosphatase activity was found to be latent, Mn²⁺-activated, responsive to PP2A inhibitors, and diminished after PP2A-immunodepletion. Use of siRNAs allowed concluding definitively that PP2A constitutively dephosphorylates dCK in cells and negatively regulates its activity.

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1. Introduction

Deoxycytidine kinase (dCK) converts deoxycytidine, deoxyguanosine and deoxyadenosine into their monophosphate form with ATP or UTP as phosphoryl donor. This reaction is the first and rate-limiting step of the deoxyribonucleoside salvage pathway that supplies cells with precursors of DNA as an alternative to *de novo* synthesis [1]. In addition, dCK phosphorylates and thereby activates several anticancer and antiviral nucleoside analogs, such as fludarabine, cytarabine, gemcitabine, zalcitabine and lamivudine [2,3].

Given the essential physiological and pharmacological role of dCK, identification of the mechanisms that regulate its activity is of particular interest. The first mechanism reported to control dCK activity was retro-inhibition by dCTP, although its physiological relevance is questionable [4–6]. Thereafter, several studies

[7–10] suggested that dCK activity could be regulated by post-translational modification, which was definitively confirmed by our group. We established that dCK is a phosphoprotein containing at least four phosphorylation sites: Thr-3, Ser-11, Ser-15 and Ser-74, the latter being the major phosphorylated residue and the only one to play a role in the regulation of dCK activity [11]. Furthermore, we found that increase in dCK activity induced by genotoxic treatments, such as etoposide, aphidicolin, UV-irradiation, was correlated to an increase of Ser-74 phosphorylation, whereas decrease of its activity induced by osmotic stress was related to Ser-74 dephosphorylation [11,12]. Insight into the mechanism by which Ser-74 phosphorylation modifies dCK activity has recently been provided through structural studies of Hazra et al. [13], who propose that phosphorylation of Ser-74 favors the adoption by dCK of an open conformation more conducive for binding of nucleoside and release of the nucleoside monophosphate product.

The finding of the phosphorylation of dCK at Ser-74 raised the question of which protein kinase(s) and phosphatase(s) directly phosphorylate(s) or dephosphorylate(s) this residue. Casein kinase 1δ was shown to be able to phosphorylate dCK on Ser-74 *in vitro*, but would not be responsible for its *in vivo* phosphorylation [14]. In contrast, the DNA-damage-response kinase ATM (ataxia telangiectasia mutated) was found to phosphorylate Ser-74 *in vitro*, but also *in vivo*, which was also associated with a role of dCK in the

Abbreviations: dCK, deoxycytidine kinase; pSer-74-dCK, dCK phosphorylated at Ser-74; ATM, ataxia telangiectasia mutated; PP, Ser/Thr phosphatase; PP1, Ser/Thr protein phosphatase 1; PP1c, catalytic subunit of PP1; PP2A, Ser/Thr protein phosphatase 2A; PP2Ac, catalytic subunit of PP2A; Fos, Fostriecin; OA, okadaic acid; siRNA, small interfering RNA; HEK, human embryonic kidney

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regulation of the G2/M checkpoint [15]. Concerning the protein phosphatase (PP) that dephosphorylates Ser-74 in intact cells, its responsiveness to okadaic acid (OA) has been established. Indeed, this PP inhibitor increased dCK activity [16] and Ser-74 phosphorylation [14] in cultured cells.

Ser/Thr specific protein phosphatases comprise three structurally unrelated superfamilies and have been implicated in the regulation of many cell events [17,18]. The vast majority of Ser and Thr dephosphorylations in eukaryotic cells are catalyzed by members of the PPP (phosphoprotein phosphatases) and PPM (protein phosphatase metal) families. PPMs are Mg^{2+}/Mn^{2+} -dependent monomeric enzymes with PP2C phosphatases as the major representatives and are insensitive to OA inhibition. PPPs comprise the large families of multimeric Ser/Thr protein phosphatase 1 (PP1), Ser/Thr protein phosphatase 2A (PP2A) and PP2B (calcineurin) phosphatases, as well as the more recently identified PP4, PP5, PP6 and PP7 phosphatases, which are expressed at relatively low abundance. PP4 and PP6 are sometimes referred to as PP2A-like phosphatases due to their high structural homology with PP2A and similar sensitivities to pharmacological phosphatase inhibitors. Among Ser/Thr phosphatases, PP2A is potently inhibited by OA in the low nanomolar range, while PP1 is around 100 times less sensitive. In contrast, PP4 and PP6 have similar IC_{50} values for OA than PP2A [19]. In the present study, we sought to determine which OA-sensitive PP is responsible for Ser-74 dephosphorylation and hence is involved in the regulation of dCK activity.

2. Material and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), ultraglutamine, fetal calf serum (FCS) and penicillin–streptomycin were purchased from Lonza. RPMI-1640 and all tissue culture reagents were from Gibco/Invitrogen. $[5-^3H]$ -deoxycytidine (30 Ci/mmol) was from Moravsek Biochemicals. OA was from Enzo Life Sciences. Anti-phospho-Ser-74 and anti-dCK antibodies were generated as described previously [11]. Anti-poly(His) monoclonal antibody, protein A Sepharose beads and NAP-5 columns were from GE Healthcare. Anti-PP1c (sc-7482), anti-PP2Ac (sc-166034) antibodies and fosfocin (Fos) were from Santa Cruz Biotechnology. Fluorescent secondary antibodies IRDye®680 or IRDye®800 and Odyssey blocking buffer were from Li-COR Biosciences. Catalytic subunit of PP2A (specific activity: 10 U/mg), purified from beef heart according to Cohen et al. [20], and GST-tagged catalytic subunit of PP1 (specific activity: 8 U/mg), purified after overexpression in *Escherichia coli*, were kindly provided by Prof. M. Rider (de Duve Institute, Brussels, Belgium). Other chemicals, materials and reagents were from Sigma–Aldrich, Merck Biosciences or Bio-Rad Laboratories.

2.2. Cell culture and incubation

CCRF-CEM cells and HEK293T cells that stably express human dCK were cultured as described [11,14]. EBV-transformed lymphoblasts GM0536 (ATM+/+) and GM1526 (ATM−/−), purchased from The NIGMS Human Mutant Cell Repository, were maintained in RPMI-1640 with Glutamax supplemented with 15% heat-inactivated FCS. OA was dissolved in DMSO and equal amounts of DMSO were added in control cells.

2.3. Preparation of cell lysates and dCK assay

CCRF-CEM, GM0536, GM1526 or HEK293T cell extracts were prepared as previously described [11,16]. dCK activity was measured with a radiochemical assay as described in [16] using

10 μ M $[5-^3H]$ -deoxycytidine and 5 mM ATP as substrates and 30–100 μ g of cellular protein or 0.05–0.1 μ g of recombinant dCK. The protein content of samples was measured by the method of Bradford [21], with BSA as a standard.

2.4. Protein phosphatase treatment of purified recombinant dCK

Recombinant dCK (0.5 μ g) overexpressed in HEK293T cells and purified as reported previously [11] was incubated at 30 °C in 50 μ l of a reaction mixture containing 50 mM Tris–HCl, pH 7.6, 2 mM dithiothreitol and 2 mM $MnCl_2$ in the absence or in the presence of 0.2 U of purified catalytic subunit of PP1 (PP1c) or catalytic subunit of PP2A (PP2Ac). After 40 min of incubation, aliquots (0.1 μ g of protein) were taken for dCK assay and western blot analysis.

2.5. Ser-74-dCK phosphatase assay in cell lysates

For analysis of dCK dephosphorylation at Ser-74 and dCK inactivation in cell lysates, cell extracts were prepared in a phosphatase assay buffer (50 mM Tris–HCl, pH 7.6, 5 mM dithiothreitol, 0.5% Nonidet P-40, 0.1 mM EDTA, 20% glycerol, 0.5 mM p-toluenesulfonyl fluoride, 5 mM benzamide, and 5 μ g/ml leupeptin and antipain). After centrifugation, supernatants were filtered on NAP-5 columns and 300 μ g of filtered protein were then incubated at 37 °C for 90 min, as such or in the presence of 0.5 μ g of purified recombinant dCK [11]. The reaction was started by the addition of Mn^{2+} at the indicated concentration. At time zero and after 90 min of incubation, aliquots were taken for analysis of dCK phosphorylation at Ser-74 and activity.

2.6. PP2A immunoprecipitation

For PP2A immunoprecipitation, protein A Sepharose beads (60 μ l) were incubated with 9 μ g of anti-PP2Ac antibody on a rocker for 1 h at 4 °C. The beads were then washed twice with the phosphatase assay buffer and incubated overnight with 900 μ g of protein extract at 4 °C. After centrifugation, the supernatant was used for Ser-74-dCK phosphatase analysis as described above.

2.7. RNA interference

HEK293T cells stably expressing dCK were plated in 10 cm-dishes (0.6×10^6 cells/dish) and transfected on the following day using negative control small interfering RNA (siRNA) (non-targeting pool: # D-001810-10, from Dharmacon), 50 nM PP2Ac siRNA (directed against the PP2Ac alpha isoform: # L-003598-010005, from Dharmacon) or 50 nM pan PP1c siRNA (# sc-43545, from Santa Cruz Biotechnology) and 20 μ l Lipofectamin 2000 (Invitrogen), according to the manufacturer's instructions. After 72 h, the cells were washed with cold PBS and cell extracts were prepared as described in [16]. After centrifugation, supernatants were used for dCK activity assay and western blot analysis.

2.8. Immunoblot analysis

Aliquots containing 40–100 μ g of cell protein or 0.05–0.1 μ g of recombinant dCK were subjected to SDS–PAGE electrophoresis in 12% (w/v) polyacrylamide gels and transferred to low fluorescence PVDF immobilon-FL membranes (Millipore). After transfer, PVDF membranes were blocked for 1 h at room temperature in Odyssey blocking buffer and then probed overnight with either anti-phospho-Ser-74 (1/1000), anti-poly(His) (1/4000), anti-dCK (1/500), anti-PP1c (1/1000), anti-PP2Ac (1/1000), or anti- β -actin (1/10,000). After washing in PBS-T (Tween 0.1%), the membranes were incubated for 1 h at room temperature with the appropriate fluorescent secondary antibody IRDye®680 or IRDye®800

(Li-COR) diluted (1/10,000) in Odyssey blocking buffer containing 0.1% Tween and 0.1% SDS. After washing, the membranes were scanned with the Odyssey Infrared Imaging System (Li-COR). Representative immunoblots are shown. When indicated, fluorescence intensities have been used to quantify dCK phosphorylation or expression [22].

2.9. Statistical analysis

dCK activities are the means $\pm$ S.E.M. of at least three independent experiments. Significance was analyzed by paired Student's *t*-tests. Changes were considered significantly different when $P < 0.05$.

3. Results and discussion

Our previous findings that dCK activity [16] and dCK phosphorylation at Ser-74 [14] were augmented in intact cells after treatment with 500 nM OA, a cell-permeable PP1/PP2A inhibitor, and the fact that purified PP2A inactivated dCK in cell lysates [16] led us to postulate that PP2A was involved in the *in vivo* regulation of dCK activity. Though OA inhibits both PP1 and PP2A, it has substantial preference *in vitro* for PP2A ($IC_{50} = 0.1$ nM) over PP1 ($IC_{50} = 10$ nM) [19]. Its selectivity for PP2A has also been demonstrated *in vivo* in intact cells in which 100 nM OA has been shown to inhibit PP2A without affecting PP1 activity [23]. Therefore, we sought to examine thoroughly the dose–response effect of OA on both dCK phosphorylation at Ser-74 and dCK activity. Analyses were performed after a 4 h-incubation in CCRF-CEM cells in which phosphorylation of dCK at Ser-74 can be easily detected by western blot [11]. Increase in Ser-74 phosphorylation (Fig. 1A) and in dCK activity (Fig. 1B) was already noticed at 25 nM OA, while maximal Ser-74 phosphorylation level and dCK activity were reached at 100 nM. These results suggested that PP2A or a PP2A-like enzyme, rather than PP1, was involved in the control of Ser-74 phosphorylation.

In cells, increase of Ser-74 phosphorylation can result from inhibition of a dCK phosphatase or from activation of a dCK kinase.

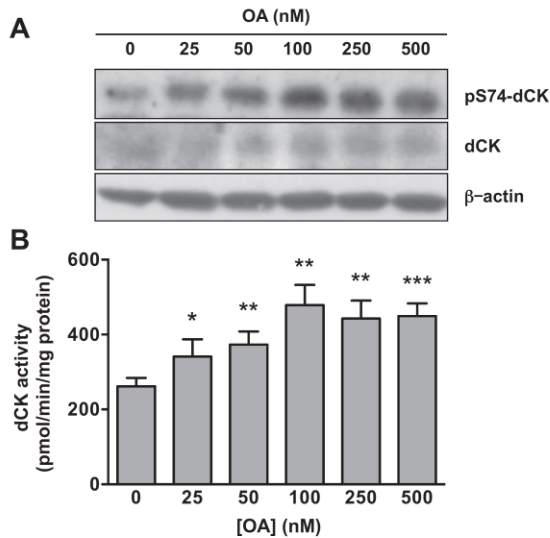


Fig. 1. Dose–response effects of OA on Ser-74-dCK phosphorylation and activity. CCRF-CEM cells were incubated for 4 h with increasing concentrations of OA. Cell lysates were analyzed for (A) dCK phosphorylation at Ser-74 and (B) dCK activity (mean $\pm$ S.E.M. of 5 independent experiments). Significance relative to the absence of OA: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Nowadays, ATM is the only protein kinase known to phosphorylate dCK at Ser-74 *in vivo* [15]. To verify that the increase of Ser-74 phosphorylation induced by OA was not due to the indirect activation of ATM, we compared the effect of OA in ATM proficient (GM0536) and deficient (GM1526) lymphoblastoid cell lines. We found that OA induced similar dCK activation in both cell lines, either at 100 (not shown) or at 500 nM (Fig. 2A and B). These results indicate that increase in Ser-74 phosphorylation induced by OA cannot be explained by ATM activation, which is consistent with the finding that OA, though inducing autophosphorylation of ATM on Ser-1981, does not increase its kinase activity [24]. Interestingly, these data also indicate that a kinase different from ATM can phosphorylate dCK at Ser-74 at least in basal conditions, which deserves further investigations.

