Biochimica et Biophysica Acta 1850 (2015) 1354–1361

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

IMP–GMP specific cytosolic 5′-nucleotidase regulates nucleotide pool and prodrug metabolism

Federico Cividini [a,1], Daniela Nicole Filoni [a,b,1], Rossana Pesi [a], Simone Allegrini [b,*], Marcella Camici [a], Maria Grazia Tozzi [a]

[a] Dipartimento di Biologia, Unità di Biochimica, Università di Pisa, Via San Zeno 51, 56127, Pisa, Italy
[b] Dipartimento di Chimica e Farmacia, Università di Sassari, Via Muroni 23A, 07100, Sassari, Italy

ARTICLE INFO

Article history:
Received 3 November 2014
Received in revised form 26 March 2015
Accepted 31 March 2015
Available online 7 April 2015

Keywords:
Chemotherapy
Resistance
cN-II
Fludarabine
Gemcitabine
Cytarabine

ABSTRACT

Background: Type II cytosolic 5′-nucleotidase (cN-II) catalyzes the hydrolysis of purine and, to some extent, of pyrimidine monophosphates. Recently, a number of papers demonstrated the involvement of cN-II in the mechanisms of resistance to antitumor drugs such as cytarabine, gemcitabine and fludarabine. Furthermore, cN-II is involved in drug resistance in patients affected by hematological malignancies influencing the clinical outcome. Although the implication of cN-II expression and/or activity appears to be correlated with drug resistance and poor prognosis, the molecular mechanism by which cN-II mediates drug resistance is still unknown.
Methods: HEK 293 cells carrying an expression vector coding for cN-II linked to green fluorescent protein (GFP) and a control vector without cN-II were utilized. A highly sensitive capillary electrophoresis method was applied for nucleotide pool determination and cytotoxicity exerted by drugs was determined with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.
Results: Over-expression of cN-II causes a drop of nucleoside triphosphate concentration and a general disturbance of nucleotide pool. Over-expressing cells were resistant to fludarabine, gemcitabine and cytarabine independently of cN-II ability to hydrolyze their monophosphates.
Conclusions: An increase of cN-II expression is sufficient to cause both a general disturbance of nucleotide pool and an increase of half maximal inhibitory concentration (IC_{50}) of the drugs. Since the monophosphates of cytarabine and gemcitabine are not substrates of cN-II, the protection observed cannot be directly ascribed to drug inactivation.
General significance: Our results indicate that cN-II exerts a relevant role in nucleotide and drug metabolism through not only enzyme activity but also a mechanism involving a protein–protein interaction, thus playing a general regulatory role in cell survival.
Sentence: Resistance to fludarabine, gemcitabine and cytarabine can be determined by an increase of cN-II both through dephosphorylation of active drugs and perturbation of nucleotide pool.

1. Introduction

The intracellular 5′-nucleotidases catalyze the dephosphorylation of ribo- and deoxyribo-nucleoside monophosphates to the corresponding nucleosides providing the catabolic arm of substrate cycles regulating the intracellular nucleotide pools and nucleoside or base excretion [1]. In the case of inosine and guanosine a further catabolic step is catalyzed by purine nucleoside phosphorylase generating the corresponding bases guanine and hypoxanthine. The anabolic arm is constituted by nucleoside kinases and, for guanine and hypoxanthine, by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) [2]. In the case of hydroxypurine (deoxy)ribonucleoside monophosphates, the rate of the cycle is regulated by cN-II (Fig. 1) [3]. This is a ubiquitous enzyme whose structure is remarkably conserved among vertebrates. Its expression is higher in cells and organs with a high turnover of nucleic acids, particularly in blasts and transformed cells [2,4,5].

cN-II is a bifunctional enzyme since it catalyzes also the transfer of phosphate from a nucleoside monophosphate to a number of natural and artificial substrates such as inosine, dideoxyinosine, and acyclovir [6–8]. The substrate cycle in which cN-II is involved has been demonstrated in systems reconstituted in vitro and in brain extracts [3] but

Abbreviations: cN-II, type II cytosolic 5′-nucleotidase; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; dCK, deoxycytidine kinase; CDA, deoxycytidine deaminase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PRPP, 5-phosphoribosyl-1-pyrophosphate; TCA, trichloroacetic acid; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; BPG, 2,3-bisphosphoglycerate; AMP-PCP, β-γ-methyleneadenosine-5′-triphosphate; dNTP, deoxynucleoside triphosphates; Ipaf, NLR family CARD domain-containing protein 4

* Corresponding author. Tel.: +39 079228716.
E-mail address: enomis@uniss.it (S. Allegrini).
[1] These authors contributed equally to the work presented in this article.

http://dx.doi.org/10.1016/j.bbagen.2015.03.017
0304-4165/

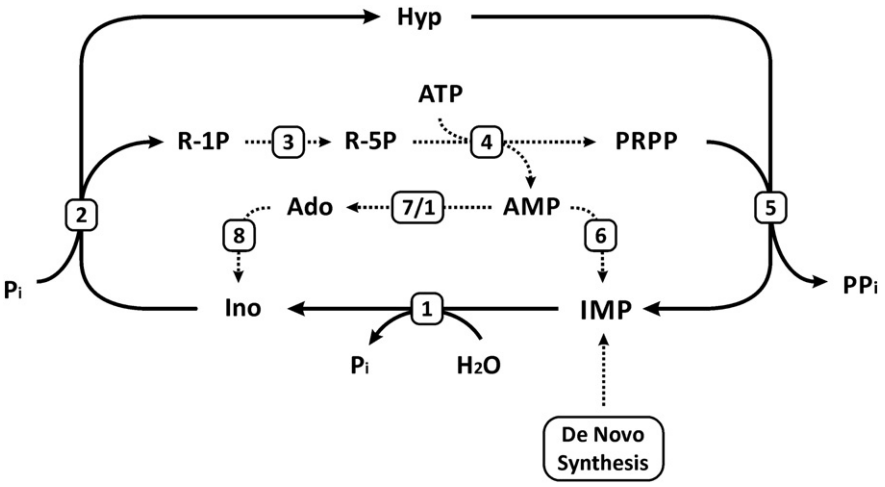

Fig. 1. Metabolic scheme. Enzymes involved in IMP substrate cycle (continuous arrows) and enzymes catalyzing connected reactions (dotted arrows): 1) cytosolic 5′-nucleotidase type II (cN-II); 2) purine nucleoside phosphorylase; 3) phosphoribomutase; 4) PRPP synthase; 5) hypoxanthine-guanine phosphoribosyl transferase; 6) AMP deaminase; 7) soluble 5′-nucleotidase type I (cN-I); 8) adenosine deaminase. 1 and 2 are the catabolic arm and 5 is the anabolic arm of the IMP substrate cycle. R-1-P: ribose-1-phosphate; R-5-P: ribose-5-phosphate.

