FEBS Open Bio 5 (2015) 634–639

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

Mycoplasma hyorhinis-encoded cytidine deaminase efficiently inactivates cytosine-based anticancer drugs

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ARTICLE INFO

Article history:
Received 8 July 2015
Revised 24 July 2015
Accepted 30 July 2015

Keywords:
Mycoplasma
Cytidine deaminase
Cancer
Nucleoside analogue
Purine nucleoside phosphorylase
Gemcitabine

ABSTRACT

Mycoplasmas may colonize tumor tissue in patients. The cytostatic activity of gemcitabine was dramatically decreased in *Mycoplasma hyorhinis*-infected tumor cell cultures compared with non-infected tumor cell cultures. This mycoplasma-driven drug deamination could be prevented by exogenous administration of the cytidine deaminase (CDA) inhibitor tetrahydrouridine, but also by the natural nucleosides or by a purine nucleoside phosphorylase inhibitor. The *M. hyorhinis*-encoded CDA_{Hyor} gene was cloned, expressed as a recombinant protein and purified. CDA_{Hyor} was found to be more catalytically active than its human equivalent and efficiently deaminates (inactivates) cytosine-based anticancer drugs. CDA_{Hyor} expression at the tumor site may result in selective drug inactivation and suboptimal therapeutic efficiency.

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1. Introduction

Mycoplasmas are considered to be the smallest self-replicating organisms, both in dimension and genome size [1]. They often lack genes that are crucial for different synthetic pathways, including the *de novo* synthesis of purine and pyrimidine bases [2,3]. Therefore mycoplasmas rely on their host tissue from which they scavenge and recycle DNA/RNA precursors using various nucleo(s)(t)ide transporters and salvage enzymes [2,4,5]. Recently, we and others showed that certain catabolic mycoplasma enzymes (i.e. pyrimidine nucleoside phosphorylase, purine nucleoside phosphorylase and cytidine deaminase) interfere with the biological (i.e. cytostatic and antiviral) activity of different therapeutic nucleoside analogues (NAs) by producing less active or inactive drug metabolites. This was demonstrated for both pyrimidine- and purine-derived antimetabolites including gemcitabine, floxuridine, trifluridine, cladribine, and others [6–10]. There have been several reports that mycoplasmas have been shown to preferentially colonize tumor tissue in patients [11–20]. If this phenomenon can be broadly confirmed and since nucleoside-derived drugs are established cornerstones in the chemotherapy of several cancers [21], the presence of such prokaryotes in the tumor microenvironment may be a confounding factor for the efficiency of anticancer nucleoside analogues and of importance for optimization of nucleoside-based cancer treatment [22,23].

Recently, we reported efficient CDA-catalyzed deamination of gemcitabine (2′,2′-difluoro-2′-deoxycytidine; dFdC) resulting in a dramatically decreased cytostatic activity (up to 60-fold) of this drug in different *Mycoplasma hyorhinis*-infected tumor cell cultures [10]. Similarly, the response of *M. hyorhinis*-infected tumor xenografts in mice to gemcitabine treatment was significantly lower compared with uninfected control tumors [10]. The biological function of CDA is to catalyze the irreversible deamination of the natural pyrimidine nucleosides cytidine (Cyd) and 2′-deoxycytidine (dCyd) to uridine (Urd) and 2′-deoxyuridine (dUrd), respectively [24]. However, several clinical anticancer (d)Cyd analogues, including gemcitabine and cytarabine (cytosine arabinoside; ara-Cyd) (Fig. 1), can be catabolized by (cellular) drug deamination producing the corresponding, less active, (2′-deoxy)uridine metabolites. These molecules therefore show a decreased cytostatic activity in CDA-overexpressing tumor cells [25,26]. In the present study we biochemically and kinetically characterized *M. hyorhinis*-encoded CDA and report on a surprising interaction between mycoplasma CDA and purine nucleoside phosphorylase (PNP) activity in mycoplasma-infected tumor cells.