To corroborate the hypothesis that PP2A rather than PP1 is involved in Ser-74 dephosphorylation, we compared the ability of purified catalytic subunits of PP1 (PP1c) and PP2A (PP2Ac) to dephosphorylate Ser-74-dCK. Recombinant dCK overexpressed in HEK293T cells, which is highly phosphorylated at Ser-74 [11], was used as substrate. Incubation of the latter with PP1c induced neither Ser-74 dephosphorylation (Fig. 3A) nor decrease of its activity (Fig. 3B) in comparison with the control condition, whereas PP2Ac used at the same specific activity as PP1c decreased Ser-74 phosphorylation (Fig. 3A) by $70.9 \pm 3.9\%$ ($n = 3$) and dCK activity (Fig. 3B) by $75.2 \pm 7.1\%$ ($n = 5$). We verified that PP1c was active by checking its capacity to dephosphorylate histone H1 32 P-prelabeled by protein kinase A as described in [19] (not shown). We conclude that PP2Ac, but not PP1c, dephosphorylates dCK at Ser-74 *in vitro*.

The next step was to analyze Ser-74-dCK phosphatase activity from cell lysates. For this purpose, CCRF-CEM cell extracts were prepared in a buffer containing no phosphatase inhibitors and then gel-filtered to remove small molecular weight molecules. First, we examined the ability of a cell lysate to dephosphorylate the recombinant dCK. Quite unexpectedly, we found that neither the phosphorylation of recombinant dCK at Ser-74 (Fig. 4A) nor

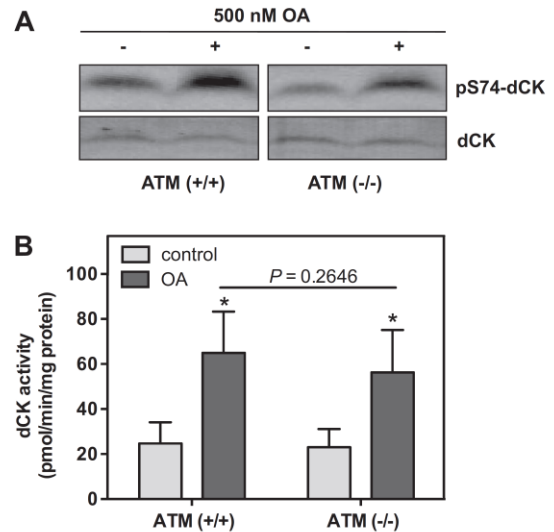


Fig. 2. Comparison of Ser-74 phosphorylation and dCK activation induced by OA in GM0536 (ATM+/+) and GM1526 (ATM–/–) cells. Cells were incubated for 4 h in the absence or the presence of 500 nM OA. Cell lysates were analyzed for (A) dCK phosphorylation at Ser-74 and (B) dCK activity (mean $\pm$ S.E.M. of 4 independent experiments). Significance relative to the absence of OA: * $P < 0.05$. Increase of dCK activity induced by OA in ATM (+/+) cells was 2.86 ± 0.37 -fold in comparison with 2.5 ± 1.18 -fold in ATM (–/–) cells.

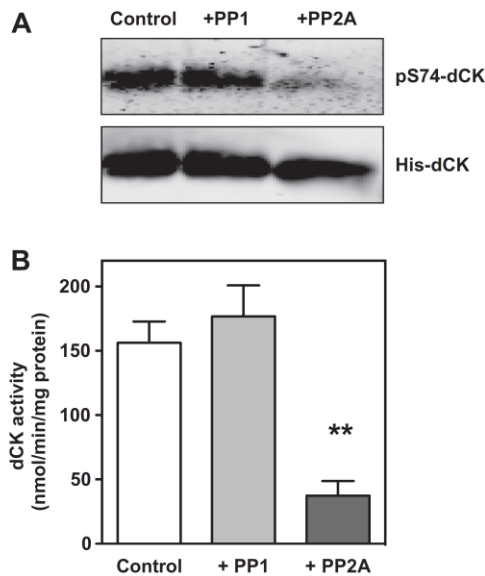


Fig. 3. PP2Ac, but not PP1c, dephosphorylates dCK at Ser-74 *in vitro*. Recombinant dCK, was incubated at 30 °C in the absence or in the presence of purified PP1c or PP2Ac, as described in the Methods. (A) dCK phosphorylation at Ser-74 and (B) dCK activity (mean $\pm$ S.E.M. of 5 independent experiments) were measured after 40 min of treatment. Significance relative to the control condition: ** $P < 0.01$.

its activity (Fig. 4B) were significantly modified by a 90 min-incubation at 37 °C in the presence of the cell lysate, unless Mn^{2+} was added in the assay mixture at a concentration exceeding 0.1 mM. Similar results were obtained with unfiltered cell lysates (not shown), confirming that Ser-74-dCK phosphatase requires Mn^{2+}

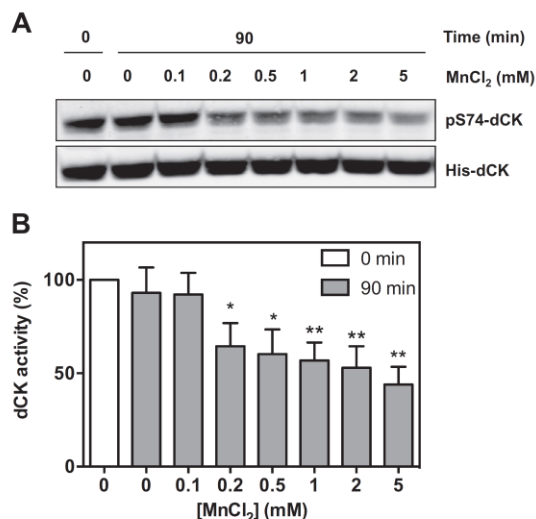


Fig. 4. Mn^{2+} activates Ser-74-dCK phosphatase activity in cell lysates. Recombinant dCK was incubated at 37 °C in the presence of 300 μ g of CCRF-CEM cell lysate protein, in the absence or in the presence of $MnCl_2$ at increasing concentrations. (A) Phosphorylation of dCK at Ser-74 and (B) dCK activity (mean $\pm$ S.E.M. of 3 independent experiments) were analyzed before (0 min) and after 90 min of incubation. Activity of dCK at 0 min was 197 ± 35 nmol/min/mg protein. Significance relative to the absence of $MnCl_2$: * $P < 0.05$; ** $P < 0.01$.

to be active. Analysis of the effect of other divalent metal showed that Fe^{2+} or Mg^{2+} could replace Mn^{2+} albeit with a lower efficiency (not shown). Following these experiments, 2 mM Mn^{2+} was included in all *in vitro* Ser-74-dCK phosphatase assays. To verify whether this Mn^{2+} -activated Ser-74-dCK phosphatase could be PP2A, we analyzed whether PP2A inhibitors could inhibit it. We found that OA completely prevented dephosphorylation of the recombinant dCK (Fig. 5A) by the cell lysate and strongly reduced its inactivation (Fig. 5B). This allows to exclude the possibility that Ser-74-dCK phosphatase is a type 2C phosphatase (PP2C), which is characterized by dependence on divalent cations, but insensitivity to OA [25,26]. Fostriecin, a highly selective inhibitor of PP2A [27], also clearly reduced the Mn^{2+} -dependent Ser-74-dCK phosphatase activity from cell lysates (Fig. 5A and B).

Although it is generally accepted that PP2A is not dependent on Mn^{2+} or other divalent cations for activity, metal ions are required in the catalysis of PPP phosphatases through the activation of a water molecule for the dephosphorylation reaction [17]. In addition, some studies reported that PP2A can exist in a latent form, which can be activated *in vitro* by treatment with Mn^{2+} [28–31]. Inactivation of PP2A, which has especially been observed after prolonged storage but also under other conditions, was explained by the loss of metal ions from its catalytic site. The Mn^{2+} -independent PP2Ac has indeed been reported to be a Zn^{2+} - and Fe^{2+} -metalloenzyme [30]. In our hands, the Mn^{2+} -dependency of Ser-74-dCK phosphatase activity was not correlated to the storage and was not due to gel-filtration, but was present as soon as cell lysates were prepared.

To further confirm that Ser-74-dCK phosphatase is PP2A, we compared Ser-74-dCK phosphatase activity of CCRF-CEM cell lysates from which PP2A had been, or not, removed by immunoprecipitation. As observed with recombinant dCK, dephosphorylation (Fig. 6A) and inactivation (Fig. 6B) of endogenous dCK in whole cell lysates were strongly inhibited by OA and fostriecin. Dephosphorylation and inactivation of dCK were also reduced following immunodepletion of PP2A, showing that Ser-74-dCK phosphatase

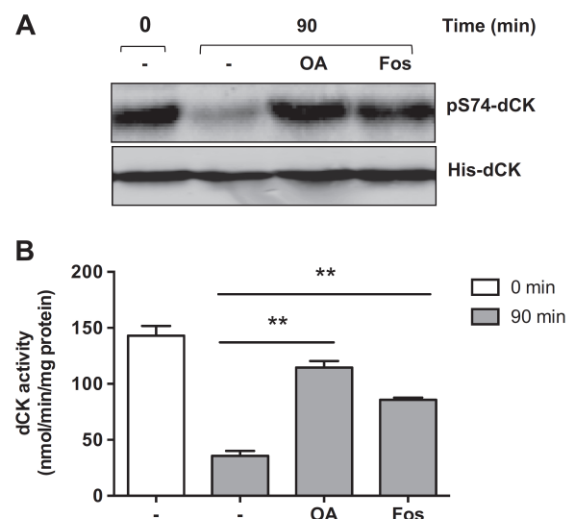


Fig. 5. Ser-74-dCK phosphatase from cell lysate is sensitive to PP2A inhibitors. Recombinant dCK was incubated at 37 °C in the presence of 300 μ g of CCRF-CEM cell lysate protein, in the absence or in the presence of 500 nM OA or 500 nM fostriecin (Fos). Mn^{2+} was present at 2 mM. (A) Phosphorylation of dCK at Ser-74 and (B) dCK activity (mean $\pm$ S.E.M. of 3 independent experiments) were analyzed before (0 min) and after 90 min of incubation. Significance relative to the absence of PP2A inhibitors: ** $P < 0.01$.

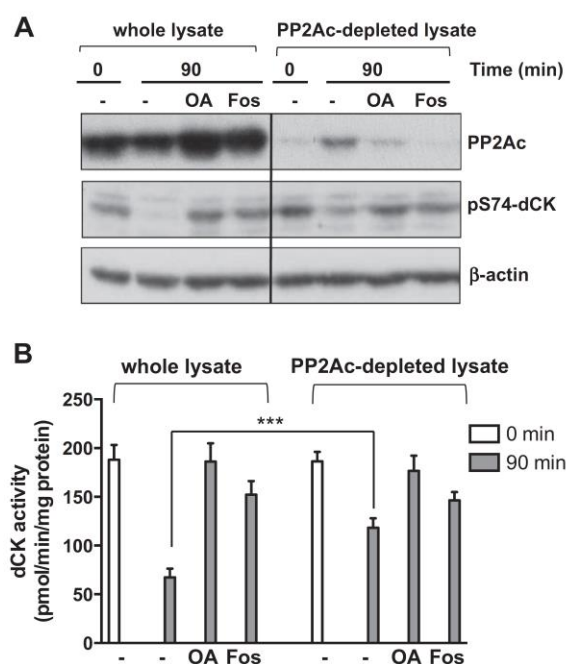


Fig. 6. Effect of PP2Ac immunodepletion on Ser-74-dCK phosphatase activity. CCRF-CEM cell lysates were submitted, or not, to PP2Ac immunoprecipitation and then incubated at 37 °C in the absence or in the presence of 500 nM OA or 1000 nM fostriecin (Fos). Mn^{2+} was present at 2 mM. (A) PP2Ac level, phosphorylation of dCK at Ser-74 and (B) dCK activity (mean $\pm$ S.E.M. of 3 independent experiments) were analyzed before (0 min) and after 90 min of incubation. Significance relative to whole cell lysate: *** P < 0.001.

activity was diminished in the PP2A-immunodepleted lysates. Addition of OA or fostriecin to PP2A-immunodepleted cell lysates inhibited more completely the dephosphorylation of dCK at Ser-74 and its inactivation. This indicates that Ser-74-dCK phosphatase activity was not completely eliminated by PP2A immunodepletion, which could be explained by residual PP2Ac presence in the supernatant (Fig. 6A). In contrast to what was observed after PP2A immunodepletion, PP1 immunodepletion did not influence the dephosphorylation of Ser-74-dCK and the inactivation of dCK in CCRF-CEM cell lysates (not shown).