its effective role in the metabolism of natural nucleotides and their analogs has never been investigated in vivo or in cell cultures. It has been demonstrated that nucleoside analogs are phosphorylated intracellularly by the same kinases involved in substrate cycles and the resulting monophosphates may be dephosphorylated by intracellular 5′-nucleotidase activities [1,9]. Therefore, the possibility for a nucleoside analog to accumulate inside cells as cytotoxic nucleoside monophosphate depends mainly on the efficiency of nucleoside transport, and on the ratio between kinase and nucleotidase activities. Deoxycytidine kinase (dCK) appears to be responsible for the phosphorylation of cytarabine and gemcitabine and also of fludarabine [10–12], while the ability to dephosphorylate the monophosphate of the three analogs is shared among different cytosolic nucleotidases [13]. For all the above-mentioned analogs, the resistance was demonstrated to be accompanied by a decrease of dCK expression, and, at least for cytarabine and gemcitabine by an increase of cN-II expression [14,15]. Furthermore, multiple reports have suggested involvement of cN-II in drug resistance in hematological neoplasias and in solid tumors [16–19]. Curiously, high cN-II expression strongly correlates with poor outcome of therapies utilizing analogs whose monophosphates are poor or to no extent substrates in in vitro tests. To explain these results it was postulated that cN-II could be a marker of tumor aggressiveness. Therefore, the enzyme might be considered a biological marker of poor prognosis and not a predictive marker of resistance to prodrugs [20]. More recently, the expression of genetic variants of cN-II was found to drive chemical resistance both in acute lymphoblastic leukemia and in acute myeloid leukemia. Such variants resulted in a higher cN-II expression in terms of mRNA and sometimes in terms of higher enzyme activity [21–24]. Although the implication of cN-II expression and/or activity appears to be certainly correlated with drug resistance and poor prognosis in a number of hematological malignancies, no proofs have been produced so far demonstrating the molecular mechanisms by which cN-II mediates drug resistance. In this paper, we demonstrate that cN-II over-expression heavily impacts on cellular nucleotide metabolism. Furthermore, in the absence of any alteration of the enzymes involved in IMP substrate cycle or prodrug metabolism, cN-II over-expression is sufficient to promote drug resistance.

2. Materials and methods

2.1. Reagents

Cytarabine, fludarabine, gemcitabine and ponasterone were purchased from Santa Cruz Biotechnology (Dallas, USA). [8-^{14}C] inosine was from Moravek Biochemicals and Radiochemicals (Brea, USA) iScript cDNA Synthesis Kit and Protein Assay Dye Reagent were from Bio-Rad Laboratories (Hercules, USA). PerfectPure total cell RNA isolation system was from 5 PRIME (Hilden, Deutschland). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum, L-glutamine, Penicillin–Streptomycin Mixture, Trypsin–Versene (EDTA) Mixture were purchased from Lonza Group Ltd (Basel, Switzerland). QuantiFluor dsDNA System and QuantiFluor RNA System were from Promega Corporation (Madison, USA). Deoxynucleoside triphosphates (dNTP) mix, miTaq DNA Polymerase and all primers were provided by Metabion International AG (Martinsried, Germany). Mouse primary monoclonal Ab anti-NT5C2, clone 3C1, secondary Ab goat anti-mouse IgG, horseradish peroxidase-conjugated, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), xanthine oxidase, ATP, 2,3-bisphosphoglycerate (BPG), 5-phosphoribosyl-1-pyrophosphate (PRPP), adenosine, guanine, thymidine and Protease Inhibitor Cocktail were purchased from Sigma-Aldrich (St. Louis, USA). ECL kit Immobilon Western-Chemoluminescence HRP Substrate and PVDF membrane Immobilon-P, 0.45 μm were from Millipore Corporation (Billerica, USA). 4-deoxyuridine (zebularine) and deoxycytidine were purchased from Carbosynth (Berkshire, UK). All other reagents were of reagent grade. All solutions were prepared in MilliQ water.

2.2. Cell lines and growth conditions for over-expression and cell viability

cN-II hyper-expressing cell lines (HKiG2) derived from human embryonic kidney HEK 293 cells were kindly provided by Prof. Vera Bianchi from University of Padova [25,26]. HKiG2 cells were carrying the vector pNTm-4 with the coding sequence for cN-II linked to the 5′-end of the GFP cDNA. As control, pIND-GFP (vector pIND with the complete cDNA for GFP) cells were used. Both systems were ponasterone inducible. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and grown at 37 °C in a humidified 5% CO_2/95% air atmosphere.

For cN-II over-expression evaluation, 10^6 cells (pIND-GFP and HKiG2) were plated in triplicate. After 20 h, medium was withdrawn and replaced with medium without or with 4 μM ponasterone. Exposure was for 0, 24 and 48 h.

For viability evaluation of pIND-GFP and HKiG2 cells 10^4 cells were plated in triplicate. After 20 h, medium was withdrawn and replaced with medium without or with 4 μM ponasterone. Exposure was for 48 h.

2.3. mRNA extraction and evaluation

Total RNA was isolated from cells using PerfectPure total cell RNA isolation system following the manufacturer's instructions and quantified using Quantifluor-ST Fluorometer according to the protocols described in the respective detection kits. 1 μg of total RNA was retrotranscribed into cDNA using iScript cDNA Synthesis Kit in a total volume of 20 μl. 1 μl of each cDNA preparation was assayed for cN-II expression in a total of 20 μl in the presence of deoxynucleoside triphosphates (50 μM each final concentration), 1 μM of forward and reverse primer specific for cN-II, 1 × PCR reaction buffer containing 1.5 mM $MgCl_2$, and 1 U mi-Taq DNA Polymerase. For normalization of RNA loading, the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was also amplified for each cDNA sample. cN-II and GAPDH primers and PCR amplification procedures are those described by Careddu et al. [27].

2.4. Protein concentration determination

Protein Assay Dye Reagent was used, according to the provider instructions, for the determination of protein concentration if not differently stated.

2.5. Immunoblot analysis

SDS-PAGE was performed following the method described by Laemmli [28]. Sample volumes corresponding to 10 μg of proteins from each sample were applied to a 10% SDS polyacrylamide gel. After electrophoresis, Western blotting was performed using PVDF membrane and processed for immune detection as described by Allegrini et al. [29].

2.6. Crude extract preparation and enzyme activities

For the evaluation of enzyme activities in pIND-GFP and HKiG2 cells, 10^6 cells were plated in triplicate. After 20 h, medium was withdrawn and replaced with medium with 4 μM ponasterone. After 48 h incubation pIND-GFP and HKiG2 were harvested and the pellet was resuspended in Tris–HCl 100 mM pH 7.4 in the presence of protease inhibitor cocktail. For dCK assay, 10 mM sodium fluoride as phosphatase inhibitor was also added to the resuspension buffer. Crude extracts were obtained by 3 freeze/thaw cycles followed by centrifugation at 10,000 ×*g* at 4 °C for 40 min to remove the cell debris. Supernatant was used for enzyme activities.

Phosphotransferase activity of cN-II in crude extracts was measured as the rate of [8-^{14}C]IMP formation from 1.4 mM [8-^{14}C] inosine, in the presence of 2 mM IMP (or GMP), 20 mM $MgCl_2$, 4.5 mM ATP and 5 mM dithiothreitol and crude extract (100 μg of protein) as previously described by Pesi et al. [30].

