Mycoplasma hyorhinis-encoded purine nucleoside phosphorylase: kinetic properties and its
effect on the cytostatic potential of purine-based anticancer drugs

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List of non-standard abbreviations:

(d)Ado, (2’-deoxy)adenosine; (d)Guo, (2’-deoxy)guanosine; (d)Ino, (2’-deoxy)inosine; 2-CAde, 2-chloroadenine; 2-CAdo, 2-chloroadenosine; 2-CdA, 2-chloro-2’-deoxyadenosine; dCK, 2’-deoxycytidine kinase; 2-FAde, 2-fluoroadenine; 5-FU, 5-fluorouracil; 6-MeP, 6-methylpurine; 6-MeP-dR, 6-methylpurine-2’-deoxyriboside; Ade, adenine; AMPK, adenylate kinase; DADMe-Imm-H, DADMe-Immucillin-H; F-ara-A, 2-fluoroadenine-arabinofuranoside; Gua, guanine; Hx, hypoxanthine; Imm-H, Immucillin-H; methyl(talo)-F-Ado, 9-[6-deoxy-α-L-talofuranosyl]-2-F-adenine; methyl(talo)-MeP-R, 9-[6-deoxy-α-L-talofuranosyl]-6-methylpurine; NDPK, nucleoside diphosphate kinase; NP, nucleoside phosphorylase; PNP, purine nucleoside phosphorylase; PyNP, pyrimidine nucleoside phosphorylase; TFT, 5-triflorothymidine; TP, thymidine phosphorylase; UP, uridine phosphorylase
Abstract

A mycoplasma-encoded purine nucleoside phosphorylase (designated PNP_{Hyor}) has been cloned and characterized for the first time. Efficient phosphorolysis of natural 6-oxopurine and 6-aminopurine nucleosides was observed with adenosine being the preferred natural substrate (K_m = 61 µM). Several cytostatic purine nucleoside analogues proved to be susceptible to PNP_{Hyor}-mediated phosphorolysis and a markedly decreased or increased cytostatic activity was observed in *Mycoplasma hyorhinis*-infected human breast carcinoma MCF-7 cell cultures (MCF-7.Hyor), depending on the properties of the released purine base. We demonstrated a ~10 fold loss of cytostatic activity of cladribine in MCF-7.Hyor cells and observed a rapid and complete phosphorolysis of this drug when exposed to the supernatant of mycoplasma-infected cells. This conversion (inactivation) could be prevented by a specific PNP inhibitor. These findings correlated well with the high efficiency of PNP_{Hyor}-catalyzed phosphorolysis of cladribine to its less-toxic base 2-chloroadenine (K_m = 80 µM). In contrast, the cytostatic activity of nucleoside analogues carrying a highly toxic purine base and being a substrate for PNP_{Hyor}, but not human PNP, was substantially increased in MCF-7.Hyor cells (~130-fold for fludarabine and ~ 45-fold for 6-methylpurine-2'-deoxyriboside). Elimination of the mycoplasmas from the tumor cell cultures or selective inhibition of PNP_{Hyor} by a PNP inhibitor restored the cytostatic activity of the purine-based nucleoside drugs. Since several studies suggest a high and preferential colonization/association of tumor tissue in cancer patients with different prokaryotes (including mycoplasmas), the data presented here may be of relevance for the optimization of purine nucleoside-based anti-cancer drug treatment.
Introduction

After cellular uptake, nucleoside analogues often require enzymatic activation (e.g. phosphorylation) to exert biological activity. However they may also be subject to enzymatic inactivation. Therefore, the anticancer activity of purine- and pyrimidine-based antimetabolites is highly dependent on the expression of nucleoside-metabolizing enzymes in tumor cells (Galmarini et al., 2002; Parker, 2009). The reversible phosphorolysis of purine and pyrimidine nucleosides to their respective nucleobases and sugar derivatives [(2’-deoxy)-ribose-1-phosphate] is catalyzed by nucleoside phosphorylases (NPs). In vivo, nucleoside phosphorolysis by NPs is highly favoured over nucleoside synthesis. Structural studies revealed two distinct classes of NPs: the NP-I family containing purine nucleoside phosphorylases (PNPs), uridine phosphorylases (UPs) and 5’-methylthioadenosine phosphorylases; and the NP-II family containing pyrimidine nucleoside phosphorylases (PyNPs) and thymidine phosphorylases (TPs) (Pugmire and Ealick, 2002). Mammalian PNP (E.C. 2.4.2.1) accepts 6-oxopurines (e.g. hypoxanthine and guanine) and their nucleosides [e.g. (2’-deoxy)inosine and (2’-deoxy)guanosine] but not 6-aminopurines (e.g. adenine), whereas prokaryotic PNPs may additionally accept 6-aminopurines and their nucleosides [e.g. (2’-deoxy)adenosine] (Bzowska et al., 2000).

NP activity may affect the efficiency of nucleoside-based chemotherapy. Blocking TP activity has been shown to increase the anticancer activity of 5-trifluorothymidine (TFT) by preventing TP-mediated breakdown of the drug to its inactive nucleobase (5-trifluorothymine) (Temmink et al., 2007). A phase III clinical trial to investigate the benefits of the co-administration of a TP inhibitor and TFT as a treatment modality for colorectal cancer (designated TAS-102) is in progress (Yoshino et al., 2012). In contrast, capecitabine, an
orally-administered prodrug for the treatment of metastatic breast and colorectal cancer, requires TP activity for its conversion to the active metabolite 5-fluorouracil (5-FU) (Miwa et al., 1998; Fernández-Martos et al., 2012). Similarly, the cytostatic activity of several adenosine analogues (e.g. 6-methylpurine-2’-deoxyriboside, 2-fluoro-2’-deoxyadenosine and 2-fluoroadenine arabinofuranoside (fludarabine)) is markedly increased after (prokaryotic) PNP-mediated phosphorolysis. Since (2’-deoxy)adenosine and its analogues are generally not accepted as substrates for mammalian PNP, suicide gene therapy introducing prokaryotic PNP at the tumor site has been investigated (Parker et al., 1997; Parker et al., 2003; Sorscher et al., 2012). However, due to PNP activity of commensal bacteria in healthy tissues, toxicity often limits such treatment options. Therefore, efforts have been made to generate mutant PNPs with different substrate specificity than human and wild-type prokaryotic PNP, in order to increase the chemotherapeutic selectivity at the tumor site (Bennett et al., 2003; Ardiani et al., 2012). It can be concluded that the expression of NPs at the tumor site may have a profound effect on the chemotherapeutic efficiency, depending on the nature and mode of action of the particular nucleoside analogue.

Apart from tumor cells, the tumor microenvironment also encompasses the surrounding normal cells (inflammatory cells, cancer-associated fibroblasts, etc.) and blood vessels. Additionally, recent research has revealed that certain prokaryotes also associate with different types of cancer. Although some prokaryotic species such as Helicobacter pylori are clearly linked with carcinogenesis, the role of bacteria in cancer is often uncertain (reviewed by Mager, 2006; Cummins and Tangney, 2013). However, mapping the diversity of microorganisms associated with the different tissues of the human body (referred to as the microbiome) leads to new insights in several diseases (e.g. diabetes and inflammatory bowel disease) and the metabolic exchange between human tissues and microbiota was found to
affect the bioavailability of several types of drugs (e.g. acetaminophen) (reviewed by Nicholson et al., 2005; Clayton, 2009). Using metabolic phenotypes to predict the metabolism or toxic effect of drugs has been proposed as a strategy towards a more personalized healthcare and was defined by Clayton et al. (2006) as pharmacometabonomics.