To definitively prove that Ser-74-dCK is a physiological substrate for PP2A and not for PP1, we used siRNA to down-regulate the expression of the catalytic subunit of PP2A or PP1. As previously described, we used HEK293T cells stably expressing dCK as experimental model for siRNA transfection [14]. Analysis of PP2Ac and PP1c by immunoblotting showed that after a 72 h-transfection, PP2Ac and PP1c siRNA decreased PP2Ac and PP1c protein levels by 73.3 ± 1.8 and $82.9 \pm 5.5\%$ ($n = 4$), respectively. Down-regulation of PP2Ac was accompanied by a significant increase of both dCK phosphorylation at Ser-74 and dCK activity, whereas down-regulation of PP1c did not influence any of them (Fig. 7A and B). These results demonstrate that PP2A is a physiological protein phosphatase for dCK phosphorylated at Ser-74 (pSer-74-dCK).

In conclusion, the present study shows that PP2A is a negative regulator of dCK activity in cells and so adds new information about the signaling pathway that controls dCK activity *in vivo*. Use of PP2A inhibitors could thus be considered as a potential strategy to enhance dCK activity and hence the activation and clinical efficacy of anticancer nucleoside analogs that are substrates of dCK, especially as PP2A inhibitors may be, under certain condi-

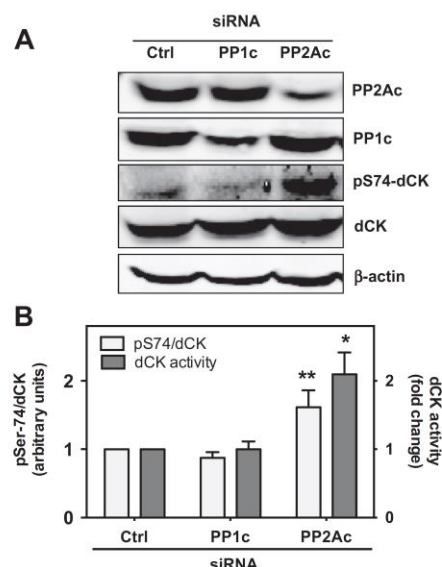


Fig. 7. Effect of PP1c or PP2Ac silencing on Ser-74-dCK phosphorylation and activity in HEK293T cells stably expressing dCK. Cells were transfected with 50 nM control siRNA, PP1c siRNA or PP2Ac siRNA. Cell lysates were analyzed 72 h after transfection for (A) PP2Ac, PP1c, pSer-74-dCK, dCK, β -actin levels and (B) dCK activity (mean $\pm$ S.E.M. of 4 independent experiments). In (B) are also shown the pSer-74-dCK/dCK ratios calculated after densitometric quantification of immunoblots (mean $\pm$ S.E.M. of 4 independent experiments). Significance relative to control siRNA: * P < 0.05; ** P < 0.01.

tions, promising therapeutic drugs for the treatment of cancer [32–34].

Acknowledgements

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CHAPTER IV:

General discussion and perspectives

1. Overview

Nucleoside analogs are anticancer or antiviral compounds that require intracellular activation to exert their therapeutic action. For several of them, phosphorylation catalyzed by dCK constitutes the first and rate-limiting step of this process. Therefore, dCK plays a pivotal role in nucleoside analog efficacy, as well demonstrated by the influence of dCK protein level or activity on the sensitivity of cells to chemotherapeutic nucleoside analogs (Hapke *et al.*, 1996; Kroep *et al.*, 2002).

Since low dCK activity is often associated with resistance to nucleoside analogs, it is attractive to search for strategies that allow increasing its activity. Knowledge about the mechanisms that regulate dCK activity is thus of particular interest. Some years ago, several studies showed that dCK activity could be increased up to 3-fold in human normal or leukemic lymphocytes by various genotoxic agents, including inhibitors of DNA synthesis, UV or γ -irradiation, and some therapeutic nucleoside analogs themselves (Sasvari-Szekely *et al.*, 1998; Spasokoukotskaja *et al.*, 1998; Spasokoukotskaja *et al.*, 1999; Cardoen *et al.*, 2001; Csapo *et al.*, 2001; Csapo *et al.*, 2003; Van Den Neste *et al.*, 2003). Activation of dCK by these different treatments occurred without change in dCK protein level, leading to the hypothesis that dCK activity could be regulated by post-translational modification. Indeed, it was demonstrated by our group that dCK is a phosphoprotein and that increase of its activity by genotoxic agents results from the increase of Ser-74 phosphorylation (Smal *et al.*, 2006). It was also shown that positive correlation exists in primary CLL cells between dCK phosphorylation at Ser-74 and dCK activity (Smal *et al.*, 2007b). Taken together, these results suggested that increasing phosphorylation of dCK at Ser-74 could be a good strategy to enhance the activation of nucleoside analogs and their efficacy in cancer cells. My PhD thesis was initiated to investigate whether this hypothesis was plausible as well as to know more about the enzyme(s) that regulate Ser-74 phosphorylation, which could also help to select conditions allowing highest dCK activity.

We will discuss first about the influence of Ser-74 phosphorylation on the kinetic properties of dCK, next about the consequences of this phosphorylation for activation and cytotoxicity of nucleoside analogs in cancer cells, and finally about the kinases and phosphatases that control the level of Ser-74 phosphorylation.

2. Effects of Ser-74 phosphorylation on the kinetic properties of dCK

Although it was definitely demonstrated that Ser-74 phosphorylation and/or S74E mutation increases dCK activity when it was assayed with dCyd (Smal *et al.*, 2006; Smal *et al.*, 2007a; McSorley *et al.*, 2008) or dCyd analogs (Ara-C and dFdC) (McSorley *et al.*, 2008), S74E mutation was found to decrease dCK activity toward its natural purine substrates dAdo and dGuo. However, the influence of Ser-74 phosphorylation on the activity of dCK toward purine analogs, which are drugs largely used in the treatment of leukemia, had not yet been investigated. Contrary to what was observed for natural purines, results of our study showed that S74E mutation increased the K_{cat} values for the purine analogs CdA and CAFdA. However, given that S74E mutation also increased the K_m values for these substrates, the catalytic efficiency (K_{cat}/K_m) of dCK for CdA or CAFdA was only slightly increased. As for F-Ara-A, S74E mutation decreased the K_{cat} , at least with ATP as phosphoryl donor, so that the S74E mutation decreased the catalytic efficiency of dCK for this analog, as observed for dAdo and dGuo.

Nucleoside	K_{cat}		K_m		K_{cat}/K_m	
dCyd	11.3	12.4	5.2	3.9	2.2	2.9
dAdo	0.9		4.4		0.2	
dGuo	0.5		2.5		0.2	
dFdC	2.8		2.4		1.1	
AraC	3.3		4.1		0.8	
CdA		8.1		6.5		1.2
CAFdA		2.2		1.5		1.5
F-Ara-A		0.8		1.2		0.6

Table 6: Ratio S74E/WT for K_{cat} , K_m and K_{cat}/K_m values determined for various substrates of dCK. This table combines values obtained by McSorley *et al.* (2008) (dark blue) and Amsailale *et al.* (2012) (light blue).

We can conclude from the study performed by McSorley *et al.* (2008) and by our group (Amsailale *et al.*, 2012) that it is very difficult, if not impossible, to predict the effect of

Ser-74 phosphorylation on dCK activity toward a particular substrate. Indeed, as illustrated in Table 6, which put together the kinetic parameters determined in the two studies, the effect of S74E mutation on K_{cat} , K_m and K_{cat}/K_m varies importantly from nucleoside to nucleoside. S74E mutation can greatly increase K_{cat} values by around 10-fold (dCyd, CdA), but also decrease it by 50% (dGuo). However as a rule, S74E mutation decreases the affinity of dCK for all natural or analog substrates, with K_m values increasing from 1.2- (F-Ara-A) to 6.5-fold (CdA). Therefore, even if S74E mutation induces an increase of K_{cat} , this positive effect could not be detected at substrate concentrations $\leq K_m$. The large variability regarding the effects of S74E mutation on the kinetic constants of dCK for its different substrates cannot be easily explained. Nevertheless, they could be related to the great ability of dCK to adopt different conformation in function of the substrate and hence to variable effects of S74E mutation on this conformation. For instance, it has been shown that the chlorine atom at carbon 2 of the purine ring, which distinguishes CdA from dAdo, was sufficient to induce distinct dCK conformations (Sabini *et al.*, 2008b).

Our observation that S74E mutation increases the K_m of dCK for its substrates probably explains why K_m values determined in previous studies with bacterially produced dCK were generally lower than those obtained with dCK purified from animal or human tissues (see section 2.4.2). Indeed, recombinant dCK overexpressed in bacteria is *a priori* devoid of post-translational modification, contrary to what can be observed in eukaryotic cells. Increase of the K_m by S74E mutation would be due to stabilization of the open conformation of dCK. Indeed, open conformation of dCK is compatible with binding of the nucleoside and release of the products, but not with phosphoryl transfer, which increases the energy barrier to the formation of the catalytically competent closed state and explains the decreased affinity of dCK for its substrates (Sabini *et al.*, 2008b; Hazra *et al.*, 2011).

As UTP is often considered as the physiological phosphoryl donor (Hughes *et al.*, 1997; Godsey *et al.*, 2006) for dCK, we have also analyzed the effect of S74E mutation in the presence of this nucleotide instead of ATP. Comparison of the results showed that the changes induced in K_m and K_{cat} values by S74E mutation were less pronounced with UTP than with ATP. But changes in catalytic efficiency were similar with both ATP and UTP, meaning that Ser-74 phosphorylation should induce similar changes in dCK activity, whether ATP or UTP is used as phosphoryl donor.

To close our kinetic study, we compared inhibition of WT and S74E dCK by dCTP, a potential dCK inhibitor in cells (Kim *et al.*, 1989). Although inhibition was relatively weak at physiological concentrations of ATP and UTP, we noted that S74E mutation could sensitize dCK to inhibition by dCTP. As dCTP functions as a multi-substrate analog, binding and bridging both the nucleoside and the nucleotide site (Jansson *et al.*, 1990), we propose that S74E mutation, which favors the binding of nucleoside substrates, also favors the binding of the nucleoside moiety of dCTP, and hence the inhibition of dCK.

The main conclusion of this part of the study is that the influence of Ser-74 phosphorylation on dCK activity will have to be analyzed for each NA individually, to identify which are positively influenced by this phosphorylation and hence to select the best candidates for potential combination therapy. dCyd is by far the deoxynucleoside for which Ser-74 phosphorylation should influence the most its metabolism by dCK. Nevertheless, analysis of Table 6 suggested that intracellular activation of dFdC, CdA and CAFdA by dCK could also be enhanced by increase of Ser-74 phosphorylation. To confirm these results obtained with recombinant dCK, we studied the influence of *in vivo*-induced increase of Ser-74 phosphorylation on endogenous dCK activity toward different NAs. It was indeed important to verify whether Ser-74 phosphorylation and replacement of serine (S) by glutamate (E) produced similar effects. Although the activity of dCK toward dAdo, CdA or F-Ara-A were not modified by increase of Ser-74 phosphorylation, its activity toward dFdC was about doubled, suggesting that this NA could really be a good candidate for combination with agents that increase Ser-74 phosphorylation. The reason why we did not find an activation of endogenous dCK with CdA, whereas the S74E mutation strongly increased K_{cat} of recombinant dCK for CdA, is presently unclear.

3. Effects of Ser-74 phosphorylation on activation and cytotoxicity of nucleoside analogs in cancer cells

The next and most important step was to analyze whether an increase of the phosphorylation of dCK at Ser-74 could really lead to higher accumulation of the active triphosphate form of NAs, and to higher cytotoxicity. Indeed, in intact cells, dCK is not the only enzyme to be involved in the activation of NAs. As explained in section 1.3.2, the net

accumulation of the monophosphate compound will result from equilibrium between dCK and 5'-nucleotidase activities. In addition, for pyrimidine nucleoside analogs, their monophosphate level is also restricted by dCyd and dCMP deaminases.

To increase dCK activity *via* Ser-74 phosphorylation in cells, we choose the DNA polymerase inhibitor aphidicolin, which was known to efficiently increase Ser-74 phosphorylation and dCK activity (Smal *et al.*, 2007b). In addition, this compound as single agent had very low cytotoxicity in CLL cells in which we first investigated the combination with NAs. While aphidicolin increased dCK activity from CLL cells by 2- to 3-fold when assayed with 10 μ M dCyd as substrate, intracellular conversion of dCyd to dCTP was increased by 1.6-fold. These results demonstrate that increase of dCK activity induced by Ser-74 phosphorylation can really increase the salvage of dCyd in intact cells. By contrast, as expected from the results obtained with endogenous dCK, the conversion of CdA and F-Ara-A, used at pharmacological concentrations, into their triphosphate form was not modified in intact CLL cells by aphidicolin. These results indicated that combination of fludarabine or CdA, which are drugs of choice for CLL treatment, with a compound that increases phosphorylation of dCK on Ser-74 would not translate into improved clinical efficacy for CLL treatment.

Results obtained with Ara-C and particularly dFdC were more encouraging. Indeed, higher activation of these compounds was induced by aphidicolin in CLL cells, but this effect was only significant for dFdC. We have then examined whether this higher activation of dFdC was associated with higher cytotoxicity. Using MTT assay, we noted that combination of dFdC with aphidicolin was nearly 6-fold more cytotoxic than dFdC alone, whereas aphidicolin itself reduced only slightly CLL cell viability (< 20%). As dFdC is not used in CLL, but in solid tumor treatments, we analyzed the effect of this combination in HCT-116 cells, a colon cancer cell line that was available in the lab. Nevertheless, we modified the experimental protocol used for CLL cells because HCT-116 cells, contrary to CLL cells, are replicating cells. Since aphidicolin is a DNA polymerase inhibitor, it can indeed block cell proliferation, which could complicate the interpretation of MTT assays, and also prevent incorporation of dFdCTP into DNA, which could reduce dFdC cytotoxicity. To avoid these “side” effects, HCT-116 cells were pre-incubated for 24 h without or with aphidicolin, washed with fresh medium to remove aphidicolin and then incubated with dFdC. Cytotoxicity of dFdC was found to be higher in cells in which dCK had been activated by aphidicolin.