Phosphatase activity of cN-II was assayed measuring the release of phosphate according to Chifflet [31]. The reaction mixture contained 2 mM IMP, 20 mM $MgCl_2$, 5 mM BPG, 500 μM β-γ-methyleneadenosine-5′-triphosphate (AMP-PCP) as ecto-nucleotidase inhibitor, Tris–HCl 100 mM pH 7.4 and crude extract (100 μg of protein).

HGPRT assay was performed spectrophotometrically, as previously described [32]. The reaction mixture contained 90 μM guanine, 0.2 mM PRPP, 10 mM $MgCl_2$, Tris–HCl 100 mM pH 7.4 and crude extract (10 μg of protein).

Adenosine deaminase (ADA) was assayed spectrophotometrically according to Kalckar [33]. The reaction mixture contained 100 μM adenosine, Tris–HCl 100 mM pH 7.4 and crude extract (40 μg of protein).

Purine nucleoside phosphorylase (PNP) was assayed spectrophotometrically according to Kalckar [34]. The reaction mixture contained 300 μM inosine, 5 mM Pi, 100 mU of xanthine oxidase as ancillary enzyme, Tris–HCl 100 mM pH 7.4 and crude extract (40 μg of protein).

dCK was determined using a radioactive assay: 20 μM [2-^{14}C]deoxycytidine (53 mCi/mmol), 1 mM ATP, 10 mM $MgCl_2$, 30 μM thymidine as competitive inhibitor of thymidine kinase 2, 20 μM 4-deoxyuridine (Zebularine) as deoxycytidine deaminase (CDA) inhibitor, 100 mM Tris–HCl pH 7.4 and crude extract (40 μg of protein). At different times (0′, 20′, 40′ and 60′) 8 μl of assay mixture was withdrawn and spotted on DE-81 paper disks. After extensive washing in 1 mM ammonium formate and water, the disks were dried and the radioactivity was determined.

CDA was measured by determining the amount of deoxyuridine formed in the presence of labeled deoxycytidine. The reaction mixture contained 0.4 mM [2-^{14}C]deoxycytidine (53 mCi/mmol), Tris–HCl 100 mM pH 7.4 and crude extract (10 μg of protein). At different time intervals (0′, 20′, 40′ and 60′), 10 μl of the assay mixture was spotted on PEI-cellulose precoated thin-layer plastic sheets and the chromatogram was developed in n-propanol/NH_3/trichloroacetic acid (100%)/H_2O (75:0.7:5:20, v/v) to separate deoxycytidine and deoxyuridine [35]. Deoxyuridine standard was used and detected as UV absorbing areas, which were excised and counted for radioactivity.

All the assays were performed at 37 °C.

For all the enzymes, 1 mU represents the amount of enzyme required to convert 1 nmol of substrate to product per min under assay conditions. All the assays were performed in triplicate.

2.7. Intracellular metabolite extraction

10^6 cells were plated in triplicate. After 20 h, medium was withdrawn and replaced with medium containing 4 μM ponasterone. After 48 h of exposure to 4 μM of ponasterone each replicate of plated pIND-GFP and HKiG2 cells was trypsinized. Pellets were rapidly separated from medium by centrifugation (1500 ×*g* for 5 min) and resuspended in 150 μl 1 M trichloroacetic acid (TCA). It is known that nucleotides are unstable in highly acidic solutions. To avoid degradation of nucleotides we adapted to our needs a protocol described by Friedecky et al. [36], in which the authors show that degradation of ribonucleotides incubated for 30 min in 1 M TCA is negligible. After three rounds of rapid freezing–thawing (3 min in dry ice-ethanol − 75 °C bath and 3 min in a warm 37 °C bath), pellets were separated from supernatants by centrifugation (14,000 ×*g* for 1 min at room temperature). 120 μl of each supernatant was collected in a 1.5 ml tube and immediately back-extracted three times with 1.2 ml ether vortexing for 20 s at maximum speed. This procedure produced samples at pH > 5 in which no more degradation of nucleotides is observed. Samples were kept at room temperature under a chemical hood for 5 min and later stored at − 20 °C until needed. The whole process of deproteination took no longer than 25 min. The protein content of the pellets previously precipitated with TCA was determined using a modified Lowry method described by Peterson [37].

2.8. Capillary electrophoresis analysis

All the experiments were performed using a Beckman P/ACE MDQ Capillary Electrophoresis System equipped with an UV detector. The intracellular nucleotide concentration was determined as previously described [29].

2.9. Cytotoxicity assay

In cytotoxicity experiments, 10^4 cells grown as described in Section 2.2, were threefold plated. After 20 h, medium was withdrawn and replaced with medium with 4 μM ponasterone. After 48 h, medium was withdrawn and replaced with medium with 4 μM ponasterone, and different concentrations of nucleoside analogs were added (gemcitabine, cytarabine and fludarabine from 0.32 μM to 5 mM). Exposure to the drug was for 48 h. After treatment threefold replicates for each cell line were incubated at 37 °C, 5% CO_2/95% air atmosphere for

2 h with 0.5 mg/ml of MTT previously dissolved in PBS and filtered according to Mosmann [38]. Formazan salts produced in the reaction were dissolved in 0.04 N HCl in isopropanol and quantified spectrophotometrically by reading the absorbance at 570 nm using the Sunrise™ Absorbance Reader (Tecan, Switzerland).

2.10. Statistical analysis

Variance was analyzed by ONE-WAY ANOVA and means were compared by Dunnet's test ($p < 0.05$). All statistical analyses were performed using the software InStat (ver. 3.05, GraphPad Software, Inc., La Jolla, CA 92037 USA).

3. Results

3.1. Induction of cN-II hyper-expression in HEK 293 cell cultures and impact on viability and nucleotide content

The HEK 293 cell line is widely utilized as cell model for protein over-expression. An inducible over-expression of cN-II in HEK 293 cells was previously obtained by Gazziola et al. [25]. We took advantage of the availability of this model to better understand the role of cN-II in nucleotide and prodrug metabolism. HEK 293 cells were stably transfected with a plasmid containing the ecdysone receptor and the coding sequence for the human cN-II linked to the 5′-end of the cDNA for the GFP (HKiG2 cells). Addition of ponasterone led to over-expression of cN-II assayed as phosphotransferase in an exposure time-dependent manner (Fig. 2). Evaluation of cN-II mRNA and protein is reported in Supplementary Fig. S1. Our results are similar to those reported in the papers describing the preparation of the cellular models [25,26]. Quantitation of mRNA and cN-II protein is in agreement with the measured cN-II activity indicating that fusion with GFP does not affect the catalytic properties of the enzyme (see Supplementary Fig. S1 and [25]). To ascertain if cN-II over-expression affects cell vitality in our experimental conditions, we measured the viability (MTT assay) of both pIND-GFP and HKiG2 cells grown 48 h in the absence and presence of ponasterone and found a significant decrease of cell viability upon treatment with ponasterone in both cell lines, but no difference in cell viability was found between control and over-expressing cells in these growth conditions (Fig. 3).