Studying the effect of bacterial colonization of tumor tissue on anticancer treatment efficiency is often limited by experimental constraints regarding the co-culturing of bacteria and mammalian tumor cells. However, the slow growth rate of mycoplasmas does allow an efficient and persistent infection of mammalian cell cultures by these bacteria. Mycoplasmas are characterized by their unusual small size and minimal genome (Razin et al., 1998). They are a common source of cell culture contamination, and infection often remains unnoticed. Clinically, these bacteria have been increasingly reported to preferentially colonize human tumor tissue in cancer patients (Chan et al., 1996; Kidder et al., 1998; Huang et al., 2001; Pehlivan et al., 2004, 2005; Yang et al., 2010; Apostolou et al., 2011; Urbanek et al., 2011; Barykova et al., 2011; Erturhan et al., 2013). Therefore a controlled mycoplasma infection of tumor cell cultures in vitro may serve as an assay model to study the effect of prokaryotes on cancer chemotherapy. Previously we have shown that the cytostatic and antiviral activities of different thymidine and uridine analogues are dramatically decreased in mycoplasma-infected cell cultures due to the expression of a mycoplasma-encoded PyNP (Bronckaers et al., 2008; Liekens et al., 2009; Vande Voorde et al., 2012). In the present study we report that the expression of a specific mycoplasma-encoded PNP may improve or diminish the cytostatic activity of purine nucleoside analogues depending on the nature of the drug and its mechanism of cytostatic action. A mycoplasma-encoded PNP has now been cloned, kinetically characterized for the first time and its impact on the cytostatic activity of purine nucleoside analogues investigated.
**Materials and Methods**

**Chemicals**

Nucleosides, nucleobases, nucleoside analogues and all inorganic compounds were purchased from Sigma-Aldrich (St-Louis, MO) unless stated differently. Immcillin-H (Imm-H) and DADMe-Immcillin-H (DADMe-Imm-H) were kindly provided by Dr. V. Schramm (Albert Einstein College of Medicine, Bronx, NY). Dr. W.B. Parker (Southern Research Institute, Birmingham, AL) generously provided 9-[6-deoxy-α-L-talofuranosyl]-2-F-adenine (methyl(talo)-F-Ado), 9-[6-deoxy-α-L-talofuranosyl]-6-methylpurine (methyl(talo)-MeP-R) and 6-methylpurine-2’-deoxyriboside (6-MeP-dR). 8-Aminoguanosine, 2-amino-2’-deoxy-2’-fluoro-adenosine and 5,6-dichlorobenzimidazole ribofuranoside were purchased from Carbosynth (Berkshire, UK). Radioactive [8-³H]-2-chloro-2’-deoxyadenosine ([8-³H]-2-CdA) ( radiospecificity: 7.8 Ci/mmol) was obtained from Moravek Biochemicals Inc. (Brea, CA).

**Cell cultures**

Human breast carcinoma MCF-7 cells were kindly provided by Prof. G.J. Peters (Amsterdam, The Netherlands). Human lymphocytic CEM cells were obtained from the American Tissue Culture Collection (Rockville, MD). Wild-type CEM/0 cells deficient in 2’-deoxycytidine kinase (CEM/dCK) were kindly provided by Dr. J. M. Leeds (Duke University Medical Center, Durham, NC). MCF-7, CEM/0 and CEM/dCK cells were infected with *Mycoplasma hyorhinis* (ATCC 17981). After two or more passages (to avoid bias by the initial inoculum) successful infection was confirmed using the MycoAlert™ mycoplasma detection kit (Lonza, Basel, Switzerland). Although this assay is only semi-quantitative, a maximal infection was observed three-four days after the passaging of the mycoplasma-exposed cells. Chronically-infected cell lines are further referred to as MCF-7.Hyor,
CEM.Hyor and CEM/dCK⁻.Hyor. All cells were maintained in Dulbecco’s modified Eagle medium (Invitrogen, Carlsbad, CA) with 10% foetal bovine serum (Integro, Dieren, the Netherlands), 10 mM HEPES and 1 mM sodium pyruvate (Invitrogen) and grown at 37 °C in a humidified CO₂-controlled incubator.

**Biological assays**

To compare the cytostatic activity of nucleoside analogues in mycoplasma-infected and non-infected cancer cell lines, MCF-7 and MCF-7.Hyor cells were seeded in 48-well plates (Nunc™, Roskilde, Denmark) at 10,000 cells/well. After 24 hours, an equal volume of fresh medium containing the test compounds [in the presence or absence of the PNP inhibitor DADMe-Imm-H (10 µM)] was added. Three days later (to ensure sufficient cell-proliferation and mycoplasma growth), cells were trypsinized and counted in a Coulter counter (Analis, Suallé, Belgium). The 50% inhibitory concentration (IC₅₀) was defined as the compound concentration required to reduce tumor cell proliferation by 50%.

**Cladribine metabolism and incorporation in the DNA/RNA of mycoplasma-infected and uninfected cells.**

The metabolism of cladribine was studied and compared in mycoplasma-infected and control CEM and CEM.dCK⁻ cell cultures. Cells were seeded at ~ 3 x 10⁶ cells/mL and subjected for 24 h to 2 µCi [8-³H]-2-CdA (43 nM 2-CdA). Cells were then harvested, washed twice with serum free DMEM medium and exposed to 66% cold methanol. The precipitated cell mixture was then kept on ice for 10 min and subsequently cleared by centrifugation (15 min; 16 000 g). The supernatant was used for the quantification of intracellular [8-³H]-2-CdA and its phosphorylated metabolites by HPLC analysis (Alliance 2690, Waters, Milford, MA) using a Partisphere-SAX anion exchange column (5.6 x 125 mm, Wattman International Ltd.,
Maidstone, UK). The following gradient was used: 5 min 100 % buffer A (5mM NH₄H₂PO₄, pH 5); a 15 min linear gradient of 100 % buffer A to 100 % buffer B (300 mM NH₄H₂PO₄, pH 5); 20 min 100 % buffer B; a 5 min linear gradient to 100 % buffer A; equilibration at 100 % buffer A for 5 min. Fractions of 2 mL were collected, transferred to 9 mL OptiPhase HiSafe 3 and radioactivity was counted in a liquid scintillation analyser. To quantify the amount of incorporated [8-³H]-2-CdA into nucleic acids, the pellet after MeOH extraction was washed twice with methanol 66%, resuspended in methanol 66% and transferred into 9 mL OptiPhase HiSafe 3. Radioactivity was then measured using a liquid scintillation analyzer.

Cladribine stability in the supernatant of mycoplasma-infected and uninfected cell cultures

The stability of [8-³H]-2-CdA in the cell-free supernatants of confluent MCF-7 and MCF-7.Hyor cell cultures was evaluated. Cells were seeded in 25 cm² culture flasks (TTP, Trasadingen, Switzerland) and after three days 1 mL supernatant was withdrawn and cleared by centrifugation at 300 g for 6 min to remove (debris of) mammalian cells. Subsequently, 20 µL PBS containing 2 µCi [8-³H]-2CdA was added to 480 µL of the supernatants (512 nM 2-CdA) and samples were incubated at 37 °C. At different time points (0’, 1 h and 6 h), 150 µL fractions were withdrawn and ice-cold MeOH was added to a final concentration of 66 % MeOH to terminate the enzymatic reactions. Samples were kept on ice for 10 min and cleared by centrifugation at 16,000 g for 15 min. The supernatants were withdrawn, evaporated and the resulting precipitate was resuspended in 65 µL PBS. Subsequently 50 µL was analyzed using HPLC as described in section 2.7.1 (gradient A on a RP-8 column). Fractions of 1 mL were collected, transferred to 9 mL OptiPhase HiSafe 3 (Perkin Elmer, Waldham, MA) and radioactivity was counted in a liquid scintillation analyzer (Perkin Elmer).
Also the stability of [8-³H]-2-CdA was similarly evaluated upon incubation of the compound for different time periods (t= 0’, 15’ and 1h) in the cell-free supernatant of CEM/0 and CEM.Hyor cells in the presence and absence of the PNP inhibitor Imm-H (10 µM).