Moreover, higher cytotoxicity of dFdC has been associated with higher activation of caspase-3, indicating an increase of apoptosis in this condition. Also, accumulation of dFdCTP was higher in cells preincubated with aphidicolin, confirming that increase of phosphorylation of dCK at Ser-74 phosphorylation can effectively induce higher NA activation.

In conclusion, our study provides a proof of principle that increasing phosphorylation of dCK at Ser-74 might constitute a valuable strategy to enhance the clinical efficacy of certain NAs, like dFdC. As dFdC is considered to be the reference treatment for patients with pancreatic cancer, but only with marginal clinical benefit (Heinemann, 2001), further studies should be conducted in pancreatic cancer cell lines, like PANC1 or MIAPACA, to improve the reliability of the strategy. It will be also of interest to test other agents able to increase Ser-74 phosphorylation to determine the best combination therapy that will improve the cytotoxicity of NAs for cancer cells without increasing side effects. Recently, it was reported that monogalactosyl diacylglycerol, a natural DNA polymerase inhibitor highly present in spinach, could strongly enhance cytotoxicity and apoptosis induced by dFdC in pancreatic cancer cell lines (Akasaka *et al.*, 2013). Moreover, synergy was still stronger if this DNA polymerase inhibitor was added before dFdC. The authors cannot explain these data, but we can suggest that monogalactosyl diacylglycerol could perhaps, like aphidicolin, induce increase of the phosphorylation of Ser-74, leading to increased cytotoxicity of dFdC in pancreatic cancer cell lines.

4. Regulation of Ser-74 phosphorylation

As Ser-74 phosphorylation controls dCK activity, it is important to identify the protein kinase(s) and phosphatase(s) that directly phosphorylate(s) or dephosphorylate(s) this residue.

4.1. Protein kinase(s)

After unraveling the mechanism by which dCK activity was regulated at post-translational level, identification of the protein kinase(s) that phosphorylate(s) Ser-74 became one of the major objectives of our group. This kinase should be constitutively active since dCK is phosphorylated under basal conditions in cell lines as well as in primary CLL cells

(Smal *et al.*, 2007b; Smal *et al.*, 2010b). Analysis of the sequence surrounding Ser-74 in dCK revealed that Ser-74 was localized downstream a Glu-rich region presenting similarities with the consensus sequence of constitutively active casein kinase I (CKI) family, and particularly of CKI δ . In addition, CKI δ was known to display high activity in lymphocytes (Spasokoukotskaja *et al.*, 1995; Maritzen *et al.*, 2003), to play an important role in the DNA damage response and to be activated after etoposide treatment (Knippschild *et al.*, 1997; Alsheich-Bartok *et al.*, 2008) that also induces increased Ser-74 phosphorylation and dCK activity in leukemic cells. All these data made CKI δ a good candidate for catalyzing Ser-74 phosphorylation. Our investigations (see Annex section) have allowed showing that recombinant CKI δ was indeed able to phosphorylate recombinant dCK on Ser-74 *in vitro* and to increase its activity. Like S74E mutation, the authentic phosphorylation of Ser-74 by CKI δ was found to increase K_{cat} and K_m values of dCK for dCyd. However, the increase in K_{cat} was lower and the increase in K_m higher with the true Ser-74 phosphorylation than with S74E mutation, indicating that the latter does not perfectly mimic Ser-74 phosphorylation. Despite its capability to phosphorylate and activate dCK *in vitro*, CKI δ would not be responsible for *in vivo* phosphorylation of dCK and for regulation of its activity. Indeed, neither CKI δ inhibitors nor CKI δ siRNA were able to decrease Ser-74 phosphorylation and dCK activity in cultured cells (Smal *et al.*, 2010b).

Whereas we were analyzing the possibility that dCK activity could be regulated by the DNA damage sensor ATM, a role of this kinase in Ser-74 phosphorylation was reported by another group (Yang *et al.*, 2012). Authors of this study showed that ATM was able to phosphorylate dCK on Ser-74 *in vitro*, and conclude that ATM would also be involved in the *in vivo* regulation of dCK activity, at least after γ -irradiation, since increase in Ser-74 phosphorylation induced by this treatment could not be detected in ATM-deficient cells or in ATM shRNA transfected cells (Yang *et al.*, 2012). In addition, these authors suggested that ATM-mediated dCK phosphorylation could contribute to activation of the G2/M checkpoint. Indeed, phosphorylated Ser-74-dCK could interact with Cdk1 and inhibit it (Yang *et al.*, 2012). Although this study was very interesting, a weak point is that the authors never measured dCK activity by a conventional assay. They investigated dCK activity by analyzing the conversion of Ara-C into Ara-CTP in intact cells, a protocol that does not really measure dCK activity. On the other hand, Maxime Beyaert from our group, by using a conventional dCK assay, demonstrated that dCK can be activated by Ser-74 phosphorylation after γ -irradiation in ATM-deficient cells (the same cell line as used by Yang *et al.*, 2012)

(confidential communication). In conclusion, even if ATM plays a role in dCK activation, we are convinced that another kinase, most probably the DNA damage sensor ATR, is involved in Ser-74 phosphorylation and in dCK activity regulation.

4.2. Protein phosphatase 2A

While research for Ser-74-dCK kinase was in progress in the lab, my own objective was to identify the protein phosphatase that dephosphorylates Ser-74. The previous observation that dCK activity could be increased in cells by incubation with okadaic acid (Smal *et al.*, 2004) indicated that this PP could be one of the PPPs. However, okadaic acid can inhibit several PPPs: PP1 and PP2A, but also PP4 and PP6 (Swingle *et al.*, 2007). PP1 is known to be about 100-fold less sensitive to okadaic acid than PP2A, but PP2A, PP4 and PP6 have similar IC₅₀ values for OA. The first step was to examine whether PP1 or PP2A, the most abundant PPPs in cells, could be responsible for Ser-74 dephosphorylation. Analysis of the dose-response effect on Ser-74 phosphorylation and dCK activity showed maximal effect at 100 nM okadaic acid, a concentration shown to inhibit PP2A without affecting PP1 activity in intact cells (Favre *et al.*, 1997). In addition, we have shown that purified PP2A, but not PP1, could dephosphorylate dCK at Ser-74. Taken together, these data suggested that PP1 was not implicated in Ser-74 dephosphorylation. Then, we decided to investigate Ser-74-dCK phosphatase in cell lysates, but we were first unable to detect this activity until we added Mn²⁺ to the lysate. In fact, the Ser-74-dCK phosphatase activity was found to be latent and to require Mn²⁺ to be active. This observation was a little surprising because PP2A is generally considered to be not dependent on Mn²⁺ for activity. However, this Mn²⁺-dependent Ser-74-dCK phosphatase was responsive to PP2A inhibitors, not only to okadaic acid, but also to fostriecin, the most selective PP2A inhibitor (Swingle *et al.*, 2007). Also, activity of Ser-74-dCK phosphatase was diminished in cell lysate after PP2A-immunodepletion. Finally, use of siRNAs against the catalytic subunit of PP2A allowed concluding definitively that PP2A dephosphorylates dCK in cells and negatively regulates its activity. Therefore, although the dependence on Mn²⁺ is not usual for PP2A activity, all data obtained during our study converge toward a role of PP2A in the control of dCK activity. It is worth noting here that some studies have reported that PP2A can exist in a latent form, which can be activated in vitro by treatment with Mn²⁺ (Longin *et al.*, 2004), and to point out that metal ions are

required in the catalysis of PPP through the activation of a water molecule for the dephosphorylation reaction (Xing *et al.*, 2006; Xu *et al.*, 2006; Shi, 2009).

Since our study demonstrated that dCK activity is negatively regulated by PP2A, use of PP2A inhibitors could be considered as a potential strategy to enhance dCK activity and thereby the activation and clinical efficacy of nucleoside analogs that are substrates of dCK. This perspective is interesting, especially as PP2A inhibitors have been reported to be, under certain conditions, promising therapeutic drugs for the treatment of cancer (McConnell *et al.*, 2009; Kalev *et al.*, 2011; Lambrecht *et al.*, 2013). As PP2A is a negative regulator of the cell cycle, inhibition of PP2A leads to acceleration of cell cycle progression and to abrogation of cell cycle checkpoints. Therefore, PP2A inhibitors trigger premature entry into mitosis, resulting in death of cancer cells (Lu *et al.*, 2009; Kalev *et al.*, 2011). In addition, combination of PP2A inhibitors with chemotherapeutic agents, like doxorubicin, has been shown to produce increased sensitivity of cells to DNA damage, disordered DNA replication, and increased levels of cell death (Lu *et al.*, 2009; Kalev *et al.*, 2011). From these data, it is tempting to study the combination of PP2A inhibitors with NAs, and first of all with dFdC. The challenge will be to find the most selective and least toxic PP2A inhibitor. Okadaic acid causes poisoning and could not be used. On the other hand, fostriecin has a low cytotoxicity and a high specificity for PP2A (and PP4), but is unfortunately highly susceptible to oxidation and has a short plasma half-life (Kalev *et al.*, 2011). To overcome this problem, investigators try to generate analogs of fostriecin, such as phoslactomycins. These drugs are potent and selective inhibitors of PP2A, and phoslactomycin A has been shown to inhibit lung metastasis in mice through activation of natural killer cells (Kawada *et al.*, 2003). Also, efforts have been devoted to the development of norcantharidin analogs with high selectivity for PP2A. One of these norcantharidin derivative, LB1.2, has already demonstrated anticancer properties (Lu *et al.*, 2009). Though promising as anti-cancer drugs, PP2A inhibitors directed to PP2Ac have the disadvantage of lacking specificity and to target both healthy and diseased cells, resulting in unwanted toxic side effects. Design of inhibitors that do not target PP2Ac (and thus all PP2A complexes), but only very specific PP2A holoenzymes or PP2A-substrate interactions may be a breakthrough in the field of phosphatase drug discovery. In line with this, identification of the regulatory subunit that interacts with the core PP2A enzyme to form the specific heterotrimer responsible for the dephosphorylation of dCK at Ser-74 could constitute a stimulating perspective of our studies.

Our understanding of the role of PP2A in the regulation of dCK activity could also help to unravel a yet unexplained observation, i.e. the decrease of dCK activity by osmotic stress. About ten years ago, decrease of dCK activity had been reported after sorbitol-induced osmotic stress, but was not explained (Csapo *et al.*, 2003). Our group demonstrated, in HEK293T cells overexpressing recombinant dCK, that this decreased activity was related to a decrease of Ser-74 phosphorylation (Smal *et al.*, 2006). This could be explained by a decrease of the activity of the kinase that phosphorylates Ser-74 or by an increase of the activity of the phosphatase that dephosphorylates Ser-74. Preliminary results obtained in CCRF-CEM cells (an acute lymphoblastic leukemia cell line), with sucrose as osmotic agent because it was less cytotoxic than sorbitol, showed that dephosphorylation of Ser-74 and decreased activity of dCK could be prevented by okadaic acid, suggesting the role of a PPP in these events. In contrast, none of the kinase inhibitors investigated, including inhibitors of p38 kinase and JNK (c-jun N-terminal kinase) both implicated in the response to osmotic stresses, did influence the decreased activity of dCK by osmotic stress. Now that we know the implication of PP2A in the regulation of dCK activity, it will be interesting to go further and to examine whether PP2A has been activated following osmotic stress and is responsible for Ser-74 dephosphorylation and the decreased activity of dCK observed in this situation. In line with this, a role of PP2A in osmotic stress-dependent dephosphorylation and inactivation of protein kinase B α has been reported, although the authors of this study were unable to detect direct activation of PP2A upon sorbitol treatment (Meier *et al.*, 1998).

5. General conclusions

In conclusion, we have provided new insights into the mechanisms that regulate dCK activity *via* Ser-74 phosphorylation. It is now clear that Ser-74 phosphorylation decreases the affinity of dCK for all its substrates, whereas it increases, decreases or has no effect on dCK catalytic efficiency. Therefore, whether Ser-74 phosphorylation could increase nucleoside analog activation has to be examined for each analog individually, not only *in vitro*, but especially in intact cells. Using this approach, we could show that combination of a compound that increases dCK phosphorylation at Ser-74 with the pyrimidine analog gemcitabine (dFdC) allows increasing its activation and its cytotoxicity in cancer cells. To increase Ser-74 phosphorylation, we have chosen the DNA polymerase inhibitor aphidicolin, but further investigations are needed to know whether aphidicolin is the best dCK activator

for this strategy. In addition to a better knowledge about the consequences of Ser-74 phosphorylation on nucleoside analog activation, we have been able to identify PP2A as the protein phosphatase that dephosphorylates dCK at Ser-74 in basal physiological conditions. These results allow completing the signaling pathway that regulates dCK activity and suggest new potential strategies for enhancing dCK activity and hence nucleoside analog activation and effectiveness in cancer. The current state of knowledge regarding regulation of dCK activity *via* Ser-74 phosphorylation and the ensuing potential clinical implication are illustrated in Figure 24, which also indicates my own contribution as well as the hypotheses under investigation.