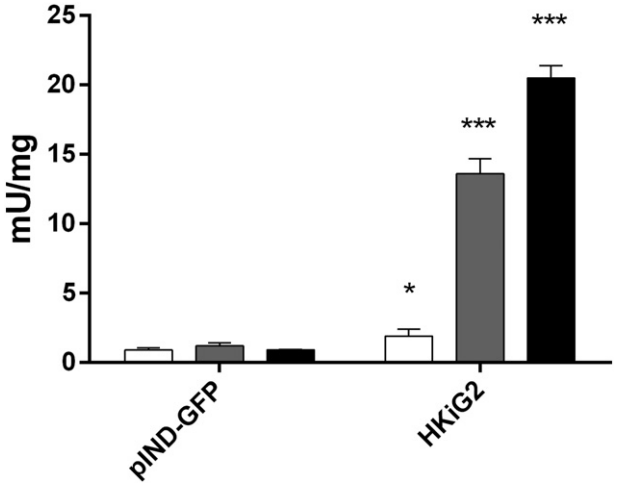

Fig. 2. cN-II phosphotransferase activity was measured in HEK 293 cell crude extracts obtained from cells grown both in the absence of ponasterone (white bars), and in the presence of 4 μM ponasterone for 24 h (gray bars) and 48 h (black bars). Data shown represent the mean ± SD and are representative of three independent experiments. Significance is related to pIND-GFP for samples not treated with ponasterone (white bars) while for those treated with ponasterone the significance is related to the respective samples without ponasterone. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

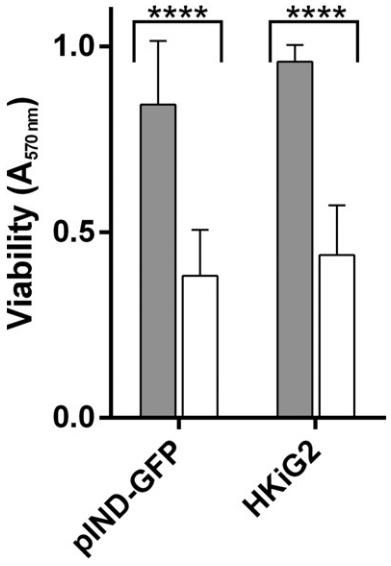

Fig. 3. Viability of pIND-GFP and HKiG2 after 48 h of growth in the presence (white) or in the absence (gray) of 4 μM ponasterone. Data shown represent the mean ± SD and are representative of three independent experiments. ****$p < 0.0001$.

Nucleotide content appears to be significantly affected by the cN-II over-expression and, as stated above, this result is not ascribable to a different sensitivity of the two cell lines to ponasterone treatment. Fig. 4 shows that purine and pyrimidine triphosphates are significantly decreased in HKiG2 cells expressing the fusion protein. Also GDP appeared significantly decreased while, among monophosphates, only IMP, the best substrate of cN-II, was significantly decreased. On the contrary, AMP levels were increased in cN-II over-expressing cells. ATP depletion was accompanied by a decrease of energy charge [(ATP + 1/2 ADP) / (AMP + ADP + ATP)] and by a loss of adenylate compounds which is not counterbalanced by the modest increase of AMP levels in HKiG2 cell. This suggests that the salvage operated by the anabolic arm of the IMP substrate cycle shown in Fig. 1 was slower than the catabolic arm when cN-II was over-expressed.

3.2. Enzyme activities in cells exposed to ponasterone

In order to ascertain if, following cN-II over-expression, compensatory mechanisms were activated, we measured the activity of several enzymes involved in the metabolic pathways shown in Fig. 1. The activity of cN-II, measured both as phosphatase and phosphotransferase, was significantly higher in HKiG2 cells. On the other hand, the specific activity of HGPRT, PNP and ADA, enzymes involved in the metabolic pathway described in Fig. 1, was not changed in our experimental conditions (Table 1). With the intent to assess the effect of cN-II over-expression on cytotoxicity exerted by fludarabine, cytarabine and gemcitabine, we also measured the specific activity of CDA and dCK in both pIND-GFP and HKiG2. CDA can deaminate, and therefore inactivate, both gemcitabine and cytarabine [39], while dCK is responsible for the activation of the three analogs [40]. CDA exhibited the same specific activity in both cell lines (Table 1). dCK requires ATP both as a substrate and as activator, being subjected to phosphorylation at Ser-74 [41,42]. To ascertain if the low ATP level found in HKGi2 cells could affect the degree of the enzyme phosphorylation, we measured dCK activity in extracts prepared with and without sodium fluoride, an inhibitor of protein phosphatases (dCK can be dephosphorylated by protein phosphatase 2A [43]). The results indicate in both cases no differences in dCK specific activities in control an over-expressing cells (Table 1). Therefore, among the enzymes assayed, only cN-II activity was altered in HKGi2 cells upon 48 h incubation with ponasterone.

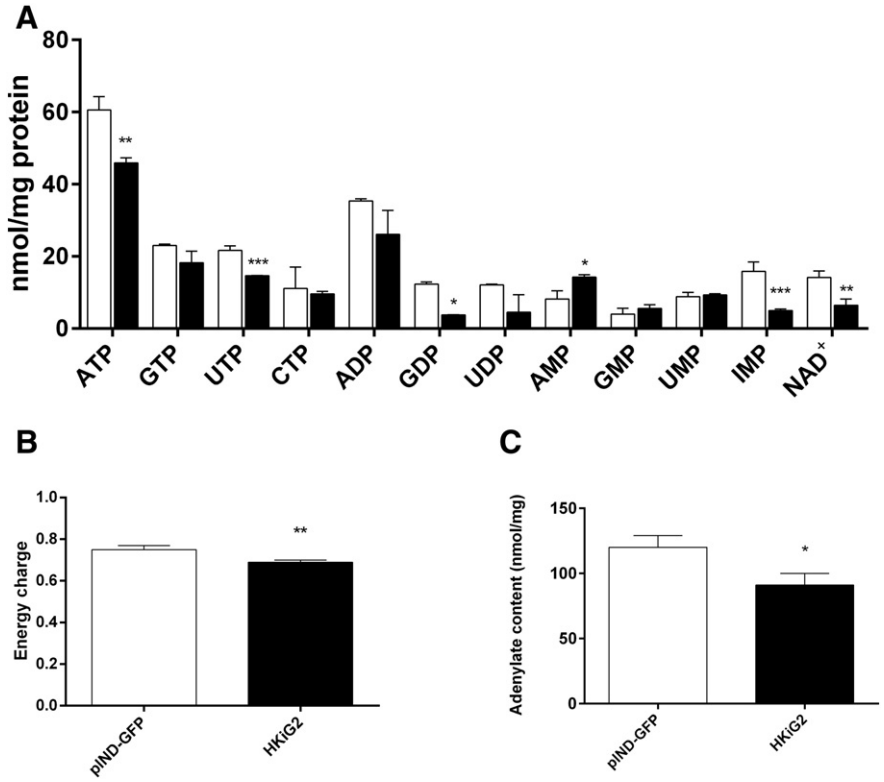

Fig. 4. Capillary electrophoresis analysis. A) Intracellular nucleotide levels (pIND-GFP white bars and HKiG2 black bars). CDP and CMP were undetectable. Energy charge (B) and adenylate intracellular content (C) after 48 h of exposure to 4 μM ponasterone. C) Data shown represent the mean ± SD and are representative of three independent experiments. Significance is related to pINDGFP. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3. Cytotoxicity tests