**Purification of *M. hyorhinis* PNP (PNPHyor)**

Genomic mycoplasma DNA was extracted from a MCF-7.Hyor cell culture and the PNP<sub>Hyor</sub> gene was isolated, based on the published *M. hyorhinis* HUB-1 genome (Liu et al., 2010). The fragment was subcloned in the pGEM-T vector (Promega, Madison, WI, USA). The forward (5′-GGATCCCCACACCACATATAAGTG) and reverse (5′-GCGGCCGCTTACTTTATTTCTAAAGC) primers (Invitrogen) introduced a BamHI and NotI site, respectively. The PNP<sub>Hyor</sub> gene was subsequently cloned between the BamHI and NotI sites of the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden). The mycoplasma PNP was then expressed in *E. coli* as a GST-fusion protein according to a procedure previously described by Liekens et al. (2002). The PNP sequence from *Mycoplasma hyorhinis* (ATCC 17981) displayed two point mutations compared with *M. hyorhinis* HUB-1 PNP, resulting in two mutated amino acids: E37K and R56K. SDS-PAGE revealed a GST-fusion PNP protein of ~50 kDa (25 kDa for PNP<sub>Hyor</sub> + 25 kDa for GST; data not shown).

**Enzyme assays**

**Determination of the pH optimum**

The pH optimum for PNP<sub>Hyor</sub>-mediated phosphorolysis of Adenosine (Ado), Inosine (Ino) and Guanosine (Guo) was determined as follows. Nucleosides (100 µM) were incubated in the presence of the enzyme (40 nM) at 37 °C at varying pH conditions (pH range between 5.5 and 8.5). Reactions were carried out in a total volume of 150 µL phosphorolysis buffer.
(50 mM MOPS; 0.2 mM EDTA; 200 mM potassium phosphate). After 10 min, the reaction was terminated by heat-inactivation of the enzyme at 95 °C for 3 min. Next, the samples were rapidly cooled on ice for 15 min and cleared by centrifugation at 16,000 g for 15 min. Nucleobases were separated from nucleosides on a reverse phase RP-8 column (Merck, Darmstadt, Germany) and quantified by HPLC analysis (Aliance 2690, Waters, Milford, MA). UV-based detection was performed at 261 nm (Ado), 245 nm (Ino) or 253 nm (Guo).

The separation of Ado from adenine (Ade) was performed by HPLC using a linear gradient A [from 98 % buffer A (50 mM NaH2PO4 (Acros Organics, Geel, Belgium); 5 mM heptane sulfonylic acid; pH 3.2) and 2 % acetonitrile (BioSolve BV, Valkenswaard, the Netherlands) to 75 % buffer A and 25 % acetonitrile] as follows: 10 min 98 % buffer A + 2 % acetonitrile; 10 min linear gradient to 90 % buffer A + 10 % acetonitrile; 10 min linear gradient to 80 % buffer A + 20 % acetonitrile; 5 min linear gradient to 75 % buffer A + 25 % acetonitrile; 5 min linear gradient to 98 % buffer A + 2 % acetonitrile followed by 10 min equilibration at 98 % buffer A and 2 % acetonitrile.

The separation of Ino from hypoxanthine (Hx) and Guo from guanine (Gua) was performed by HPLC using a linear gradient B [from 100 % buffer A to 75 % buffer A and 25 % acetonitrile] as follows: 10 min linear gradient of 100 % buffer A to 98 % buffer A + 2 % acetonitrile; 10 min linear gradient to 90 % buffer A + 10 % acetonitrile; 5 min linear gradient to 75 % buffer A + 25 % acetonitrile; 5 min linear gradient to 100 % buffer A followed by equilibration at 100 % buffer A for 10 min.

**Determination of PNP<sub>Hyr</sub> substrate specificity**

To study the phosphorolysis of different nucleosides and nucleoside analogues by PNP<sub>Hyr</sub> and human PNP (ProSpec, Rehovot, Israel) different potential substrates (100 µM) were exposed to the enzyme (20 nM PNP<sub>Hyr</sub> or 6 nM human PNP) and incubated at 37 °C in
PNP buffer (50 mM MOPS; 0.2 mM EDTA; 200 mM potassium phosphate; pH = 6.5) in a total volume of 500 µL. At different time points (0, 20, 40 and 60 min), 100 µL-fractions were withdrawn, transferred and processed as described above. Nucleobases and nucleosides were separated by HPLC analysis as described above [Gradient A for Ado, dAdo and cladribine (2-CdA); Gradient B for the other nucleosides and nucleoside analogues] and for each product UV-based detection was performed at the specific wavelength of optimal absorption. The separation of 2-fluoroadenine-arabinofuranoside (fludarabine; F-ara-A), 6-methylpurine-2’-deoxyriboside, 2-fluoro-2’-deoxyadenosine, methyl(talo)-F-Ado and 6-mercaptopurine riboside from their respective nucleobases was performed on a reverse phase RP-18 column (Merck, Darmstadt, Germany) by gradient C [from 100 % buffer A to 60 % buffer A and 40 % acetonitrile] as follows: 20 min linear gradient of 100 % buffer A to 80 % buffer A + 20 % acetonitrile; 5 min linear gradient to 60 % buffer A + 40 % acetonitrile; 5 min 60 % buffer A + 40 % acetonitrile; 5 min linear gradient to 100 % buffer A followed by equilibration at 100 % buffer A for 10 min.

**Kinetic assays**

The enzymatic activity of PNP	extsubscript{Hyor} towards different substrates [Ado, Ino, Guo, dAdo, dIno, dGuo, 2-CdA, F-ara-A] was evaluated. The nucleoside-to-nucleobase conversion at varying concentrations of substrate ranging from 25 µM to 5 mM was studied in a reaction containing 2 or 20 nM enzyme (depending on the nucleoside conversion efficiency) incubated in PNP buffer containing 5 % DMSO (10 % DMSO for F-ara-A conversion) at 37 °C for 10 min. Kinetic parameters (K\textsubscript{m} and k\textsubscript{cat}) were determined by means of non-linear regression analysis (using GraphPad Prism5). In the kinetic assays where the enzymatic activity of PNP	extsubscript{Hyor} was evaluated at varying concentrations of P\textsubscript{i} (100 µM to 50 mM), the nucleoside
substrate was kept fixed at a concentration of ~ 10 x $K_m$. After incubation, samples were processed and analyzed by HPLC as described above.

**PNP inhibition assays**

In the assays where the inhibitory effect of the PNP inhibitors Imm-H and DADMe-Imm-H was evaluated, different inhibitor concentrations (ranging between 1 and 1,000 nM) were added to a reaction mixture that contained 100 µM substrate (Ado, Ino, Guo, 2-CdA or 2-F-ara-A) in PNP buffer containing 2 mM $P_i$ (reaction mixture for human PNP) or 200 mM $P_i$ (reaction mixture for PNP$_{Hyor}$). Next, the reaction mixture was exposed to human PNP (3 nM) or PNP$_{Hyor}$ (20 nM) and after 15 min incubation at 37 °C, the substrate degradation was determined by HPLC analysis as described above.
Results

Mycoplasma PNP alters the cytostatic activity of purine nucleoside analogues

Human breast carcinoma MCF-7 cell cultures were infected with *Mycoplasma hyorhinis* (designated MCF-7.Hyor) and used to study the effect of the mycoplasma infection on the antiproliferative activity of various anticancer purine nucleoside and nucleobase analogues.