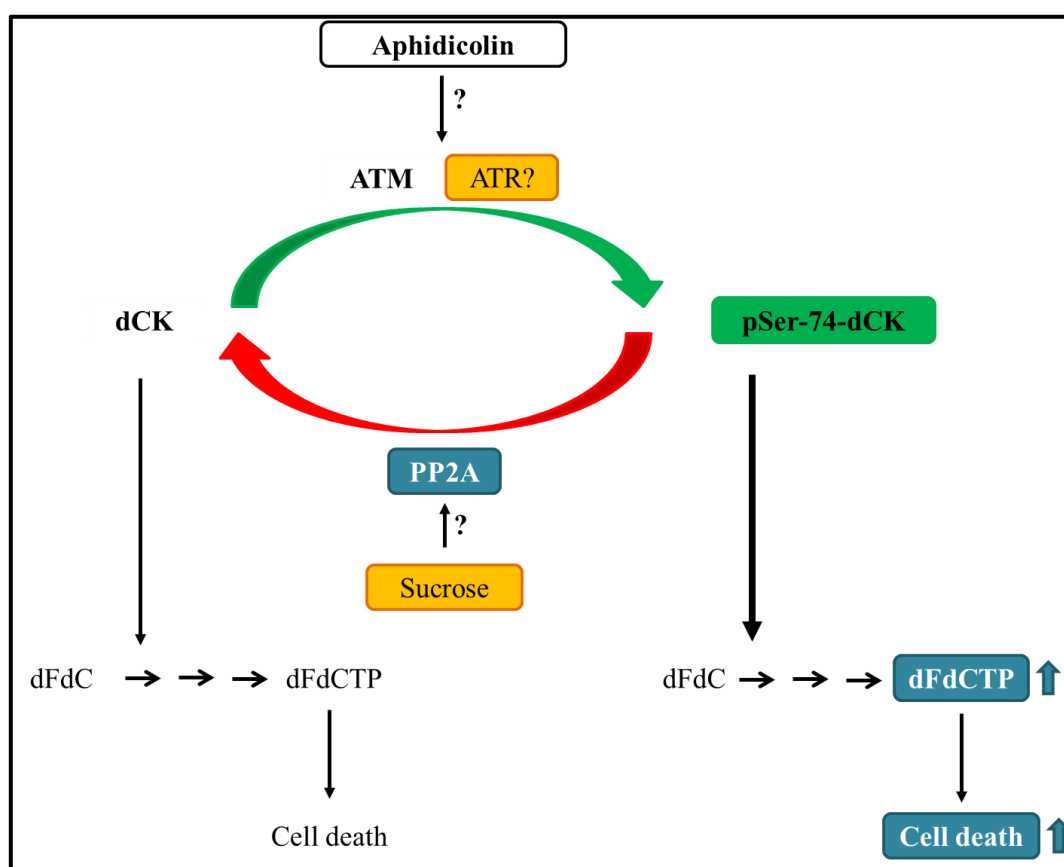


Figure 24: State of knowledge of Ser-74-dCK regulation and potential clinical implication.

dCK activity is regulated *via* Ser-74 phosphorylation. The kinase that phosphorylates Ser-74 and activates dCK is thought to be ATM, but experimental data also suggest a role for ATR. On the other hand, the PP that dephosphorylates Ser-74 is PP2A. Potential activation of PP2A by osmotic stress could perhaps explain the decreased activity of dCK observed in this situation. Besides fundamental interest, dCK also presents a clinical interest coming from its key role in the activation of chemotherapeutic nucleoside analogs, like gemcitabine (dFdC). Following aphidicolin treatment, dCK is more phosphorylated on Ser-74, probably by ATM and/or ATR, inducing increase of dCK activity. This activation of dCK can lead to higher activation and cytotoxicity of dFdC, which could be of pharmacological interest. My contribution is indicated in blue and preliminary results obtained by our group are in orange.

CHAPTER V:

Annex



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Casein kinase 1 δ activates human recombinant deoxycytidine kinase by Ser-74 phosphorylation, but is not involved in the *in vivo* regulation of its activity

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ABSTRACT

Deoxycytidine kinase (dCK) is a key enzyme in the salvage of deoxynucleosides and in the activation of several anticancer and antiviral nucleoside analogues. We recently showed that dCK was activated *in vivo* by phosphorylation of Ser-74. However, the protein kinase responsible was not identified. Ser-74 is located downstream a Glu-rich region, presenting similarity with the consensus phosphorylation motif of casein kinase 1 (CKI), and particularly of CKI δ . We showed that recombinant CKI δ phosphorylated several residues of bacterially overexpressed dCK: Ser-74, but also Ser-11, Ser-15, and Thr-72. Phosphorylation of dCK by CKI δ correlated with increased activity reaching at least 4-fold. Site-directed mutagenesis demonstrated that only Ser-74 phosphorylation was involved in dCK activation by CKI δ , strengthening the key role of this residue in the control of dCK activity. However, neither CKI δ inhibitors nor CKI δ siRNA-mediated knock-down modified Ser-74 phosphorylation or dCK activity in cultured cells. Moreover, these approaches did not prevent dCK activation induced by treatments enhancing Ser-74 phosphorylation. Taken together, the data preclude a role of CKI δ in the regulation of dCK activity *in vivo*. Nevertheless, phosphorylation of dCK by CKI δ could be a useful tool for elucidating the influence of Ser-74 phosphorylation on the structure–activity relationships in the enzyme.

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Introduction

Human deoxycytidine kinase (dCK⁴; EC 2.7.1.74) catalyzes the phosphorylation of 2'-deoxycytidine (dCyd), 2'-deoxyadenosine and 2'-deoxyguanosine to their corresponding monophosphate forms, using ATP or UTP as phosphoryl donors. This reaction is the first and rate-limiting step of the deoxyribonucleoside salvage

pathway, which provides deoxynucleoside triphosphates for DNA replication and repair as an alternative to *de novo* nucleotide synthesis [1]. In addition to recycling natural deoxynucleosides, dCK plays a key role in the activation of a number of anticancer and antiviral nucleoside analogues, including fludarabine, cladribine and cytarabine used in hematological malignancies, gemcitabine active against solid malignant tumors [2,3], and the anti-HIV compounds zalcitabine and lamivudine [4]. All these nucleoside analogues are prodrugs that need to be converted into triphosphate derivatives to exert their pharmacological action. As demonstrated by a positive correlation between dCK activity and nucleoside analogue sensitivity, this enzyme plays a critical role in their therapeutic efficacy [5].

The possibility of post-translational regulation of dCK activity was alluded to at the end of the 1990s, when it was found that several genotoxic drugs including the DNA polymerase α inhibitor aphidicolin, the topoisomerase II inhibitor etoposide, and certain nucleoside analogues, such as cladribine and cytarabine, increased dCK activity without changing its protein level [6–8]. Similar results were observed in response to UVC- and γ -irradiation, two treatments that induce DNA repair [9,10]. The presumption of a reversible phosphorylation as a mechanism for the regulation of

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⁴ Abbreviations used: dCK, deoxycytidine kinase; CKI, casein kinase I; HEK, human embryonic kidney; ESI-MS/MS, electrospray ionization tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; WT, wild-type; OA, okadaic acid; APC, aphidicolin; dCyd, deoxycytidine; HIV, human immunodeficiency virus; UV, ultraviolet; CLL, chronic lymphocytic leukemia; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LB, Luria–Bertani; DTT, dithiothreitol; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; siRNA, silencing RNA; PKC, protein kinase C; ATM, ataxia telangiectasia mutated.

dCK activity was strengthened by our observation that several protein kinase and phosphatase inhibitors could modulate dCK activity in several types of leukemic cells [11]. Also, we demonstrated that dCK overexpressed in HEK293T cells exists as a phosphoprotein that contains at least four phosphorylation sites: Thr-3, Ser-11, Ser-15 and Ser-74, the latter being the major phosphorylated residue. Site-directed mutagenesis experiments indicated that Ser-74 phosphorylation activated dCK, whereas phosphorylation of the other sites did not [12,13]. Moreover, use of a specific anti-phospho-Ser-74 antibody showed a direct correlation between the phosphorylation state of Ser-74 and dCK activity in human leukemic lymphocytes, including B-cell chronic lymphocytic leukemia (B-CLL) cells [14]. Although activation of dCK by Ser-74 phosphorylation is now well established, the signaling pathway involved in this process is not yet known. The first step toward deciphering this pathway would be to identify the protein kinase responsible for Ser-74 phosphorylation. This protein kinase should be constitutively active, since dCK is phosphorylated under basal conditions in various cultured cells [12,14]. By examining the sequence surrounding Ser-74 in dCK, we noticed that it was localized downstream a Glu-rich region presenting similarities with the consensus sequence of constitutively active casein kinase I (CKI) family, and particularly of CKI δ [15].

CKI isoforms are widely expressed and highly conserved Ser/Thr protein kinases. In mammals, at least seven distinct isoforms (α , β , γ 1, γ 2, γ 3, δ and ϵ) have been identified, having a similar structural architecture with a short N-terminal region, a highly conserved catalytic domain of ~300 amino acids, and a highly variable C-terminal domain [16]. These kinases are implicated in a variety of cellular functions, including vesicle trafficking, DNA repair, cell division and progression, circadian rhythms and apoptosis [17]. Interestingly, CKI δ , like dCK, has been shown to display high activity in lymphocytes [18,19] and to play an important role in the DNA damage response [9,20,21]. Moreover, CKI δ protein levels were found to be p53-dependently elevated upon etoposide treatment [20] that also induces increase of Ser-74 phosphorylation and dCK activity in leukemic cells [7,14]. All these data made CKI δ a candidate enzyme for catalyzing Ser-74 dCK phosphorylation. Here, we investigated whether CKI δ could activate dCK *in vitro* and modulate dCK activity *in vivo*.

Experimental

Materials

Sequencing-grade trypsin was purchased from Promega. HPLC-grade solvents and acids were from Acros Organics. Primers for cloning and site-directed mutagenesis were from Eurogentec. Reverse transcriptase, T4 polynucleotide kinase, Tag polymerase, *Pfu* DNA polymerase, restriction enzymes and PageBlue™ Protein Staining Solution were from Fermentas. [5-³H]-dCyd was from Moravsek and [γ -³²P]-ATP from Perkin Elmer. Purified recombinant CKI δ expressed in bacteria was obtained from New England Biolabs (truncated at residue 317 to prevent autophosphorylation of its C-terminal domain and inhibition of casein kinase activity *in vitro* [22]). CKI-7 (*N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide) and α -casein were purchased from Sigma. IC-261 and okadaic acid (OA) were from Calbiochem. Anti-phospho-Ser-74 antibody was generated as described previously [12]. Anti-poly(His) monoclonal antibody was from GE Healthcare. Anti-CKI δ antibody and anti-CKI δ antibody coupled to agarose beads were from Santa Cruz Biotechnology. Anti-rabbit and anti-mouse IgG conjugated to peroxidase were from Sigma. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Pierce. Fluorescent secondary antibodies IRDye®800 or IRDye®680 and Odyssey blocking buffer

were from LI-COR Biosciences. Other chemicals, materials and reagents were from Sigma, Calbiochem or Bio-Rad Laboratories.

Cell culture, transfection and treatment

B-CLL cells, isolated as previously reported [14], CCRF-CEM cells and HEK293T cells were cultured as described [12]. To generate HEK293T cells stably expressing human dCK, 4 μ g of the eukaryotic pEF6/His vector containing the cDNA of human dCK was linearized with AatII and transfected in HEK293T cells using the JetPEI procedure (PolyPlus Transfection) [12]. Forty-eight hours after transfection, cells were cultured in selective medium containing 10 μ g/ml blasticidin.

Plasmid construction and site-directed mutagenesis

The cDNA of human dCK was amplified by PCR with *Pfu* DNA polymerase using a 5'-primer containing the putative ATG codon in a NdeI site and a 3'-primer containing the putative stop codon flanked by a BamHI site. A ~800 bp product was obtained, which was subcloned in pBlueScript II SK (+) vector, checked by DNA sequencing and used for site-directed mutagenesis. Wild-type construct was used as a template (50–100 ng) to create single mutations by PCR (S74A or S74E) using the QuickChange protocol (Stratagene). The complete dCK coding region of all plasmids was sequenced on a CEQ2000 sequencer (Beckman Coulter) to verify the newly introduced mutations and the absence of random mutations. NdeI–BamHI fragments containing wild-type or mutated dCK were partially digested with NdeI and BamHI and ligated in pET-15b vector (Novagen) providing a His₆ tag at the N-terminus.

dCK expression and purification

The pET-15b vectors with the dCK constructs were used to transform *Escherichia coli* BL21 (DE3). The resulting bacteria were grown in Luria–Bertani (LB) medium containing 0.1 g/l ampicillin. The culture was grown at 37 °C until the A₆₀₀ reached 0.5–0.6. It was then cooled on ice for 20 min and the inducer IPTG was added to a final concentration of 0.4 mM. After overnight incubation at 37 °C, bacteria were collected by centrifugation, resuspended in buffer A (50 mM HEPES, pH 7.5, 0.1% (w/v) β -mercaptoethanol, 20% (w/v) glycerol, 1 mg/ml lysozyme, 1 mM *p*-toluenesulfonyl fluoride, 5 mM benzamidine, 5 μ g/ml leupeptin, 5 μ g/ml antipain) and submitted to three cycles of freezing and thawing. The bacterial extract was incubated on ice for 1 h with 0.1 mg/ml of DNaseI in the presence of 10 mM MgSO₄ and centrifugated for 30 min at 12,000g. The resulting supernatant (10 ml) was incubated with 1 ml of Talon (Co⁺⁺) affinity resin (Clontech) for 1 h at 4 °C. After three washings with buffer B (50 mM HEPES, pH 7.5, 0.1% (w/v) β -mercaptoethanol, 1 mM *p*-toluenesulfonyl fluoride, 5 mM benzamidine, 5 μ g/ml leupeptin, 5 μ g/ml antipain and 300 mM NaCl) followed by two washings with buffer B supplemented with 10 mM imidazole, recombinant dCK was eluted with buffer B containing 150 mM imidazole. Fractions containing purified protein were pooled, desalted using NAP-5 columns (GE Healthcare) equilibrated with 50 mM Tris–HCl, pH 7.6, 2 mM DTT and 5 mM benzamidine, and concentrated by vivaspin ultrafiltration spin column (Sartorius). The purified proteins were stored at –80 °C in the presence of 20% (w/v) glycerol.