The viability of the two cell lines described above was measured in the presence of increasing concentrations of fludarabine, gemcitabine and cytarabine and the effect of the ponasterone mediated over-expression of cN-II on cell viability is shown in Fig. 5 and in Table 2. pIND-GFP was susceptible to micromolar concentration of both fludarabine and gemcitabine while cytarabine exerted a modest effect at higher concentrations. The IC_{50} measured for fludarabine was of the same order of magnitude found by other authors [44,45], whereas the IC_{50} values found for gemcitabine and cytarabine are significantly higher than the values found by other authors in cultured tumor cells [45–48]. This observation might be due to an elevated CDA activity in HEK 293 cells (see Table 1) as compared to other cells and organs [49], causing a natural resistance to the cytidine analogs [50,51]. The over-expression of cN-II clearly protects (Table 2) from cytotoxicity exerted by fludarabine (IC_{50} ratio HKiG2/pIND-GFP = 82) and gemcitabine (IC_{50} ratio HKiG2/pIND-GFP = 63) and to a lower but significant extent, by cytarabine (IC_{50} ratio HKiG2/pIND-GFP = 5.5).

Table 1
Enzyme activities in pIND-GFP and HKiG2 cell extracts. Numbers indicate mU/mg of proteins ± SD.

Enzyme	pIND-GFP	HKiG2
cN-II phosphotransferase	1.03 ± 0.24	20.53 ± 0.65
cN-II phosphatase	6.79 ± 0.34	34.04 ± 0.30
HGPRT	34.17 ± 0.49	35.73 ± 4.15
PNP	52.87 ± 1.63	56.97 ± 0.95
ADA	9.47 ± 0.35	10.00 ± 0.10
dCK (with NaF)	0.21 ± 0.03	0.23 ± 0.02
dCK	0.05 ± 0.01	0.08 ± 0.03
CDA	1.5 ± 0.51	1.35 ± 0.37

4. Discussion

An increase of cN-II activity, as well as a decrease, has been reported to be toxic at least for dividing cells [25,27]. This observation indicates a pivotal role played by the enzyme in cell proliferation and surviving. Furthermore, several reports demonstrated that cN-II over-expression or hyper-activity correlate with drug resistance in both hematological malignancies and solid tumors [15,17]. The mechanism of drug resistance remains to be unraveled since the protection of cN-II over-expression is exerted also upon treatment with nucleoside analogs whose monophosphates are not substrates of the enzyme. We took advantage of the availability of an over-expressing model to investigate on the role of cN-II in both the catabolism of purine compounds and resistance to purine and pyrimidine nucleoside analogs used in cancer therapy. HEK 293 cells carrying an expression vector coding for cN-II linked to GFP (HKiG2) and a control vector without cN-II (pIND-GFP) were used. Both systems are ponasterone inducible as described by Gazziola et al. [25]. After 48 h of incubation with the inducer, HKiG2 cells expressed approximately 20 times more cN-II, measured as phosphotransferase, and 5 times more measured as phosphatase, than the pIND-GFP cells. It is conceivable that this discrepancy depends on an over-estimation of the phosphatase activity in control cells. In fact, while the phosphotransferase assay is specific for cN-II [30], the phosphatase activity, measured as the rate of IMP hydrolysis, even in the presence of an ectosolic-5′-nucleotidase inhibitor, depends not only on cN-II, but also on other cytosolic 5′-nucleotidases or aspecific phosphatases [52,53]. To assess the effect of cN-II over-expression on the viability of HEK 293 cells, we measured the viability of cells after 48 h of ponasterone exposure finding no significant differences between over-expressing and control cells. This finding is in line with that obtained by Gazziola et al. [25], who reported no difference after 48 h treatment, while a significant proliferation decrease of cN-II over-expressing cells with respect to control cells was found after several

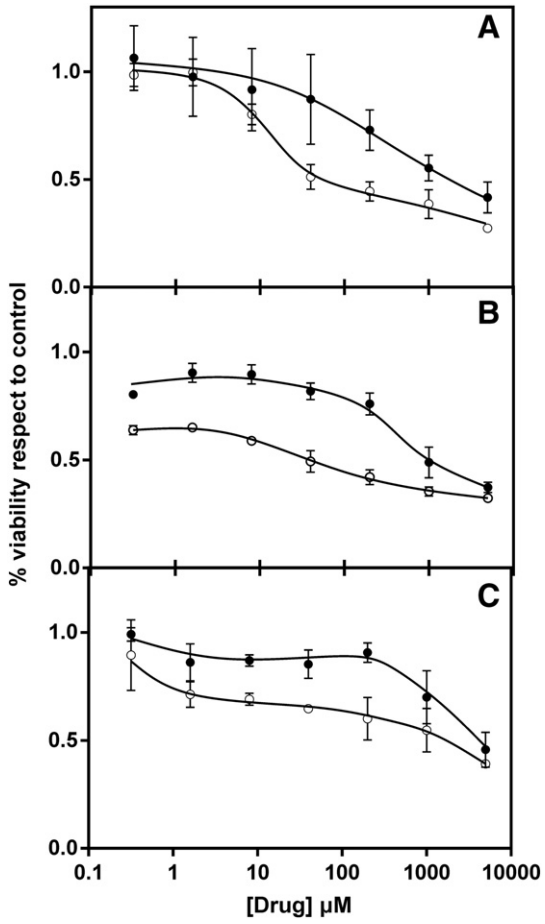

Fig. 5. HEK 293 cells exposure to nucleoside analogs: cell viability was assayed with MTT. pIND-GFP (empty circles) and HKiG2 (filled circles) were treated with 4 μM ponasterone for 48 h. Later the medium was replaced with fresh medium containing 4 μM ponasterone and the cells were exposed to μM concentrations (from 0.32 to 5000) of A) fludarabine, B) gemcitabine and C) cytarabine for 48 h. Data are reported in graphs as percentage of cell viability respect to control (no drug exposure) and are representative of three independent replicates.

days of exposure to the inducer. Our previous results demonstrated that an increase of cN-II activity was followed by PRPP consumption in tissue extracts [3] and ATP consumption in over-expressing yeast cells [29]. Accordingly, we found that cN-II over-expression in HEK 293 cells caused a decrease of all nucleoside triphosphates with a consequent drop in the adenylate content and energy charge. This result is in line with that obtained by Gazziola et al. [25] who found a 20% decrease in triphosphate content. The extraction procedure and the analytical method used in the present work allowed us to determine, besides triphosphates, also diphosphate and monophosphate nucleosides. Nucleoside diphosphates and NAD^+ were decreased in cN-II over-expressing cells, and among monophosphates, only IMP was substantially decreased while AMP increased significantly. Indeed, AMP accumulates as the result of ATP dephosphorylation, required for PRPP synthesis, necessary for the salvage of hypoxanthine, whose formation increases as a consequence of cN-II over-expression (see Fig. 1). Furthermore, both the low AMP deaminase activity at low ATP concentrations [54] and the very high K_m for AMP of cN-II [55] contribute to AMP accumulation. These results demonstrate that cells try to keep a constant pool of PRPP at the expense of ATP when the catabolic arm of the cycle of Fig. 1, as a consequence of cN-II over-expression, is running faster than in normal conditions. To ascertain if any compensatory alteration of enzyme expression occurred in cN-II over-expressing cells, we measured the activity of several enzymes involved in the metabolic pathways shown in Fig. 1, i.e., cN-II, HGPRT and PNP (IMP substrate cycle) and ADA, and found that only phosphatase and phosphotransferase activities specific of cN-II were altered upon 48 h incubation with ponasterone.