Tumor cell cultures were exposed to varying concentrations of the anti-neoplastic purine nucleoside analogue 2-chloro-2'-deoxyadenosine (2-CdA; cladribine) [within the range of the actual levels reached in leukaemia cells of cancer patients (100-400 nM plasma levels at the end of drug injection period; 40-50 µM intracellular levels) (Liliemark *et al.*, 1997)]. As shown in Table 1, the cytostatic activity of 2-CdA (used for the treatment of various lymphoproliferative diseases), was decreased by 10-fold in MCF-7.Hyor tumor cell cultures (IC₅₀ = 13 µM) compared with mycoplasma-free control MCF-7 tumor cells (IC₅₀ = 1.3 µM). Using an MTS colorimetric assay, the 50% cytotoxic concentration (CC₅₀) of 2CdA was determined to be > 50 µM and 27 ± 0.07 µM in MCF-7 and MCF-7.Hyor cells, respectively (data not shown). Pretreatment of MCF-7.Hyor tumor cell cultures with tetracycline at 1 µg/mL for 6 days efficiently eliminated the mycoplasma-infection [undetectable by the MycoAlert™ mycoplasma detection kit (Lonza)] and fully restored the cytostatic activity of 2-CdA (IC₅₀ = 0.70 ± 0.11 µM) (data not shown). Also, the co-administration of DADMe-Immuclin-H (DADMe-Imm-H; 10 µM), a specific and potent PNP inhibitor (Evans *et al.*, 2003), rescued the antiproliferative activity of 2-CdA in MCF-7.Hyor cells (IC₅₀ = 0.69 µM) but did not affect its activity in non-infected MCF-7 control tumor cell cultures (IC₅₀ = 1.1 µM) (Table 1). Thus, these results suggest that a mycoplasma-encoded PNP, specifically expressed in MCF-7.Hyor cell cultures, efficiently catalyzes the phosphorolysis of 2-CdA.
resulting in the release of the free base 2-chloroadenine (2-CAde) which has a much lower
cytotatic potential. As displayed in Table 2, 2-CAde indeed exerts a ~10-fold lower
cytotatic activity compared with 2-CdA in non-infected control MCF-7 cell cultures (IC50 =
11 µM versus 1.3 µM, respectively). The cytostatic activity of 2-CAde is neither affected by
mycoplasma infection nor by co-administration of the potent PNP inhibitor DADMe-Imm-H
(Table 2).

*M. hyorhinis* infected and non-infected tumor cell cultures were also exposed to
varying concentrations of the adenosine analogue 2-fluoroadenine-arabinofuranoside (F-ara-
A; fludarabine) within the range of the actual levels reached in leukaemia cells of cancer
patients (~3 µM plasma levels at the end of the drug infusion; 20-60 µM intracellular levels)
(Gandhi and Plunkett, 2002). In contrast to 2-CdA, the antiproliferative activity of F-ara-A
was increased ~130-fold in MCF-7.Hyor cell cultures (IC50 = 0.30 µM) compared with non-
infected MCF-7 tumor cell cultures (IC50 = 39 µM) (Table 1). The CC50 of F-ara-A was
determined using an MTS colorimetric assay and showed to be > 50 µM and 4.8 ± 0.7 µM in
MCF-7 and MCF-7.Hyor cells, respectively (data not shown). F-ara-A is administered to
patients as the 5’-monophosphate for the treatment of haematological malignancies
(Galmarini et al., 2002). The increased cytostatic activity of F-ara-A in MCF-7.Hyor cell
cultures could again be reduced by (i) elimination of the mycoplasmas from the cell cultures
using tetracycline (resulting in an IC50 of 27 ± 16 µM) (data not shown) or (ii) the co-
administration of DADMe-Immucillin-H (resulting in an IC50 of 22 µM) (Table 1). In contrast
to 2-chloroadenine, 2-fluoroadenine (nucleobase of fludarabine; 2-FAde) proved ~ 280-fold
more cytostatic (IC50 = 0.14 µM) compared with its nucleoside in non-infected MCF-7 tumor
cell cultures (Table 2). These findings strongly suggest that a mycoplasma PNP-mediated
release of the highly cytostatic 2-FAde base from the parent F-ara-A is responsible for its
increased antiproliferative activity in mycoplasma-infected tumor cell cultures. Also a ~15-
fold increased cytostatic activity of other nucleoside analogues carrying 2-FAde as a purine base [i.e. 2-fluoro-2’deoxyadenosine and methyl(talo)-F-Ado] was observed in mycoplasma-infected cell cultures and could be reversed by the addition of DADMe-Imm-H (Table 1).

Similar to fludarabine, the cytostatic activity of 6-methylpurine-2’deoxyriboside (6-MeP-dR) was increased ~45-fold in MCF-7.Hyor tumor cell cultures compared with non-infected MCF-7 tumor cell cultures presumably due to the release of the highly cytostatic 6-methylpurine base (6-MeP) (Tables 1 & 2). Co-administration of DADMe-Imm-H annihilated the increased cytostatic activity of 6-MeP-dR in the mycoplasma-infected tumor cell cultures (Table 1). As shown in Table 2, the cytostatic activity of 6-methylpurine was unaffected by (i) mycoplasma infection and (ii) co-administration of DADMe-Imm-H.

A slightly (3-fold) decreased cytostatic activity of 6-mercaptopurine riboside and 6-thioguanosine was observed in MCF-7.Hyor cell cultures (IC₅₀ = 2.2 μM and IC₅₀ = 1.3 μM, respectively) compared with non-infected cell cultures (IC₅₀ = 0.75 μM and IC₅₀ = 0.44 μM, respectively). Co-administration of DADMe-Imm-H dramatically decreased the cytostatic activity of both molecules in the non-infected MCF-7 tumor cells, suggesting that human PNP-directed metabolism (and thus, free purine base formation) is required for these molecules to exert their biological activity. Co-administration of DADMe-Imm-H also markedly decreased the cytostatic activity of 6-thioguanosine in MCF-7.Hyor cells but this effect was not observed for 6-mercaptopurine riboside. As displayed in Table 2, 6-mercaptopurine and 6-thioguanine showed a cytostatic activity comparable to their riboside counterparts and were unaffected by mycoplasma infection. The antiproliferative activity of 6-mercaptopurine was somewhat compromised by the co-administration of DADMe-Imm-H in MCF-7 cell cultures (~ 6-fold) but not in MCF-7.Hyor cell cultures. In contrast, the cytostatic activity of 6-thioguanine was not affected by PNP inhibition in both non-infected and mycoplasma-infected tumor cell cultures.
The activities of other nucleoside analogues (i.e. 8-aminoguanosine, 5,6-dichlorobenzimidazole ribofuranoside, 2-amino-2'-deoxy-2'-fluoroadenosine and adenine arabinofuranoside) were also evaluated but were not affected by mycoplasma infection nor by DADMe-Imm-H administration (Table 1).

Metabolism of cladribine in mycoplasma-infected and uninfected cell cultures

The metabolism of 2-CdA to its 5'-triphosphate metabolite and subsequent incorporation in nucleic acids (DNA and RNA) was evaluated (Fig. 1). Since 2-CdA is phosphorylated by 2'-deoxycytidine kinase (dCK), we compared the metabolic conversion of [8-³H]-2-CdA in wild-type and dCK-deficient tumor cells both in the absence and presence of a mycoplasma infection. Since no dCK-deficient MCF-7 cells were available, the experiment was performed in human lymphocyte wild-type CEM/0 and dCK-deficient CEM/dCK⁻ cell cultures. In the non-infected CEM/0 cells, pronounced levels of the 5'-triphosphate metabolite and incorporated radiolabel in methanol-insoluble material (DNA, RNA) were detected, but only small amounts of 5'-triphosphate metabolite and incorporated label in methanol-insoluble material were detected in CEM/dCK⁻ cells. In contrast, substantial amounts of drug-derived radiolabeled 5'-triphosphate and incorporated radiolabel were found in mycoplasma-infected CEM.Hyor and CEM/dCK⁻.Hyor cells. It was assumed that in case of non-infected CEM/0 cells, the 5'-triphosphate of 2-CdA was formed, whereas in case of mycoplasma-infected CEM/0 and CEM/dCK⁻ cells, 2-CdA was first mainly converted to its free base 2-CAde prior to a predominant further eventual conversion to the 5'-triphosphate derivative of its riboside 2-chloro-adenosine (2CAdo) upon adenine phosphoribosyl transferase (APRT) and adenylate (AMP)/nucleoside diphosphate (NDP) kinase activity. The retention times of the radiolabeled 5'-triphosphate peaks derived from infected and non-infected CEM/0 and CEM/dCK⁻ tumor cells were nearly identical (data not shown). Since in the HPLC separation
system ATP and dATP eluted at nearly identical retention times, we assumed that the 5’-triphosphate of 2-CdA and 2-CAdo also virtually co-eluted on the anion-exchange column, and thus, we could not discriminate between both metabolites based on their retention times.