Phosphorylation of recombinant dCK by CKI δ

Purified dCK (6 μ g of protein) was incubated at 30 °C with 8000 units of CKI δ in 120 μ l of phosphorylation buffer containing 50 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol (DTT) and 200 μ M [γ -³²P]ATP (~1000 cpm/pmol). At various times,

aliquots (20 μ l) were removed and stopped by boiling in Laemmli SDS–PAGE buffer. Samples were subjected to SDS–PAGE electrophoresis and autoradiography to visualize dCK labeling. To determine phosphorylation stoichiometry, the same gels were stained with Coomassie blue, and radioactivity of dCK bands was measured by Cerenkov counting. One unit of CKI δ corresponds to the amount of enzyme that catalyzes the incorporation of 1 pmol/min of 32 P at 30 °C.

To analyze whether CKI δ phosphorylated Ser-74 and increased dCK activity, incubation was performed as described above, except that 200 μ M unlabeled ATP and 12 μ g of purified dCK were used in a total volume of 120 μ l and bovine serum albumin (BSA) (1 mg/ml) was included in the assay. At various times, aliquots (10 μ l) were withdrawn for immediate measurement of dCK activity, as described below, or stopped in Laemmli SDS–PAGE buffer for the analysis of Ser-74 phosphorylation by immunoblotting.

Identification of phosphorylated residues by tandem mass spectrometry

For the identification of *in vitro* phosphorylated sites, purified dCK (10 μ g) was incubated for 3 h with 8000 units of CKI δ and [γ - 32 P]ATP as described above in a final volume of 30 μ l. After the addition of 10 mM DTT and 100 mM iodoacetamide, the reaction was stopped by adding 10% trichloroacetic acid (v/v). After 30 min on ice, precipitated proteins were collected by centrifugation, washed once with 200 μ l of ice-cold acetone and resuspended in 25 μ l of 50 mM NH_4HCO_3 for overnight digestion at 30 °C with 1 μ g of sequencing-grade trypsin. Peptides were separated by reverse-phase narrow-bore HPLC on a Vydac C18 column (2.1 mm $\times$ 25 cm) at a flow rate of 200 μ l/min [23]. Radioactivity of each fraction was measured by Cerenkov counting. Radioactive peaks were concentrated to $\sim$ 10 μ l, mixed with 0.1% (v/v) trifluoroacetic acid, desalted using a ZipTip C18 pipette tip (Millipore), eluted in 50% (v/v) acetonitrile, 0.3% (v/v) acetic acid, and analyzed by nanoelectrospray ionization tandem mass spectrometry (nanoESI-MS/MS), as described [12]. Fractions 19 + 20 were analyzed by MALDI TOF/TOF. One μ l of a solution containing 5 mg/ml alpha-cyano-4-hydroxycinnamic acid (alpha-cyano MALDI matrix) in 50% (v/v) acetonitrile and 0.1% (v/v) TFA was added to 1 μ l of peptide solution. One μ l of this solution was spotted onto an Applied Biosystems MALDI plate Opti-TOF™ 384 Well Insert (Foster City, USA). MS and MS/MS spectra were acquired using an Applied Biosystems 4800 MALDI-TOF/TOF™ Analyzer spectrometer using a 200 Hz solid state laser operating at a wavelength of 355 nm. MS spectra were obtained using a laser intensity of 3000 and 2000 laser shots per spot in a range of *m/z* between 800 and 4000. MS/MS spectra were obtained by selecting 15 most intense precursors ions per spot and using a laser intensity of 3500 and 2000 laser shots per precursor. The automatically selected precursors were fragmented using a collision energy of 1 kV with collision gas air at a pressure of about 1×10^6 Torr. Data were collected with the Applied Biosystems 4000 Series Explorer™ software.

dCK assay

HEK293T, B-CLL and of CCRF-CEM cell extracts were prepared as reported previously [12,14]. The protein content of samples was measured by the method of Bradford [24] with BSA as a standard. dCK was assayed on 4–40 μ g of cell extract or 0.15–1 μ g of purified dCK. Except for kinetic studies, dCK activity was assayed in the presence of 10 μ M dCyd and 5 mM ATP as described in [12]. The K_m for dCyd was determined with dCyd concentrations ranging from 0.02 to 2 μ M. The Michaelis–Menten constants were calculated using non-linear regression with the GraphPad Prism 4.0 software.

Immunoblot analysis

Purified dCK (1 μ g) or cell lysates (20–30 μ g of protein) were subjected to SDS–PAGE electrophoresis in gels containing 12% (w/v) polyacrylamide and transferred to Hybond C-extra membranes (GE Healthcare) or low fluorescence PVDF immobilon-FL membranes (Millipore) depending on chemiluminescent (for CKI δ , GAPDH and β -actin) or fluorescent (for phospho-Ser-74, poly(-His)-dCK and dCK) detection. After transfer, Hybond C-extra membranes were blocked in PBS containing 5% (w/v) fat-free milk powder and then probed overnight at 4 °C with either anti-CKI δ antibody (1/1000), anti-GAPDH antibody (1/1000) or anti- β -actin antibody (1/10,000) in PBS-T (Tween 0.1%, w/v) with 5% (w/v) fat-free milk powder. After extensive washing in PBS-T, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase (1/10,000). After further extensive washings in PBS-T, the blots were developed using (advanced) enhanced chemiluminescence (GE Healthcare). Quantification of CKI δ expression was carried out using the ImageJ 1.42q software (<http://rsb.info.nih.gov/ij>). PDVF membranes were blocked in Odyssey blocking buffer. After washing in PBS-T (Tween 0.1%) and incubation with anti-phospho-Ser-74 antibody (1/1000), anti-poly(His) antibody (1/4000) or anti-dCK antibody (1/1000), the membranes were incubated for 1 h at room temperature with the appropriate fluorescent secondary antibody IRDye®800 or IRDye®680 (LI-COR Biosciences) diluted (1/10,000) in Odyssey blocking buffer containing 0.1% Tween and 0.1% SDS. After washing, the membranes were scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences). Fluorescence intensities were used to quantify dCK expression or phosphorylation [13].

RNA interference

HEK293T cells stably expressing dCK were plated in 6 cm-dishes (0.5×10^6 cells/dish) and transfected on the following day with the siRNA transfection reagent INTERFERin™ (PolyPlus Transfection) using 5 nM control siRNA, 5 nM GAPDH siRNA or 5 nM CKI δ siRNA (synthesized by Dharmacon) and 10 μ l INTERFERin™, according to the manufacturer's instructions. After 48 h, the cells were washed with cold PBS and resuspended in lysis buffer containing 50 mM HEPES, pH 7.5, 50 mM NaF, 1 mM K_2HPO_4 , 0.1% (w/v) β -mercaptoethanol, 5 mM β -glycerophosphate, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1% (w/v) Triton, protease inhibitors (1 mM *p*-toluenesulfonyl fluoride, 5 mM benzamidine, 5 μ g/ml leupeptin and anti-pain), 1 mM sodium orthovanadate and 20% glycerol (w/v). Cells were lysed by one cycle of freeze–thawing. After centrifugation, supernatants were boiled in Laemmli SDS–PAGE buffer and subjected to electrophoresis, or used for CKI δ assay, as described below.

CKI δ assay

For each CKI δ assay, a HEK293T cell lysate (500 μ g of protein) was incubated overnight at 4 °C with 40 μ l of agarose beads conjugated to anti-CKI δ antibody (sc-6474Ac). The beads were washed twice with 1 ml of lysis buffer without glycerol and Triton and twice with 1 ml of kinase buffer (50 mM Tris–HCl, pH 7.6, 10 mM MgCl_2 , 2 mM DTT, 10 mM NaF, 1 mM β -glycerophosphate and 1 mM orthovanadate). Immunoprecipitates were incubated in 20 μ l of kinase buffer containing 10 μ g of α -casein and 50 μ M [γ - 32 P]ATP ($\sim$ 1000 cpm/pmol). After 30 min at 30 °C, the reaction was terminated by addition of SDS sample buffer. 32 P-labeled α -casein was resolved by 12% SDS–PAGE and detected with Coomassie blue. The bands corresponding to α -casein were cut from the gel and subjected to counting by Cerenkov radiation.

Data analysis

The results of experiments repeated at least three times are given as means $\pm$ SEM. Significance was assessed by using the paired Student's *t* test.

Results

CKI δ phosphorylates and activates dCK

Inspection of the sequence surrounding Ser-74 of dCK showed a Glu-rich region upstream of the phospho-acceptor site, which fits the consensus recognition motif of CKI δ (Table 1). To determine whether dCK was a substrate for CKI δ , we tested commercially available CKI δ on human recombinant dCK expressed in *E. coli*. As illustrated in Fig. 1A, dCK was phosphorylated in a time-dependent manner on incubation with CKI δ and [γ - 32 P]ATP. However, phosphorylation of dCK by CKI δ was less than with α -casein with 0.20 ± 0.03 mol of phosphate ($n = 8$) being incorporated per mol of dCK subunit after 1 h of incubation at 30 °C compared with 1.85 ± 0.41 mol of phosphate ($n = 3$) per mol of α -casein under the same conditions (not shown).

To verify whether CKI δ actually phosphorylated Ser-74, recombinant dCK was analyzed by Western blot with the specific anti-phospho-Ser-74 antibody. As illustrated in Fig. 1B, dCK was indeed phosphorylated with time on Ser-74 by CKI δ . In parallel, we observed that dCK activity progressively increased to attain a ~ 4 -fold activation after 4 h (Fig. 1C). Neither phosphorylation of dCK on Ser-74 nor dCK activation appeared to be complete at this time. Additional experiments confirmed that dCK activation persisted at the same rate at least up to 6 h (not shown). No change in dCK activity was observed in the absence of CKI δ (Fig. 1C). IC-261, a selective CKI δ inhibitor, was found to reduce 32 P incorporation as well as Ser-74 phosphorylation and dCK activation by more than 80% (not shown).

CKI δ phosphorylates dCK on several residues

As the phosphorylation of protein substrate by CKI is not strictly dependent on the consensus sequence, depending also on the tertiary structure of its substrate [25], and can occur on multiple residues [26], we wanted to verify whether Ser-74 of dCK was the only site phosphorylated by CKI δ . Therefore, recombinant dCK was incubated for 3 h with CKI δ in the presence of [γ - 32 P]ATP, precipitated with trichloroacetic acid and digested with trypsin. Peptides were separated by reverse-phase HPLC and fractions were counted by Cerenkov radiation. As the mixture also contained CKI δ peptides, we performed in parallel an analysis of a sample containing BSA instead of recombinant dCK, to confirm the absence of autophosphorylation sites in truncated CKI δ . No peak was found in this condition (not shown), whereas four peaks were detected with dCK (Fig. S1), indicating that CKI δ phosphorylated more than one residue in recombinant dCK. All the peaks were screened for phosphopeptides by neutral loss of H_3PO_4 (98 Da) by nano-ESI-MS/MS, as previously described [12].

In peak I, no phosphopeptide was detected. Peak II corresponded to a peptide of dCK containing one or two phosphates, which explains the detection of two different monoisotopic masses for this peptide (Table 2). Fragmentation of the tryptic peptides identified Ser-11 and Ser-15 as the phosphorylated residues. No phosphopeptide could be detected in peak III by nano-ESI-MS/MS. However, using MALDI TOF/TOF, a peptide corresponding to residues located upstream of the initiator Met and containing the poly(His) tag preceded by one or two phosphorylated Ser residues, was identified in peak III. Therefore, this phosphopeptide did not belong to native dCK. As peak IV was relatively broad, fractions 31–32 and 33–34 were analyzed separately. In fractions 31–32, a phosphopeptide was detected and Thr-72 was identified as the phosphorylated residue. Fractions 33–34 contained the same peptide, but phosphorylated on Ser-74. This phosphopeptide was also present with two different monoisotopic masses because of oxidation of one or two residues. Taken together, these results show that CKI δ phosphorylates recombinant dCK on at least four residues, Ser-11, Ser-15, Thr-72 and Ser-74. Peak IV that contains both phosphorylated Ser-74 and Thr-72 represented only $24.2 \pm 2.5\%$ of total 32 P incorporation, indicating that the phosphorylation of Ser-74 accounted for only a minor part of the total phosphorylation of dCK by CKI δ .