Table 2
IC_{50} values ± SD of the three analogs in pIND-GFP (control) and HKiG2 (over-expressing) cells.

Drug	pIND-GFP (μM)	HKiG2[a] (μM)	HKiG2/pIND-GFP
Fludarabine	44 ± 4	3615 ± 617***	82
Gemcitabine	30 ± 19	1905 ± 398**	63
Cytarabine	714 ± 272	3929 ± 1428*	5.5

[a] Significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Over-expressing and control cells were incubated in the presence of the analogs fludarabine, gemcitabine and cytarabine. IC_{50} of control cells for fludarabine was in line with the values found by other authors with different cell lines [44,45], while pIND-GFP sensitivity for gemcitabine and cytarabine was significantly lower [45–48]. In fact CDA, that can inactivate both drugs, in HEK 293 cells exhibits a specific activity higher than that reported for many other cells and organs, (in many tumoral tissues/cells, CDA activity is lower than 0.1 mU/mg) [49]. This finding may account for the high IC_{50} measured for cytotoxicity exerted by gemcitabine and cytarabine on these cells.

Cells over-expressing cN-II resulted to be protected from cytotoxic effect exerted by fludarabine and gemcitabine and, to a lower extent, by cytarabine. The extent of this protective effect does not correlate with the enzyme ability to dephosphorylate the active phosphorylated form of the drug analogs. Indeed fludarabine monophosphate has been demonstrated to be substrate of cN-II [56], while gemcitabine and cytarabine monophosphates are not substrates of the enzyme [13]. We might therefore infer that the extent of protection can depend on the perturbance of purine and pyrimidine nucleotide pool determined by cN-II over-expression, which might interfere at different levels with the transport and intracellular metabolism of the drugs. It has been demonstrated that the rate limiting step in the metabolism of the analogs used in the present work is their phosphorylation mediated by dCK [40], which can accept as triphosphate substrate either ATP or UTP [57]. Furthermore, dCK is regulated by phosphorylation, resulting in enzyme activation [41]. However, dCK activity was the same both in pIND-GFP and in HKiG2 cells even when the activating phosphorylation of the enzyme was preserved using a protein phosphatase inhibitor for the preparation of the extracts, indicating that the decrease of ATP in over-expressing cells does not interfere with dCK activation. Furthermore, being the K_m for the best phosphate donor (UTP) very low (1 μM), the observed decrease in the level of this nucleotide in over-expressing cells is unlikely to affect dCK activity. On the other hand, dCK is a very complex allosteric enzyme [58] and therefore, the possibility that a profound alteration of nucleotide pool might negatively reflect on dCK activation and activity, cannot be ruled out. The observation that a modest cN-II silencing causes activation of the apoptotic program, without any measurable alteration of nucleotide pool [27], suggests that cN-II plays some regulatory role on important cellular mechanisms. Indeed, we recently demonstrated that cN-II interacts with the cytosolic protein NLR family CARD domain-containing protein 4 (Ipaf) involved in innate immunity and inflammation [59]. Ipaf, upon activation exerted by extracellular or intracellular signals, promotes interleukin production and apoptosis activation [60]. It is therefore conceivable that the interaction of Ipaf with cN-II may play a modulatory role, possibly reflecting on prodrug metabolism and cytotoxicity.

In conclusion, our results demonstrate that cN-II, besides being directly involved in the inactivation of drugs, is also determinant for the intracellular nucleotide concentration, by regulating the rate of the metabolic pathways shown in Fig. 1. This might reflect on the rate of drug intracellular metabolism. Finally, our findings demonstrate that

over-expression of cN-II is sufficient to cause cell resistance and might explain why a high level of cN-II expression is protective against drugs whose monophosphates are poor or to no extent substrates of the enzyme.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2015.03.017.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

The Authors wish to thank Prof. Vera Bianchi, University of Padova Italy, for providing pIND-GFP and HKiG2 cells.

This work was financially supported by a grant from the REGIONE AUTONOMA DELLA SARDEGNA, L.R. 07/08/2007, grant number CRP3360 (TITLE: Modulation of expression by inducible silencing; heterologous expression in S. cerevisiae; two-hybrid system. Three models to make clear the physiological role of cytosolic 5′nucleotidase II), and by local grants from the University of Pisa. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