**Stability of cladribine in the medium of mycoplasma-infected and uninfected cell cultures**

Radiolabeled [8-³H]-2-CdA (2 µCi) was exposed to the cell-free medium of three day-old mycoplasma-infected and uninfected MCF-7 and CEM tumor cell cultures. Cells and cellular debris but not mycoplasmas were removed by a low-speed centrifugation step prior to the experiment. An efficient and time-dependent conversion of [8-³H]-2-CdA to its free base [8-³H]-2-CAdo was found in the supernatant of MCF-7.Hyor and CEM.Hyor cell cultures with virtually complete phosphorolysis being observed after 6 h and 1 h incubation, respectively (Fig. 2 A,B). In contrast, no degradation of [8-³H]-2-CdA could be observed when exposing the drug to the cell-free medium of uninfected MCF-7 or CEM/0 cell cultures (Fig. 2 A,B). Since the co-administration of Imm-H to the cell-free medium of CEM.Hyor cell cultures could markedly prevent the phosphorolysis of cladribine (data not shown), PNP activity is likely responsible for the efficient cladribine breakdown in mycoplasma-infected cells.

**Substrate selectivity and kinetic properties of PNP<sub>Hyor</sub>**

Due to the strong evidence that a mycoplasma-encoded PNP is responsible for the marked changes in cytostatic activity of several purine nucleoside analogues, including cladribine and fludarabine, we decided to clone and express the annotated PNP gene in the genome of *M. hyorhinis* HUB-1. The obtained enzyme was designated as PNP<sub>Hyor</sub> and found to catalyse not only phosphorolysis of inosine (Ino) and guanosine (Guo) but also adenosine
(Ado). All three purine nucleosides were found to be optimally phosphorolysed at slightly acidic conditions (pH = 6 - 6.5) (Fig. 3).

By exposing different purine nucleosides and nucleoside analogues to PNP_{Hyor} and human PNP in the presence of P_i (inorganic phosphate) the substrate selectivity of both enzymes could be determined and compared site-by-site (Table 3). An enzyme-catalyzed time-dependent conversion of (2’-deoxy)Ino and (2’-deoxy)Guo to their respective nucleobases (hypoxanthine and guanine) and phosphorylated sugars (2-deoxyribose-1-phosphate and ribose-1-phosphate) could be demonstrated for PNP_{Hyor} and human PNP. Phosphorolysis of (2’-deoxy)Ado was observed for PNP_{Hyor} only and not for human PNP. In contrast, xanthosine was found to be a substrate for human PNP but not for the prokaryotic PNP_{Hyor}.

Using non-linear regression analysis the kinetic parameters (K_m and k_cat) of PNP_{Hyor}-catalyzed nucleoside conversion were determined for different substrates and the phosphorolytic capacity (k_cat/K_m) was calculated as an estimate for the catalytic efficiency of the enzyme (Table 4). Ado was the preferred substrate for PNP_{Hyor} since Ado phosphorolysis [k_cat/K_m = 18 (min*µM)^{-1}] occurred almost twice as efficient as Ino [k_cat/K_m = 9.7 (min*µM)^{-1}] and four times as efficient as Guo phosphorolysis [k_cat/K_m = 4.4 (min*µM)^{-1}]. The phosphorolysis of 2’-deoxynucleosides proved less efficient compared with their riboside counterparts, and 2’-deoxyadenosine (dAdo) phosphorolysis [k_cat/K_m = 7.8 (min*µM)^{-1}] was found to be ~10 and ~3 times more efficient compared with 2’-deoxyinosine (dIno) [k_cat/K_m = 0.7(min*µM)^{-1}] and 2’-deoxyguanosine (dGuo) phosphorolysis [k_cat/K_m = 2.7 (min*µM)^{-1}], respectively.

Comparable catalytic efficiencies of PNP_{Hyor} with regard to inorganic phosphate were found when varying P_i concentrations were used in the presence of saturating concentrations of the different co-substrates (Ado, Ino, Guo, dAdo or dGuo) (Table 4). The experiments were
not performed for dIno because saturating conditions of this nucleoside could not be obtained due to its poor solubility in the reaction mixture.

Next, several purine-based nucleoside analogues with reported cytostatic or antiviral activity were evaluated as potential substrates for PNP_{Hyor} and human PNP (Table 3). Cladribine was efficiently converted into 2-CAde by PNP_{Hyor}. This reaction occurred with a catalytic efficiency \([K_m = 80 \mu M; k_{cat}/K_m = 5.7 \text{ (min}^\ast\mu M)^{-1}]\) comparable to dAdo phosphorolysis. Also PNP_{Hyor}-mediated phosphorolysis of fludarabine was observed although the catalytic efficiency of this reaction (\(K_m = 1120 \mu M; k_{cat}/K_m = 0.2 \text{ (min}^\ast\mu M)^{-1}\)) was much lower compared with Ado or dAdo phosphorolysis. No conversion of cladribine or fludarabine was observed when exposing this drug to human PNP. Similarly, 2-FAde formation could be confirmed when exposing 2-fluoro-2′-deoxyadenosine or methyl(talo)-F-Ado to PNP_{Hyor} but not when exposing these compounds to human PNP. Also 6-methylpurine-2′-deoxyriboside, lacking measurable conversion by human PNP, was efficiently converted into the highly toxic 6-MeP base by PNP_{Hyor}.

In contrast, 6-mercaptopurine-riboside, 6-thioguanosine, 8-aminoguanosine [a well-known PNP inhibitor (Kazmers et al., 1981)] and the RNA synthesis inhibitor 5,6-dichlorobenzimidazole ribofuranoside (Chodosh et al., 1989), were found to be substrates for both human PNP and PNP_{Hyor}. PNP_{Hyor}-catalyzed phosphorolysis of 2-amino-2′-deoxy-2′fluoroadenosine and the antiherpetic adenine-arabinofuranoside (ara-A; vidarabine; Pavan-Langston and Buchanan, 1976) (a very poor substrate) was observed but both compounds were not accepted as substrates by human PNP.