Phosphorylation of Ser-74 only plays a role in the activation of dCK by CKI δ

To determine whether phosphorylation of residues other than Ser-74 could contribute to the activation of dCK by CKI δ , we produced a mutated His-tagged dCK, in which Ser-74 was replaced by Ala (S74A mutant) to prevent phosphorylation at this locus. We also constructed the phosphomimetic S74E mutant, in which Glu mimics phosphorylation at Ser-74, to see whether phosphorylation at other sites might influence the activity of this activated form of dCK. The two mutated dCK proteins were produced in *E. coli* and purified by affinity chromatography, as for wild-type dCK. For S74A dCK, the specific activity was 38.4 ± 1.6 nmol/min/mg ($n = 3$), which was not drastically different from the activity of wild-type dCK (25.7 ± 2.8 nmol/min/mg, $n = 6$). By contrast, activity of the S74E mutant was 278.3 ± 17.6 nmol/min/mg ($n = 3$) and about 11-fold higher than the activity of wild-type dCK, as previously reported [27]. As illustrated in Fig. 2A, the activities of S74A and S74E dCK proteins were not significantly modified during the course of incubation with CKI δ (Fig. 2A). However, both mutants were clearly phosphorylated under these conditions (Fig. 2B). As expected from the absence of phosphorylatable Ser-74, labeling of S74A dCK was slightly lower ($\sim 10\%$ to 25%) than that of wild-type dCK, but more surprisingly, it was markedly lower for S74E dCK ($\sim 50\%$) than for S74A dCK (Table S1). Both labeled mutated dCKs were subjected to analysis by nano-ESI-MS/MS, as described for wild-type dCK and the same phosphorylation sites, apart from Ser-74, were identified (not shown). These results indicate that phosphorylation of Ser-74 only plays a role in the activation of dCK by CKI δ and that phosphorylation of other sites does not inhibit the activity of S74E dCK.

Table 1

Comparison of sequence surrounding Ser-74 in dCK with the optimal sequence recognition motif of CKI δ determined by the peptide library approach [15]. Identical residues are shown in boldface. CKI δ is a negative charge-directed protein kinase, which needs a cluster of acidic amino acids located upstream the phosphorylated residue. dCK contains two Glu (E) residues at the -4 and -5 positions.

	Amino acid position										
	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5
Residues surrounding Ser-74 in dCK	E	E	L	T	M	S ⁷⁴	Q	K	N	G	G
Optimal consensus sequence for CKI δ	E	F/E	D	T/A/G	G	S	I	I/I/F/Y/G	I/G/F	F/G	F/P/L

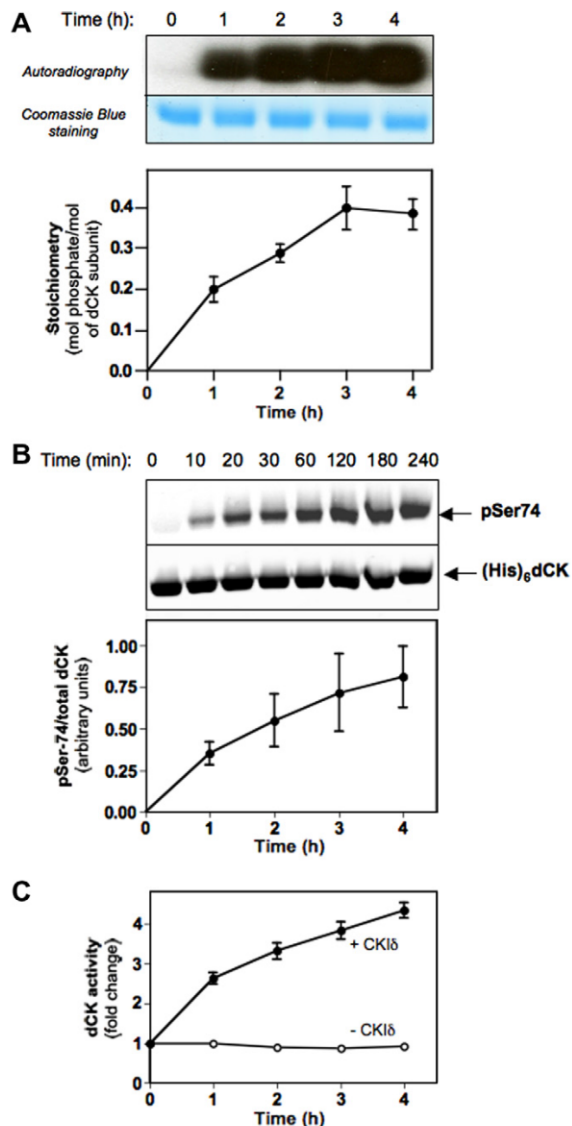


Fig. 1. Phosphorylation and activation of dCK by CKI δ *in vitro*. Purified human recombinant dCK was incubated in the presence of CKI δ and radioactive (A) or unlabeled (B and C) ATP, as described in the Methods. (A) At the indicated times, aliquots containing 1 μ g of dCK were removed for SDS-PAGE, Coomassie blue staining and autoradiography (upper panels). The stoichiometry of phosphorylation was calculated after counting the radioactivity of dCK bands visualized by staining (lower panel). The results are means $\pm$ SEM of eight separate experiments. (B) At the indicated times, recombinant dCK phosphorylation by CKI δ was analyzed using anti-phospho-Ser-74 and anti-poly(His) antibodies by immunoblotting. One representative immunoblot and the pSer-74-dCK/total dCK ratios (means $\pm$ SEM of three independent experiments) are shown. (C) At various times of incubation in the presence or absence of CKI δ , dCK activity was measured with 10 μ M dCyd as substrate. The results are the means $\pm$ SEM of 6 independent experiments. The specific activity of dCK before incubation was 25.7 ± 2.8 nmol/min/mg of protein ($n = 6$).

Phosphorylation of dCK by CKI δ changes its kinetic properties

The kinetic properties of dCK phosphorylated by CKI δ were studied and compared with those of unphosphorylated wild-type

and S74E dCK. As shown in Table 3, phosphorylation of wild-type dCK by CKI δ for 3 h increased its K_{cat} , but which did not attain the K_{cat} value of S74E dCK. Moreover, phosphorylation of wild-type dCK by CKI δ markedly decreased its affinity for dCyd, the K_m value increasing about 4.5-fold. An increase in K_m , although lower, was also seen with the S74E mutant compared with the wild-type, confirming that phosphorylation of Ser-74 decreases the affinity of dCK for dCyd. As a result of parallel increases in K_{cat} and K_m , the catalytic efficiency (K_{cat}/K_m) of dCK after a 3 h-phosphorylation by CKI δ was not modified in comparison with unphosphorylated dCK, whereas it was increased about 3-fold for S74E dCK.

dCK activity is likely not regulated by CKI δ in cultured cells

To determine whether CKI δ phosphorylated dCK on Ser-74 and regulated its activity in intact cells, we first analyzed the effects of cell-permeable inhibitors of CKI δ . We incubated primary B-CLL lymphocytes with IC-261, a specific inhibitor of CKI δ/ϵ [28] or CKI-7, a general CKI inhibitor [29], at concentrations known to inhibit CKI δ in intact cells and we examined their influence on Ser-74 phosphorylation and on dCK activation induced by aphidicolin [14]. We observed (Fig. 3A) that Ser-74 phosphorylation, nearly undetectable in basal conditions, was clearly enhanced by aphidicolin, both in the absence and presence of IC-261 or CKI-7. Accordingly, these CKI inhibitors did not modify basal dCK activity or dCK activation induced by aphidicolin. Similar results were obtained in the leukemia cell lines CCRF-CEM and EHEB (not shown). We also analyzed the effect of CKI inhibitors in HEK293T cells, in which dCK is highly phosphorylated on Ser-74 after transient transfection [12]. Our strategy was to investigate whether CKI inhibition could prevent reactivation of dCK following its inactivation. To inactivate dCK, HEK293T cells were treated for 30 min with 0.5 M sorbitol which, as previously reported [12], induced a decrease in Ser-74 phosphorylation and dCK activity (Fig. 3B). The cells were then washed to remove sorbitol and incubated again for 90 min in the presence or absence of 200 μ M CKI-7. At the end of this incubation period, Ser-74 phosphorylation had returned to its initial value in the absence, but also in the presence of CKI-7 (Fig. 3B), indicating that CKI is not responsible for the constitutive phosphorylation of dCK on Ser-74 in HEK293T cells.

A second approach to evaluate the contribution of CKI δ to Ser-74 phosphorylation in intact cells was to down-regulate the kinase by siRNA. As co-transfection of plasmids encoding dCK and CKI δ siRNA was unsuccessful in our hands, down-regulation of CKI δ was performed in HEK293T cells that stably expressed dCK. Unfortunately, these cells contained a much lower (at least 100-fold) level of dCK than HEK293T cells that transiently overexpressed dCK, so that Ser-74 phosphorylation was barely detectable in this model (Fig. 4A). Nevertheless, the level of Ser-74 phosphorylation could be increased and became detectable in these cells after incubation with okadaic acid (OA), a protein phosphatase 2A inhibitor (Fig. 4A). Therefore, we analyzed the consequences of CKI δ down-regulation in the presence of OA. Efficacy of siRNA treatment was assessed by analyzing the level of CKI δ protein by immunoblotting 48 h after transfection. Compared with control siRNA or GAPDH siRNA (that reduced GAPDH levels by about 50%), CKI δ siRNA decreased CKI δ levels by about 60% (Fig. 4B and C). A marked decrease in CKI δ electrophoretic mobility in the presence of OA was observed, which could be explained by an increase in autophosphorylation on its carboxy-terminal domain. Indeed, CKI δ , like the closely related CKI ϵ , autophosphorylates on C-terminal Ser and Thr residues, creating an autoinhibitory domain for its kinase activity [22,30]. In fact, OA induced a marked decrease in CKI δ activity in HEK293T cells (Fig. 4D). CKI δ siRNA reduced CKI δ activity by about 60% in parallel with the decrease in its protein

Table 2

Sequences of dCK phosphopeptides obtained by mass spectrometry after phosphorylation by CKI δ . HPLC peaks (Fig. S1) were analyzed by nano-ESI-MS/MS or MALDI TOF/TOF. Phosphopeptides were identified by loss of 98 Da upon collision-induced dissociation, and the phosphorylated residue was further identified by fragmentation. c, iodoacetamide (carboxamidomethyl)-modified cysteine; p, phosphorylated residue; M_{ox}, oxidized Met; ND, not determined.

Peptide	Fractions	Sequence	Measured average masses [M+H] ⁺	Theoretical monoisotopic masses [M+H] ⁺
I	10	ND		
II	13 + 14	⁸ ScPpSFSApSSEGTR ²⁰	1532.4 (avg)	1532.5
	15	⁸ ScPpSFSApSSEGTR ²⁰	1452.5	1452.5
III	19 + 20	⁻¹⁹ GpSSHSHHHHHSSGLVPR ⁻⁴	1848.86	1848.81
		⁻¹⁹ GpSpSHSHHHHHSSGLVPR ⁻⁴	1928.83	1928.78
IV	31 + 32	⁵⁸ WcNVQSTQDEFEELpTMpSQK ⁷	2520.1 (avg)	2519.9
	33 + 34	⁵⁸ WcNVQSTQDEFEELTMoxpSQK ⁷⁶	2455.8	2455.9
		⁵⁸ WoxcNVQSTQDEFEELTMoxpSQK ⁷⁶	2459.8	2460.0

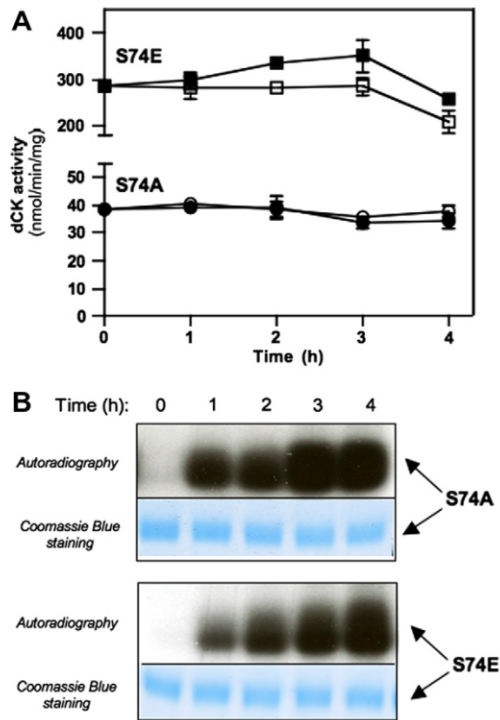


Fig. 2. Effect of mutation of Ser-74 on dCK activity after phosphorylation by CKI δ . (A) Recombinant S74A and S74E dCK proteins were incubated with unlabeled ATP in the presence (●, ■) or absence (○, □) of CKI δ . dCK activity was measured at the indicated times. The results are the means $\pm$ SEM of 3 independent experiments. (B) The recombinant S74A and S74E proteins were incubated in the presence of CKI δ and [γ -³²P]ATP. At the indicated times, aliquots corresponding to 1 μ g of dCK were taken for SDS-PAGE and autoradiography. Representative data are shown.

Table 3

Kinetic parameters of wild-type dCK, dCK phosphorylated by CKI δ and S74E dCK. Wild-type dCK was incubated for 3 h at 30 °C with or without CKI δ and ATP. Kinetic measurements were performed with 5 mM ATP and increasing concentrations of dCyd from 0.02 to 2 μ M. For comparison, the kinetic parameters of S74E dCK, also incubated for 3 h at 30 °C, were determined. The results are the means $\pm$ SEM of three or five separate experiments, as indicated.