[1] V. Bianchi, J. Spychala, Mammalian 5′-nucleotidases, J. Biol. Chem. 278 (2003) 46195–46198.
[2] M.G. Tozzi, R. Pesi, S. Allegrini, On the physiological role of cytosolic 5′-nucleotidase II (cN-II): pathological and therapeutical implications, Curr. Med. Chem. 20 (2013) 4285–4291.
[3] C. Barsotti, R. Pesi, F. Felice, P.L. Ipata, The purine nucleoside cycle in cell-free extracts of rat brain: evidence for the occurrence of an inosine and a guanosine cycle with distinct metabolic roles, Cell. Mol. Life Sci. 60 (2003) 786–793.
[4] R. Itoh, IMP–GMP 5′-nucleotidase, Comp. Biochem. Physiol. B 105 (1993) 13–19.
[5] M.G. Tozzi, M. Camici, S. Allegrini, R. Pesi, M. Turriani, A. Del Corso, P.L. Ipata, Cytosolic 5′-nucleotidase/phosphotransferase of human colon carcinoma, Adv. Exp. Med. Biol. 309B (1991) 173–176.
[6] S. Banditelli, C. Baiocchi, R. Pesi, S. Allegrini, M. Turriani, P.L. Ipata, M. Camici, M.G. Tozzi, The phosphotransferase activity of cytosolic 5′-nucleotidase; a purine analog phosphorylating enzyme, Int. J. Biochem. Cell Biol. 28 (1996) 711–720.
[7] M.A. Johnson, A. Fridland, Phosphorylation of 2′,3′-dideoxyinosine by cytosolic 5′-nucleotidase of human lymphoid cells, Mol. Pharmacol. 36 (1989) 291–295.
[8] M. Turriani, R. Pesi, A. Nardone, G. Turchi, F. Sgarrella, P.L. Ipata, M.G. Tozzi, Cytosolic 5′-nucleotidase/nucleoside phosphotransferase: a nucleoside analog activating enzyme? J. Biochem. Toxicol. 9 (1994) 51–57.
[9] H. Kawasaki, C.J. Carrera, L.D. Piro, A. Saven, T.J. Kipps, D.A. Carson, Relationship of deoxycytidine kinase and cytoplasmic 5′-nucleotidase to the chemotherapeutic efficacy of 2-chlorodeoxyadenosine, Blood 81 (1993) 597–601.
[10] R. Garcia-Carbonero, D.P. Ryan, B.A. Chabner, Cytidine analogs, in: C. B.A., L. D.L. (Eds.), Cancer Chemotherapy and Biotherapy, Lippincott-Raven, Philadelphia 1996, pp. 265–294.
[11] W. Plunkett, P.P. Saunders, Metabolism and action of purine nucleoside analogs, Pharmacol. Ther. 49 (1991) 239–268.
[12] C.M. Galmarini, M.L. Clarke, L. Jordheim, C.L. Santos, E. Cros, J.R. Mackey, C. Dumontet, Resistance to gemcitabine in a human follicular lymphoma cell line is due to partial deletion of the deoxycytidine kinase gene, BMC Pharmacol. 4 (2004) 8.
[13] C. Mazzon, C. Rampazzo, M.C. Scaini, L. Gallinaro, A. Karlsson, C. Meier, J. Balzarini, P. Reichard, V. Bianchi, Cytosolic and mitochondrial deoxyribonucleotidases: activity with substrate analogs, inhibitors and implications for therapy, Biochem. Pharmacol. 66 (2003) 471–479.
[14] T. Yamauchi, E. Negoro, S. Kishi, K. Takagi, A. Yoshida, Y. Urasaki, H. Iwasaki, T. Ueda, Intracellular cytarabine triphosphate production correlates to deoxycytidine kinase/cytosolic 5 ′-nucleotidase II expression ratio in primary acute myeloid leukemia cells, Biochem. Pharmacol. 77 (2009) 1780–1786.
[15] P. Seve, J.R. Mackey, S. Isaac, O. Tredan, P.J. Souquet, M. Perol, C. Cass, C. Dumontet, cN-II expression predicts survival in patients receiving gemcitabine for advanced non-small cell lung cancer, Lung Cancer 49 (2005) 363–370.
[16] E. Giovannetti, V. Mey, L. Loni, S. Nannizzi, G. Barsanti, G. Savarino, S. Ricciardi, M. Del Tacca, R. Danesi, Cytotoxic activity of gemcitabine and correlation with expression profile of drug-related genes in human lymphoid cells, Pharmacol. Res. 55 (2007) 343–349.
[17] C.M. Galmarini, E. Cros, X. Thomas, L. Jordheim, C. Dumontet, The prognostic value of cN-II and cN-III enzymes in adult acute myeloid leukemia, Haematologica 90 (2005) 1699–1701.
[18] C.M. Galmarini, X. Thomas, K. Graham, A. El Jafaari, E. Cros, L. Jordheim, J.R. Mackey, C. Dumontet, Deoxycytidine kinase and cN-II nucleotidase expression in blast cells predict survival in acute myeloid leukaemia patients treated with cytarabine, Br. J. Haematol. 122 (2003) 53–60.
[19] A.K. Mitra, M.N. Kirstein, A. Khatri, K.M. Skubitz, A.Z. Dudek, E.W. Greeno, R.A. Kratzke, J.K. Lamba, Pathway-based pharmacogenomics of gemcitabine pharmacokinetics in patients with solid tumors, Pharmacogenomics 13 (2012) 1009–1021.
[20] C.M. Galmarini, What does over-expression of cN-II enzyme signify in haematological malignancies? Leuk. Res. 31 (2007) 1325–1326.
[21] A.K. Mitra, K.R. Crews, S. Pounds, X. Cao, T. Feldberg, Y. Ghodke, V. Gandhi, W. Plunkett, M.E. Dolan, C. Hartford, S. Raimondi, D. Campana, J. Downing, J.E. Rubnitz, R.C. Ribeiro, J.K. Lamba, Genetic variants in cytosolic 5′-nucleotidase II are associated with its expression and cytarabine sensitivity in HapMap cell lines and in patients with acute myeloid leukemia, J. Pharmacol. Exp. Ther. 339 (2011) 9–23.
[22] J.A. Meyer, W.L. Carroll, T. Bhatla, Screening for gene mutations: will identification of NT5C2 mutations help predict the chance of relapse in acute lymphoblastic leukemia? Expert. Rev. Hematol. 6 (2013) 223–224.
[23] J.A. Meyer, J. Wang, L.E. Hogan, J.J. Yang, S. Dandekar, J.P. Patel, Z. Tang, P. Zumbo, S. Li, J. Zavadil, R.L. Levine, T. Cardozo, S.P. Hunger, E.A. Raetz, W.E. Evans, D.J. Morrison, C.E. Mason, W.L. Carroll, Relapse-specific mutations in NT5C2 in childhood acute lymphoblastic leukemia, Nat. Genet. 45 (2013) 290–294.
[24] G. Tzoneva, A. Perez-Garcia, Z. Carpenter, H. Khiabanian, V. Tosello, M. Allegretta, E. Paietta, J. Racevskis, J.M. Rowe, M.S. Tallman, M. Paganin, G. Basso, J. Hof, R. Kirschner-Schwabe, T. Palomero, R. Rabadan, A. Ferrando, Activating mutations in the NT5C2 nucleotidase gene drive chemotherapy resistance in relapsed ALL, Nat. Med. 19 (2013) 368–371.
[25] C. Gazziola, M. Moras, P. Ferraro, L. Gallinaro, R. Verin, C. Rampazzo, P. Reichard, V. Bianchi, Induction of human high K_M 5′-nucleotidase in cultured 293 cells, Exp. Cell Res. 253 (1999) 474–482.
[26] C. Rampazzo, C. Gazziola, P. Ferraro, L. Gallinaro, M. Johansson, P. Reichard, V. Bianchi, Human high-Km 5′-nucleotidase effects of overexpression of the cloned cDNA in cultured human cells, Eur. J. Biochem. 261 (1999) 689–697.
[27] M.G. Careddu, S. Allegrini, R. Pesi, M. Camici, M. Garcia-Gil, M.G. Tozzi, Knockdown of cytosolic 5′-nucleotidase II (cN-II) reveals that its activity is essential for survival in astrocytoma cells, Biochim. Biophys. Acta 1783 (2008) 1529–1535.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[29] S. Allegrini, D.N. Filoni, A. Galli, A. Collavoli, R. Pesi, M. Camici, M.G. Tozzi, Expression of bovine cytosolic 5′-nucleotidase (cN-II) in yeast: nucleotide pools disturbance and its consequences on growth and homologous recombination, Plos One 8 (2013) e63914.
[30] M.G. Tozzi, M. Camici, R. Pesi, S. Allegrini, F. Sgarrella, P.L. Ipata, Nucleoside phosphotransferase activity of human colon carcinoma cytosolic 5′-nucleotidase, Arch. Biochem. Biophys. 291 (1991) 212–217.
[31] S. Chifflet, A. Torriglia, R. Chiesa, S. Tolosa, A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases, Anal. Biochem. 168 (1988) 1–4.
[32] M.P. Giovannitti, R. Pesi, G. Cercignani, Spectrophotometric assay for hypoxanthine-guanine phosphoribosyltransferase in human-erythrocytes, Ital. J. Biochem. 37 (1988) A44–A46.
[33] H.M. Kalckar, Differential spectrophotometry of purine compounds by means of specific enzymes; determination of adenine compounds, J. Biol. Chem. 167 (1947) 445–459.
[34] H.M. Kalckar, Differential spectrophotometry of purine compounds by means of specific enzymes; determination of hydroxypurine compounds, J. Biol. Chem. 167 (1947) 429–443.
[35] C. Barsotti, R. Pesi, M. Giannecchini, P.L. Ipata, Evidence for the involvement of cytosolic 5′-nucleotidase (cN-II) in the synthesis of guanine nucleotides from xanthosine, J. Biol. Chem. 280 (2005) 13465–13469.
[36] D. Friedecky, J. Tomkova, V. Maier, A. Janost'akova, M. Prochazka, T. Adam, Capillary electrophoretic method for nucleotide analysis in cells: application on inherited metabolic disorders, Electrophoresis 28 (2007) 373–380.
[37] G.L. Peterson, Determination of total protein, in: C.H.W. Hirs (Ed.), Methods Enzymol — Enzyme Structure. Part I, vol. 91, Academic Press 1983, pp. 95–104.
[38] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[39] C.M. Galmarini, J.R. Mackey, C. Dumontet, Nucleoside analogues: mechanisms of drug resistance and reversal strategies, Leukemia 15 (2001) 875–890.
[40] B. Munch-Petersen, J. Piskur, Deoxynucleoside kinases and their potential role in deoxynucleoside cytotoxicity, in: G.J. Peters (Ed.), Deoxynucleoside Analogs in Cancer Therapy, Humana Press Inc., Totowa, New Jersey, United States 2006, pp. 53–79.
[41] C. Smal, D. Vertommen, L. Bertrand, S. Ntamashimikiro, M.H. Rider, E. Van Den Neste, F. Bontemps, Identification of in vivo phosphorylation sites on human deoxycytidine kinase — role of Ser-74 in the control of enzyme activity, J. Biol. Chem. 281 (2006) 4887–4893.
[42] C. Smal, E. Van Den Neste, M. Maerevoet, X. Poire, I. Theate, F. Bontemps, Positive regulation of deoxycytidine kinase activity by phosphorylation of Ser-74 in B-cell chronic lymphocytic leukaemia lymphocytes, Cancer Lett. 253 (2007) 68–73.
[43] R. Amsailale, M. Beyaert, C. Smal, V. Janssens, E. Van Den Neste, F. Bontemps, Protein phosphatase 2A regulates deoxycytidine kinase activity via Ser-74 dephosphorylation, FEBS Lett. 588 (2014) 727–732.
[44] R. Silber, B. Degar, D. Costin, E.W. Newcomb, M. Mani, C.R. Rosenberg, L. Morse, J.C. Drygas, Z.N. Canellakis, M. Potmesil, Chemosensitivity of lymphocytes from patients with B-cell chronic lymphocytic leukemia to chlorambucil, fludarabine, and camptothecin analogs, Blood 84 (1994) 3440–3446.