As displayed in Table 3, the following relevant purine nucleoside analogues were tested and found to be no substrate for PNP_{Hyor} or human PNP: clofarabine, used in the treatment of relapsed or refractory lymphoblastic leukaemia in pediatric patients (Hijiya et al., 2012); 7-deaza-adenosine (Tubercidin), an adenosine analogue with cytotoxic (Acs et al.,
Inhibitor studies

To explain the restoring effect of a PNP inhibitor on the compromised cytostatic activity of the purine nucleoside analogues in the mycoplasma-infected tumor cell cultures, we examined the susceptibility of PNP\textsubscript{Hyor} to two well-studied transition state analogue inhibitors of PNP: Immucillin-H (Imm-H) and DADMe-Imm-H. These compounds are studied in phase II clinical for their use in the treatment of T-cell malignancies and gout, respectively (Balakrishnan \textit{et al.}, 2010; Schramm, 2013). We compared the capacity of the inhibitors to block the catalytic activity of PNP\textsubscript{Hyor} and human PNP. As shown in Table 5, both compounds efficiently inhibited human PNP-catalyzed Ino and Guo phosphorolysis in the low nanomolar range. PNP\textsubscript{Hyor} was also found to be highly susceptible to inhibition by both compounds. Imm-H efficiently blocked PNP\textsubscript{Hyor}-catalyzed Ino and Guo phosphorolysis (IC\textsubscript{50} = 69 nM and 76 nM, respectively). In addition, the PNP\textsubscript{Hyor}-catalysed phosphorolysis of Ado, which is not a substrate for human PNP, was also efficiently prevented (IC\textsubscript{50} = 90 nM). The PNP\textsubscript{Hyor}-mediated phosphorolysis of cladribine and fludarabine, both not recognized as substrate for human PNP, was also inhibited by Imm-H (IC\textsubscript{50} = 405 nM and 60 nM, respectively). DADMe-Imm-H proved to be more efficient than Imm-H in inhibiting PNP\textsubscript{Hyor}-catalyzed breakdown of Ado, Ino and Guo (IC\textsubscript{50} = 18, 8.4 nM and 24 nM, respectively). DADMe-Imm-H also efficiently inhibited the PNP\textsubscript{Hyor}-mediated breakdown of cladribine and fludarabine at low nanomolar concentrations (IC\textsubscript{50} = 45 nM and 40 nM, respectively).
Discussion

Mycoplasmas are common cell culture contaminants, causing infections that often remain unnoticed. Previously, we described the inactivation of several cytostatic/antiviral pyrimidine nucleoside analogues in mycoplasma-infected tumor cell cultures due to the expression of a broad-spectrum mycoplasma-encoded pyrimidine nucleoside phosphorylase (PyNP) (Bronckaers et al., 2008; Vande Voorde et al., 2012). We now show that the presence of mycoplasmas in tumor cell cultures also severely affects the antiproliferative effect of several purine-based nucleoside analogues provided that they are a substrate for mycoplasma PNP but not for human PNP. Depending on the properties of the purine nucleobase, mycoplasma infection of the tumor cell cultures resulted in a significantly increased or decreased cytostatic activity of these drugs, which could be restored by (i) suppression/elimination of the mycoplasmas by antibiotics or (ii) administration of DADMe-Imm-H, a potent and selective PNP inhibitor. These observations support the view that PNP$_{Hyor}$ plays an instrumental role in the altered cytostatic potential of a variety of purine nucleoside analogues. Since mycoplasmas (Chan et al., 1996; Kidder et al., 1998; Huang et al., 2001; Pehlivan et al., 2004, 2005; Yang et al., 2010; Apostolou et al., 2011; Urbanek et al., 2011; Barykova et al., 2011; Erturhan et al., 2013) and other prokaryotes (reviewed by Mager, 2006; Cummins and Tangney, 2013) have been found to preferentially associate with tumor cell tissue in cancer patients, our findings may be of relevance for cancer chemotherapy and may argue for a personalized treatment schedule based on the microbiotic tumor environment. A drug administration protocol combining certain anticancer purine derivative drugs with a PNP inhibitor could therefore increase therapeutic efficiency. Alternatively, the administration of a mycoplasma-targeting antibiotic, prior to cancer therapy, to eliminate the mycoplasma infection could be considered. Given the potential role of mycoplasma-encoded
PNP in the altered cytostatic activity of several purine nucleoside analogues, we decided to characterize this enzyme more in detail.

Whereas mammalian PNP did not catalyze the conversion of (2‘-deoxy)adenosine [(d)Ado] to adenine, PNP\textsubscript{Hyor} was found to accept not only the (deoxy)nucleoside derivatives of 6-oxopurines (hypoxanthine and guanine) but also of 6-aminopurines (adenine). Based on our kinetic studies, it could even be concluded that Ado is the preferred substrate of PNP\textsubscript{Hyor}. Phosphorolysis proceeded optimally under slightly acidic conditions as has also been reported before for other prokaryotic and mammalian PNPs (Bzowska et al., 2000). We found that, alike human PNP, PNP\textsubscript{Hyor} was efficiently inhibited by the well-known PNP transition state inhibitors Imm-H and DADMe-Imm-H.

We could demonstrate a ~10-fold decreased cytostatic activity of the anti-neoplastic purine nucleoside analogue cladribine (2-CdA) in mycoplasma-infected tumor cell cultures compared with uninfected control cultures. This drug is used for the treatment of various lymphoproliferative diseases and cannot be catabolized by human PNP (Bzowska and Kazimierczuk, 1995; our observations). After cellular uptake, 2-CdA is phosphorylated by deoxycytidine kinase (dCK; EC 2.7.1.74) to 2-chloro-2’-deoxy-AMP and then further converted into 2-chloro-2’-deoxy-ADP and 2-chloro-2’-deoxy-ATP (Beutler, 1992). The latter is a good substrate for DNA polymerases, competes with intracellular dATP for incorporation in the cellular DNA and ultimately induces apoptosis. Furthermore, a self-potententiating effect of cladribine occurs due to inhibition of ribonucleotide reductase by 2-chloro-2’-deoxy-ATP, thereby depleting intracellular dNTP pools (Beutler, 1992; Parker, 2009). As a result, 2-chloro-2’-deoxy-ATP incorporation in cellular DNA is favoured due to the lower levels of the natural substrate dATP. Using the recombinant enzyme, we here show that 2-CdA is indeed an excellent substrate for PNP\textsubscript{Hyor}-catalyzed phosphorolysis, resulting in the release of the free nucleobase 2-chloro-adenine (2-CAde), which has a lower cytostatic
potential than 2-CdA. We were able to confirm the time-dependent phosphorolysis of radiolabeled [8-³H]-2-CdA in the cell-free supernatant of mycoplasma-infected MCF-7 cell cultures, an activity that was absent in uninfected control cell cultures. As fast as 1 h post incubation of the compound, we observed virtual complete conversion of [8-³H]-2-CdA into [8-³H]-2-CAde. Upon cellular uptake, 2-CAde is most likely phosphoribosylated by adenine phosphoribosyltransferase (APRT; EC 2.4.2.7) ultimately leading to accumulation of 2-chloro-ribo-ATP, a substrate for RNA incorporation (Bontemps et al., 2000). Evidence for the formation of 2-chloro-2’-deoxy-ATP in uninfected control cells versus 2-chloro-ribo-ATP in mycoplasma-infected cells was provided by studying the metabolism of [8-³H]-2-CdA in wild-type and dCK-deficient CEM cell cultures in the presence or absence of mycoplasmas. Indeed, incorporated radiolabel derived from [8-³H]-2-CdA was markedly found in the nucleic acids of uninfected CEM/0 cells but not in CEM.dCK cells since 2-CdA requires dCK activity for its eventual metabolic conversion to its 5’-triphosphate metabolite. In contrast, substantial radiolabel was found in the nucleic acids of both CEM/0 and CEM.dCK cells upon mycoplasma-infection. These results strongly indicate the formation and RNA incorporation of 2-chloro-ribo-ATP in the mycoplasma-infected cells and explain the less efficient inhibition of mycoplasma-infected tumor cell proliferation by 2-CdA, compared with non-infected tumor cell cultures (Fig. 4).