	Wild-type (n = 5)	Wild-type + CKI δ (n = 3)	S74E (n = 3)
K_{cat} (s ⁻¹)	0.016 $\pm$ 0.001	0.078 $\pm$ 0.010	0.153 $\pm$ 0.024
K_m (nM)	59 $\pm$ 3	268 $\pm$ 31	157 $\pm$ 27
K_{cat}/K_m (M ⁻¹ s ⁻¹)	281 $\pm$ 12	289 $\pm$ 13	983 $\pm$ 40

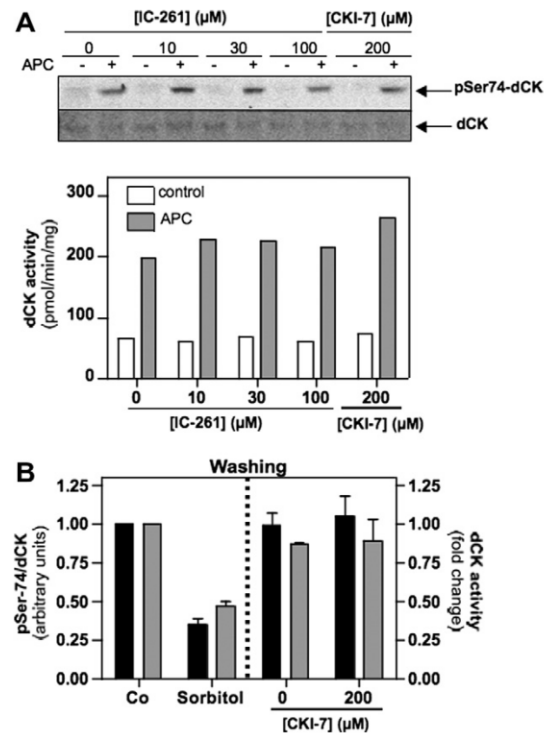


Fig. 3. Effect of CKI inhibitors on Ser-74 phosphorylation and dCK activity *in vivo*. (A) Freshly isolated B-CLL cells were pre-treated, or not, with increasing concentrations of IC-261 or with 200 μ M CKI-7 for 30 min, and then incubated for 2 h in the presence or absence of 10 μ M aphidicolin (APC). Cell lysates were analyzed for dCK phosphorylation and expression by immunoblotting with the anti-phospho-Ser-74 and anti-dCK antibodies (upper panel) and for dCK activity (lower panel). A representative experiment is shown. (B) HEK293T cells were transiently transfected with the PEF₆/His vector containing wild-type dCK. Forty hours after transfection, HEK293T cells were incubated for 30 min in the presence or absence of 0.5 M sorbitol to decrease Ser-74 phosphorylation and dCK activity. Then, cells were washed to remove sorbitol and incubated for 90 min with or without 200 μ M CKI-7. Before washing and after incubation with or without CKI-7, cells were lysed and analyzed for dCK Ser-74 phosphorylation by immunoblotting (black bars) and for dCK activity (gray bars). The results are the means $\pm$ SEM of 3 separate experiments.

level. A combination of both siRNA CKI δ and OA treatment led to more than 90% inhibition of CKI δ activity (Fig. 4D). Despite this acute decrease in activity, neither phosphorylation of Ser-74 nor dCK activity were modified (Fig. 4E), indicating that CKI δ was likely not responsible for dCK Ser-74 phosphorylation in intact cells.

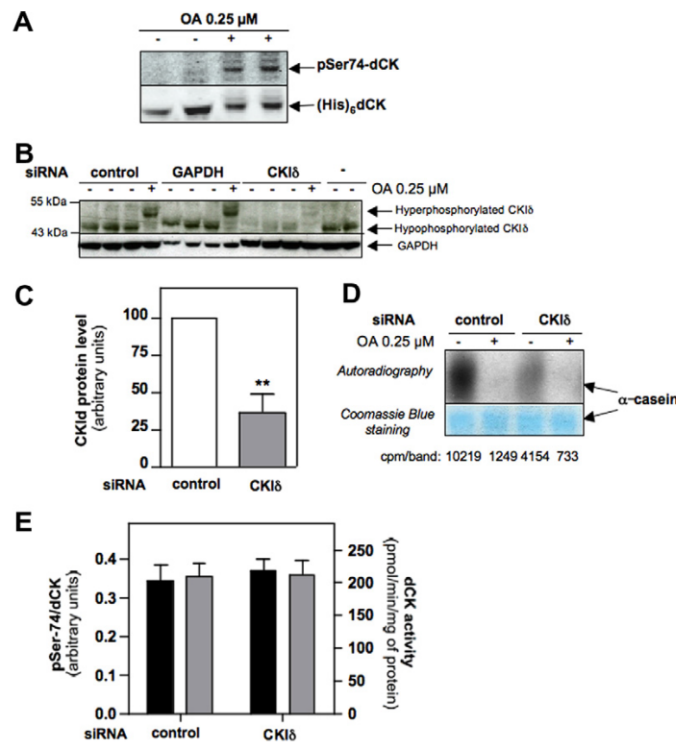


Fig. 4. Effect of CKI δ down-regulation by siRNA on Ser-74 phosphorylation and dCK activity in HEK293T cells stably expressing dCK. (A) Cells were incubated in the presence or absence of 0.25 μ M okadaic acid (OA) for 4 h. Cell lysates were probed for Ser-74 dCK phosphorylation and expression with anti-phospho-Ser-74 and anti-poly(His) antibodies by immunoblotting. A representative immunoblot is shown. (B) Cells were transfected with 5 nM control siRNA, 5 nM GAPDH siRNA or 5 nM CKI δ siRNA for 48 h in quadruplicate. In one, 0.25 μ M OA was added 4 h before the end of incubation. CKI δ and GAPDH expression were analyzed by immunoblotting after loading 30 μ g of protein, as illustrated in a representative immunoblot. (C) CKI δ and GAPDH expression was quantified by ImageJ software. The results shown are the means $\pm$ SEM of three independent experiments. Significant relative to control siRNA: $P < 0.01$. (D) HEK293T cells were transfected with control siRNA or CKI δ siRNA for 48 h and 0.25 μ M OA was added 4 h before the end of incubation. After cell lysis and immunoprecipitation, CKI δ activity was measured as explained in the Methods. A representative experiment is shown. (E) HEK293T cells were transfected with 5 nM control siRNA or 5 nM CKI δ siRNA for 48 h and 0.25 μ M OA was added 4 h before the end of incubation. Lysates were probed for Ser-74 dCK phosphorylation (black bars) and activity (gray bars). The results are the means $\pm$ SEM of three independent experiments.

Discussion

Regulation of dCK activity by Ser-74 phosphorylation was discovered by our group four years ago [12]. However, a protein kinase able to phosphorylate this residue has not been identified since. Similarities between the sequence surrounding Ser-74 in dCK and the recognition motif of CKI δ led us to investigate the potential role of this kinase in the regulation of dCK activity. Our study shows that dCK can be phosphorylated by CKI δ *in vitro* on Ser-74 and that phosphorylation correlated with dCK activation. However, CKI δ is unlikely to be the kinase responsible for Ser-74 phosphorylation and control of dCK activity in intact cells.

Initial studies characterizing the substrate specificity of CKI isoforms led to the identification of the consensus sequence pS/T-X-X-S/T, where pS/T refers to a phospho-Ser or a phospho-Thr, X refers to any amino acid and the underlined residues refer to the target site [31,32], indicating that phosphorylation of a Ser/Thr residue by CKI requires priming by another kinase. Nevertheless, further studies showed that CKI also phosphorylates a related unprimed site, which optimally contains a cluster of acidic amino acids N-terminal to the target S/T including an acidic residue at the n-3 position [33,34]. The sequence upstream of Ser-74 does not possess an acidic residue at n-3 position, but nevertheless contains two Glu at n-5 and n-4 positions in addition to a Thr at the n-2 position, which are all three found in the optimal consensus sequence of CKI δ (Table 1). We indeed demonstrated that CKI δ was able to phos-

phorylate dCK (Fig. 1A) and more precisely to phosphorylate Ser-74 (Fig. 1B), as confirmed by use of a specific anti-phospho-Ser-74 antibody. Moreover, as revealed by tryptic digestion and mass spectrometry, residues other than Ser-74 were phosphorylated by CKI δ , namely Ser-11, Ser-15 and Thr-72 (Fig. S1 and Table 2). The HPLC peak IV that contained Ser-74, but also Thr-72 (Fig. S1 and Table 2), represented $24.2 \pm 2.5\%$ ($n = 3$) of total 32 P incorporation, indicating that Ser-74 phosphorylation does not exceed $\frac{1}{4}$ of the overall stoichiometry. Although the sequences surrounding Ser-11 and Ser-15 do not fit the CKI consensus as well as that of Ser-74, they were phosphorylated by CKI δ presumably reflecting dependence on the tertiary structure of the substrate [25]. This emphasizes that the consensus sequences found in databases are not always strictly adhered to. It is noteworthy that phosphorylation of Ser-11 and Ser-15 in addition to Ser-74 of dCK has been detected *in vivo* [12]. dCK activity clearly increased in a time-dependent manner on incubation with CKI δ , reaching more than 4-fold activation after a 4 h-incubation (Fig. 1C). This corresponds to the maximal activation of dCK induced by DNA-damaging agents in leukemic cells [11,14]. As sites other than Ser-74 were phosphorylated by CKI δ , it was essential to investigate the role of the phosphorylation of these sites in the activation of dCK by CKI δ . Studies with S74A dCK indicated that dCK activation by CKI δ resulted only from Ser-74 phosphorylation, since no activation of S74A dCK was observed, despite phosphorylation at other sites (Fig. 2B). These results corroborate our previous finding that

phosphorylation of Ser-74 plays a crucial role in the control of dCK activity and confirm that phosphorylation of Ser-11 and Ser-15 does not influence dCK activity [13]. Also, phosphorylation of Thr-72, which had not been detected *in vivo*, seems not to influence dCK activity. When S74E dCK was phosphorylated by CKI δ , no major change in dCK activity was noted (Fig. 2A). However, a ~50% reduction in its labeling was observed (Table S1), suggesting that phosphorylation of dCK at Ser-74 by CKI δ could hamper phosphorylation at the other sites. Another possibility is that the three-dimensional conformation of S74E dCK is less favorable to phosphorylation by CKI δ than that of S74A.

Kinetic studies performed by McSorley et al. [27] had shown that replacement of Ser-74 by Glu in dCK increased both the K_m (~5-fold) and the K_{cat} (~11-fold) of dCK, measured with dCyd as substrate, resulting in a ~2-fold increase in catalytic efficiency (K_{cat}/K_m) [27]. We found that phosphorylation of dCK by CKI δ also increased the K_m for dCyd (4.5-fold), and the K_{cat} (4.9-fold). However, the catalytic efficiency of dCK phosphorylated at Ser-74 by CKI δ did not attain that of S74E dCK, so that the catalytic efficiency of dCK was not modified by phosphorylation. This discrepancy could be explained by the fact that dCK was not maximally phosphorylated by CKI δ after a 3 h-incubation or by the difference between a Glu and phosphorylated Ser residue.

Since dCK could be activated *in vitro* by CKI δ , we thought that dCK could also be activated by this kinase *in vivo*, which was tested by two different approaches. First, CKI-7, a general CKI inhibitor, and IC-261, considered as a more selective CKI δ inhibitor, were unable at effective concentrations [28,35–37] to decrease or to prevent Ser-74 dCK phosphorylation in various cultured cells (HEK293T cells transiently overexpressing dCK, B-CLL or CCRF-CEM cells) either under basal or dCK activating conditions (Fig. 3). Also, the two inhibitors did not influence basal dCK activity or dCK activation. Second, specific down-regulation of CKI δ by RNA interference did not decrease Ser-74 phosphorylation or dCK activity in HEK293T cells that stably expressed dCK (Fig. 4). However, CKI δ activity was reduced by more than 90% in our experimental model because of both a reduction in CKI δ protein level and treatment with OA. The latter was added to allow detection of Ser-74 dCK phosphorylation by immunoblotting, but also induced hyperphosphorylation of CKI δ and inhibition of its activity, as previously reported [22,30]. Taken together, the results indicate that CKI δ is not implicated in the control of dCK activity or its activation by DNA-damaging agents such as aphidicolin, most probably because Ser-74 of dCK is a poor substrate of this protein kinase. A role of another member of the CKI family is also unlikely since the general CKI inhibitor, CKI-7, did not affect Ser-74 phosphorylation or dCK activity. The protein kinase responsible for Ser-74 phosphorylation and for the control of dCK activity *in vivo* thus remains to be identified. Several years before identification of Ser-74 as a crucial phosphorylation site for the control of dCK activity, it was reported that dCK purified from leukemic blasts could be phosphorylated and activated by protein kinase C (PKC) α [38]. However, this was not the case when bacterially expressed human recombinant dCK was used as substrate of PKC [39]. Furthermore, specific activators or inhibitors of PKC did not modify dCK activity in intact leukemic cells [11], precluding a role of PKC in the control of dCK activity *in vivo*. As suggested by a proteomic study, Ser-74 could be a target of ATM (ataxia telangiectasia mutated) kinase [40], but this remains to be demonstrated. We are currently trying to identify the Ser-74-dCK kinase by fractionating cell extracts by chromatography techniques.

In conclusion, CKI δ can phosphorylate dCK *in vitro* at several sites. Among these, only phosphorylation at Ser-74 controls dCK activity. To date, CKI δ is the only known protein kinase that can phosphorylate dCK on Ser-74. Although, it is unlikely to be responsible for the control of dCK activity *in vivo*, it could be used as a tool

to produce a phosphorylated and active form of dCK for the study of structure–activity relationships via phosphorylation on Ser-74.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2010.07.009.

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CHAPTER VI:

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