[45] L.P. Jordheim, E. Cros, M.H. Gouy, C.M. Galmarini, S. Peyrottes, J. Mackey, C. Perigaud, C. Dumontet, Characterization of a gemcitabine-resistant murine leukemic cell line: reversion of in vitro resistance by a mononucleotide prodrug, Clin. Cancer Res. 10 (2004) 5614–5621.
[46] Y. Nakano, S. Tanno, K. Koizumi, T. Nishikawa, K. Nakamura, M. Minoguchi, T. Izawa, Y. Mizukami, T. Okumura, Y. Kohgo, Gemcitabine chemoresistance and molecular markers associated with gemcitabine transport and metabolism in human pancreatic cancer cells, Br. J. Cancer 96 (2007) 457–463.
[47] H. Achiwa, T. Oguri, S. Sato, H. Maeda, T. Niimi, R. Ueda, Determinants of sensitivity and resistance to gemcitabine: the roles of human equilibrative nucleoside transporter 1 and deoxycytidine kinase in non-small cell lung cancer, Cancer Sci. 95 (2004) 753–757.
[48] M. Hewish, S.A. Martin, R. Elliott, D. Cunningham, C.J. Lord, A. Ashworth, Cytosine-based nucleoside analogs are selectively lethal to DNA mismatch repair-deficient tumour cells by enhancing levels of intracellular oxidative stress, Br. J. Cancer 108 (2013) 983–992.
[49] J. Sigmond, R.J. Honeywell, T.J. Postma, C.M. Dirven, S.M. de Lange, K. van der Born, A.C. Laan, J.C. Baayen, C.J. Van Groeningen, A.M. Bergman, G. Giaccone, G.J. Peters, Gemcitabine uptake in glioblastoma multiforme: potential as a radiosensitizer, Ann. Oncol. 20 (2009) 182–187.
[50] C.M. Galmarini, J.R. Mackey, C. Dumontet, Nucleoside analogues and nucleobases in cancer treatment, Lancet Oncol. 3 (2002) 415–424.
[51] M.H. Tattersall, K. Ganeshaguru, A.V. Hoffbrand, Mechanisms of resistance of human acute leukaemia cells to cytosine arabinoside, Br. J. Haematol. 27 (1974) 39–46.
[52] R. Pesi, M. Camici, V. Micheli, L. Notarantonio, G. Jacomelli, M.G. Tozzi, Identification of the nucleotidase responsible for the AMP hydrolysing hyperactivity associated with neurological and developmental disorders, Neurochem. Res. 33 (2008) 59–65.
[53] R. Pesi, V. Micheli, G. Jacomelli, L. Peruzzi, M. Camici, M. Garcia-Gil, S. Allegrini, M.G. Tozzi, Cytosolic 5′-nucleotidase hyperactivity in erythrocytes of Lesch–Nyhan syndrome patients, Neuroreport 11 (2000) 1827–1831.
[54] C. Barsotti, P.L. Ipata, Metabolic regulation of ATP breakdown and of adenosine production in rat brain extracts, Int. J. Biochem. Cell Biol. 36 (2004) 2214–2225.
[55] R. Itoh, Enzymatic properties and physiological roles of cytosolic 5′-nucleotidase II, Curr. Med. Chem. 20 (2013) 4260–4284.
[56] C.M. Galmarini, L. Jordheim, C. Dumontet, Role of IMP-selective 5′-nucleotidase (cN-II) in hematological malignancies, Leuk. Lymphoma 44 (2003) 1105–1111.
[57] E.S. Arner, S. Eriksson, Mammalian deoxyribonucleoside kinases, Pharmacol. Ther. 67 (1995) 155–186.
[58] T.L. Hughes, T.M. Hahn, K.K. Reynolds, D.S. Shewach, Kinetic analysis of human deoxycytidine kinase with the true phosphate donor uridine triphosphate, Biochemistry 36 (1997) 7540–7547.
[59] F. Cividini, M.G. Tozzi, A. Galli, R. Pesi, M. Camici, C. Dumontet, L. Jordheim, S. Allegrini, Cytosolic 5′-nucleotidase II interacts with the leucin rich repeat of NLR family member Ipaf, PLoS One 10 (2015) e0121525.
[60] F.S. Sutterwala, R.A. Flavell, NLRC4/IPAF: a CARD carrying member of the NLR family, Clin. Immunol. 130 (2009) 2–6.