Likewise, we demonstrated that the prokaryotic PNP_Hyor cleaves the relatively nontoxic adenosine analogues [i.e. fludarabine (F-ara-A), 2-fluoro-2’-deoxyadenosine and 6-methylpurine-2’-deoxyriboside (6-MeP-dR)] to release highly toxic adenine analogues [i.e. 2-fluoroadenine (2-FAde) and 6-methylpurine (6-MeP)], which explains the markedly increased cytostatic potential of these drugs in mycoplasma-infected tumor cell cultures. Mycoplasma-induced cytotoxicity of 6-MeP-dR due to phosphorolysis was already earlier proposed as a detection method for the presence of mycoplasmas in cell cultures in a study by McGarrity...
and Carson (1982). In contrast to 2-CAde (the free base of cladribine), 2-FAde and 6-MeP are highly toxic after APRT-mediated intracellular metabolism (Parker et al., 1998). F-ara-A, 2-fluoro-2′-deoxyadenosine and 6-MeP-dR were confirmed to be no substrate for human PNP and therefore display an increased cytostatic activity in mycoplasma-infected tumor cell cultures compared with non-infected cell cultures. Thus, relatively non-toxic adenosine analogues carrying a toxic base component may gain therapeutic antitumor selectivity when used to treat tumors that are associated with prokaryotes such as mycoplasmas showing efficient adenosine phosphorylase activity.

The introduction of prokaryotic (E. coli) PNP into human tumors has been explored as a selective antitumor suicide gene therapy approach (Zhang et al., 2005). Low level expression of E. coli PNP (< 1% of transfected tumor cells) was shown to lead to pronounced bystander killing of non-transfected tumor cells due to diffusion of 6-MeP, released from 6-MeP-dR, to adjacent tissue. A marked anticancer activity has been observed using this approach in a variety of tumors (reviewed by Ardiani et al., 2012). In this respect we believe that mycoplasma PNPs should be added to the suicide gene armamentarium to be considered as potential gene constructs useful for specific cancer gene therapy. In search of an ideal PNP/prodrug combination, studies have also explored the possibility to generate rationally engineered prokaryotic PNPs that display a higher affinity for the newly-designed non-toxic purine-based analogues. Parker et al. (2005) reported an increased phosphorolysis of 5′-modified nucleoside analogues by the E. coli PNP mutant (M64V) compared with wild-type E. coli PNP. Molecules such as 9-[6-deoxy-α-L-talofuranosyl]-6-methylpurine (methyl(talo)-MeP-R) and 9-[6-deoxy-α-L-talofuranosyl]-2-F-adenine [methyl(talo)-F-Ado] were shown to release their cytotoxic bases more efficiently in the presence of the mutated PNP. We observed an increased cytostatic activity of Me(talo)-F-Ado in mycoplasma-infected cells and identified this purine nucleoside analogue as a PNP_{Hyor} substrate. In contrast, PNP_{Hyor} did not
catalyze the release of 6-MeP from Me(talo)-MeP-R and, accordingly, the cytostatic activity of this molecule remained unchanged in mycoplasma-infected cell cultures (data not shown). Therefore, a comparative study between human PNP, different prokaryotic PNPs and mycoplasma PNPs such as PNP_{Hyor} might lead to the identification of molecules that are selectively activated in the presence of mycoplasma-encoded PNPs.

Our findings also revealed that co-culturing well-defined mycoplasmas with mammalian cell lines represents a convenient and useful model to study the effect of these, often commensal, prokaryotes on the chemotherapeutic treatment of cancer cells. Our in vitro models show selective mycoplasma-mediated catabolism of nucleoside-based drugs which may be prevented by the administration of a specific enzyme inhibitor (or antibiotic). Since a preferential colonization of human tumors by bacteria (including mycoplasmas) has been suggested in several studies, investigating the effect of such prokaryotes on the biological activity of nucleoside analogues may ultimately optimize current cancer chemotherapy. Our results therefore argue for a careful clinical investigation of this phenomenon. Although we showed a markedly decreased activity of cladribine in mycoplasma-infected cell cultures, the mycoplasma presence has only been studied in solid tumors and their relation (if any) with haematological malignancies remains to be elucidated. Additionally, very little information is available on the extent of a mycoplasma infection in tumors (i.e. actual numbers present at the tumor site). A single study has determined the amount of Mycoplasma hominis 16s rRNA present in prostate cancer tissues by quantitative real-time PCR and reported to be \( \sim 9.1 \times 10^5 \) copies/g tissue (Barykova et al., 2011), but it is unclear whether this also applies to an infection of different mycoplasmas (e.g. M. hyorhinis) in other tumors. Nevertheless, a rational choice for drugs that display an increased cytostatic activity after metabolism by mycoplasma-encoded enzymes present in the local environment of a tumor could result in a more pronounced therapeutic selectivity. A mycoplasma infection may thus increase the
therapeutic index of drugs such as fludarabine (which is currently used for the treatment of haematological malignancies) towards solid tumors. A pharmacometabonomic approach of cancer therapy could therefore address inter-individual responses to nucleoside analogue-based anticancer treatment and may possibly improve drug efficacy and reduce adverse drug reactions.
Acknowledgments

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Authorship Contributions

Participated in research design: Vande Voorde, Liekens and Balzarini

Conducted experiments: Vande Voorde

Performed data analysis: Vande Voorde, Liekens and Balzarini

Wrote or contributed to the writing of the manuscript: Vande Voorde, Liekens and Balzarini
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Footnotes

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Figure Legends

Figure 1: Conversion of [8-³H]-2-CdA into the 5’-triphosphate metabolite of [8-³H]-2-CdA or [8-³H]-2-CAdo and subsequent incorporation into methanol-insoluble macromolecules (RNA/DNA).

Radiolabel in the fractions 24-26 of the HPLC chromatogram is derived from [8-³H]-2-CdA and/or [8-³H]-2-CAdo upon prior phosphorolysis to [8-³H]-2-CAde by PNP_Hyor.

Figure 2: Cladribine stability in the medium of M.hyorhinis-infected and non-infected MCF-7 and CEM cells.

Time-dependent phosphorolysis of [8-³H]-2-CdA, incubated in the cell-free supernatant of MCF-7 and MCF-7.Hyor cell cultures (A) or CEM/0 and CEM.Hyor cell cultures (B).

Data are the mean of at least 2 independent experiments ± S.D.

Figure 3: pH dependence of PNP_Hyor-mediated phosphorolysis

pH-dependent Adenosine (A), Inosine (B) and Guanosine (C) degradation after 10 min incubation of the substrate (100 µM) in the presence of PNP_Hyor

Data are the mean of 2 independent experiments ± S.D.

Figure 4: Proposed metabolic fate of 2-CdA in M.hyorhinis–infected and non-infected tumor cell cultures

In non-infected cell cultures, 2-CdA is taken up and converted by dCK and AMP kinase/NDP kinase to its 5’-triphosphate prior to incorporation into DNA. In mycoplasma-infected cell cultures, a substantial amount of 2-CdA is already converted by PNP_Hyor to its free base 2-CAde prior to uptake. Once inside the cell, APRT can anabolize 2-CAde to 2-CAMP which is further converted by nucleoside kinases to its 5’-triphosphate prior to incorporation into RNA.
Tables

Table 1: Cytostatic activity of purine nucleoside analogues in MCF-7 and MCF-7.Hyor cells in the absence or presence of the selective PNP inhibitor DADMe-Imm-H

Results are the means ± SD of at least two independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀μM (MCF-7/0)</th>
<th>IC₅₀μM (MCF-7.Hyor)</th>
</tr>
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<tbody>
<tr>
<td>2-chloro-2’-deoxyadenosine (Cladribine)</td>
<td>1.3 ± 0.4</td>
<td>1.1 ± 0.3</td>
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<tr>
<td>2-fluoroadenine-arabinofuranoside (Fludarabine)</td>
<td>39 ± 12</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>2-fluoro-2’-deoxyadenosine</td>
<td>2.9 ± 0.6</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>methyl(talo)-F-Ado</td>
<td>46 ± 24</td>
<td>78 ± 9</td>
</tr>
<tr>
<td>6-methylpurine-2’-deoxyriboside</td>
<td>55 ± 5</td>
<td>57 ± 10</td>
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<tr>
<td>6-mercaptopurine-riboside</td>
<td>0.75 ± 0.22</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>6-thioguanosine</td>
<td>0.44 ± 0.21</td>
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<tr>
<td>8-aminoguanosine</td>
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<tr>
<td>5,6-dichlorobenzimidazole ribofuranoside</td>
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<td>12 ± 3</td>
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<tr>
<td>2-amino-2’-deoxy-2’-fluoro-adenosine</td>
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<td>&gt; 100</td>
</tr>
<tr>
<td>adenine-arabinofuranoside</td>
<td>&gt; 100</td>
<td>-</td>
</tr>
</tbody>
</table>

a 50% inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%

- not determined
Table 2: Cytostatic activity of purine nucleobase analogues in MCF-7 and MCF-7.Hyor cells in the absence or presence of the PNP inhibitor DADMe-Imm-H

Results are the mean ± SD of at least two independent experiments.

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<thead>
<tr>
<th></th>
<th>IC₅₀ᵃ (µM)</th>
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<th>IC₅₀ᵃ (µM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MCF-7</td>
<td>+ DADMe-Imm-H</td>
<td>MCF-7.Hyor</td>
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<td>2-chloroadenine</td>
<td>11 ± 1</td>
<td>14 ± 3</td>
<td>17 ± 6</td>
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<td>2-fluoroadenine</td>
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<td>6-thioguanine</td>
<td>1.0 ± 0.1</td>
<td>3.5 ± 1.9</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

ᵃ 50% inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%
Table 3: Substrate specificity of PNP<sub>Hyor</sub> and human PNP for natural purine nucleosides and nucleoside analogues

<table>
<thead>
<tr>
<th>Natural purine nucleosides</th>
<th>Substrate for PNP&lt;sub&gt;Hyor&lt;/sub&gt;</th>
<th>Substrate for human PNP</th>
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</thead>
<tbody>
<tr>
<td>adenosine</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>inosine</td>
<td>+</td>
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<tr>
<td>guanosine</td>
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<td>+</td>
</tr>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2’-deoxyinosine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2’-deoxyguanosine</td>
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<td>+</td>
</tr>
<tr>
<td>xanthosine</td>
<td>-</td>
<td>+</td>
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<table>
<thead>
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<th>Purine nucleoside analogues</th>
<th>Substrate for PNP&lt;sub&gt;Hyor&lt;/sub&gt;</th>
<th>Substrate for human PNP</th>
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<td>2-chloro-2’-deoxyadenosine (cladribine)</td>
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<td>-</td>
</tr>
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</tr>
<tr>
<td>methyl(talo)-F-Ado</td>
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</tr>
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<td>6-methylpurine-2’-deoxyriboside (6-MeP-dR)</td>
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</tr>
<tr>
<td>6-mercaptopurine riboside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6-thioguanosine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8-aminoguanosine</td>
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<td>+</td>
</tr>
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<td>5,6-dichlorobenzimidazole ribofuranoside</td>
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</tr>
<tr>
<td>adenine arabinofuranoside (araA)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2-amino-2’-deoxy-2’-fluoroadenosine</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>clofarabine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>tubercidin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7-deaza-2’-deoxyadenosine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3-deaza-adenosine</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+: substrate activity detected  
- : no substrate activity detected
Table 4: Kinetic parameters of PNP_Hyor

The $K_m$ and $k_{cat}$ values ± SD for the natural substrates of PNP_Hyor, for 2-chloro-2’-deoxyadenosine (cladribine) and 2-fluoroadenine-arabinofuranoside (fludarabine), and for P_i were determined using non-linear regression analysis (using GraphPad Prism 5) from data obtained in at least two independent experiments.

<table>
<thead>
<tr>
<th>Substrate Type</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (min*µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Natural nucleoside substrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adenosine</td>
<td>61 ± 4</td>
<td>1099 ± 62</td>
<td>18</td>
</tr>
<tr>
<td>inosine</td>
<td>283 ± 7</td>
<td>2757 ± 263</td>
<td>9.7</td>
</tr>
<tr>
<td>guanosine</td>
<td>405 ± 135</td>
<td>1777 ± 419</td>
<td>4.4</td>
</tr>
<tr>
<td><strong>Natural 2’-deoxy nucleoside substrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2’-deoxyadenosine</td>
<td>47 ± 2</td>
<td>365 ± 1</td>
<td>7.8</td>
</tr>
<tr>
<td>2’-deoxyinosine</td>
<td>2287 ± 455</td>
<td>1502 ± 213</td>
<td>0.7</td>
</tr>
<tr>
<td>2’-deoxyguanosine</td>
<td>315 ± 46</td>
<td>832 ± 22</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Nucleoside analogues</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-chloro-2’-deoxyadenosine (cladribine)</td>
<td>80 ± 9</td>
<td>457 ± 59</td>
<td>5.7</td>
</tr>
<tr>
<td>2-fluoroadenine-arabinofuranoside (fludarabine)</td>
<td>1120 ± 98</td>
<td>251 ± 131</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Inorganic phosphate P_i</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>co-substrate adenosine</td>
<td>672 ± 123</td>
<td>1134 ± 292</td>
<td>1.7</td>
</tr>
<tr>
<td>co-substrate inosine</td>
<td>978 ± 51</td>
<td>2793 ± 797</td>
<td>2.9</td>
</tr>
<tr>
<td>co-substrate guanosine</td>
<td>1252 ± 645</td>
<td>1101 ± 123</td>
<td>0.9</td>
</tr>
<tr>
<td>co-substrate 2’-deoxyadenosine</td>
<td>180 ± 10</td>
<td>487 ± 106</td>
<td>2.7</td>
</tr>
<tr>
<td>co-substrate 2’-deoxyguanosine</td>
<td>1573 ± 207</td>
<td>1209 ± 4</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Table 5: Inhibition of PNP<sub>Hyor</sub> and human PNP by Immucillin-H and DADMe-Immucillin-H

Data are the mean ± SD of at least two independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Immucillin-H</th>
<th>DADMe-Immucillin-H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PNP&lt;sub&gt;Hyor&lt;/sub&gt;</td>
<td>Human PNP</td>
</tr>
<tr>
<td>Adenosine</td>
<td>90 ± 10</td>
<td>-</td>
</tr>
<tr>
<td>Inosine</td>
<td>69 ± 1</td>
<td>9.0 ± 1.7</td>
</tr>
<tr>
<td>Guanosine</td>
<td>76 ± 0.3</td>
<td>32 ± 14</td>
</tr>
<tr>
<td>2-chloro-2'-deoxyadenosine (cladribine)</td>
<td>405 ± 21</td>
<td>-</td>
</tr>
<tr>
<td>2-fluoroadenine-arabinofuranoside (fludarabine)</td>
<td>60 ± 13</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inhibitor concentration needed to reduce the enzymatic phosphorolysis by 50 %

- : no substrate
Figure 2
Figure 3
Figure 4

Tumor cell cultures

2-CdA

\[ \downarrow \]

2-CdA
dCK

\[ \downarrow \]

2-CdAMP
dCK

\[ \downarrow \]

2-CdADP

\[ \downarrow \]

2-CdATP

DNA pol

DNA
(high cytostatic event)

Cell membrane

Tumor cell cultures infected with *M. hyorhinis*

2-CdA

\[ \xrightarrow{PNP_{Hyor}} \]

2-CdA

\[ \downarrow \]

2-CdA

\[ \downarrow \]

2-CdAMP

\[ \downarrow \]

2-CdADP

\[ \downarrow \]

2-CdATP

DNA pol

DNA
(high cytostatic event)

2-CAde

\[ \downarrow \]

2-CAdde

APRT

2-CAMP

\[ \downarrow \]

2-CADP

RNA pol

2-CATP

RNA
(poor cytostatic event)