Abbreviations: 3TC, 2′,3′-dideoxy-3′-thiacytidine; ara-Cyd, cytosine arabinoside; CDA, cytidine deaminase; (d)Ado, (2′-deoxy)adenosine; (d)Guo, (2′-deoxy)guanosine; (d)Ino, (2′-deoxy)inosine; (d)Urd, (2′-deoxy)uridine; ddC, 2′,3′-dideoxycytidine; dThd, thymidine; dFdC, gemcitabine; dFdU, 2′,2′-difluoro-2′-deoxyuridine; Imm-H, Immucillin-H; NA, nucleoside analogue; PNP, purine nucleoside phosphorylase

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http://dx.doi.org/10.1016/j.fob.2015.07.007
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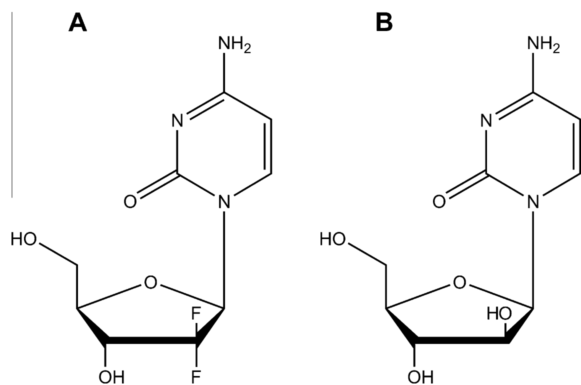

Fig. 1. Molecular structure of the (2′-deoxy)cytidine analogues gemcitabine (A) and cytarabine (B).

2. Materials and methods

2.1. Chemicals

Nucleosides, nucleoside analogues and inorganic agents were purchased from Sigma–Aldrich (St-Louis, MO) unless stated differently. Gemcitabine (2′,2′-difluoro-2′-deoxycytidine; dFdC) was purchased from Carbosynth (Berkshire, UK). Radioactive [5-^{3}H]-gemcitabine ([5-^{3}H]-dFdC) (radiospecificity: 12 Ci/mmol) was obtained from Moravek Biochemicals Inc. (Brea, CA). Immucillin-H (Imm-H) was kindly provided by Dr. V. Schramm (Albert Einstein College of Medicine, Bronx, NY).

2.2. Cell cultures

Human breast carcinoma MDA-MB-231 cells and *M. hyorhinis* were obtained from the American Tissue Culture Collection (Rockville, MD). Human breast carcinoma MCF-7 cells were kindly provided by Prof. G.J. Peters (Amsterdam, The Netherlands). Cells were infected with *M. hyorhinis* and 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 to four days after subculturing the mycoplasma-exposed cells. Chronically *M. hyorhinis*-infected tumor cells are further referred to as MDA-MB-231.Hyor and MCF-7.Hyor. All cells were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented 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 CO_2-controlled incubator.

2.3. Biological assays

The cytostatic activity of dFdC (gemcitabine) was compared in mycoplasma-infected and uninfected tumor cells. MDA-MB-231 and MDA-MB-231.Hyor cells were seeded in 48-well plates (Nunc™, Roskilde, Denmark) at 10,000 cells/well. After 24 h, an equal volume of fresh medium containing gemcitabine [in the presence or absence of natural purine nucleosides (100 μM) or the PNP inhibitor 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, Suarlée, Belgium). The 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce tumor cell proliferation by 50%.

2.4. Gemcitabine stability in the supernatant of mycoplasma-infected and uninfected cell cultures

The stability of gemcitabine in spent cell-free but mycoplasma-containing culture medium of confluent MDA-MB-231, MDA-MB-231.Hyor, MCF-7 and MCF-7.Hyor tumor cells was evaluated. Tumor cells were seeded in 75 cm^2 culture flasks (TTP, Trasadingen, Switzerland). After five days, supernatant was withdrawn and cleared by centrifugation at 300*g* for 6 min to remove (debris of) the tumor cells. Reactions were performed in a final volume of 300 μL containing dFdC (5 μM), [5-^{3}H]dFdC (1 μCi), different concentrations of thymidine (dThd), uridine (Urd), adenosine (Ado) or inosine (Ino) and 240 μL spent culture medium. Samples were incubated at 37 °C and after 60 min incubation, 100 μL was withdrawn and ice-cold MeOH was added to a final concentration of 66% MeOH to terminate the enzymatic reactions and to precipitate (remove) macromolecules such as DNA, RNA and proteins. Samples were kept on ice for 10 min and cleared by centrifugation at 16,000*g* for 15 min. The supernatants were withdrawn and analyzed on a reverse phase RP-8 column (Merck, Darmstadt, Germany) using HPLC (Alliance 2690, Waters, Milford, MA). The following gradient (further referred to as gradient A) was used: 10 min linear gradient of 100% buffer A [50 mM NaH_2PO_4 (Acros Organics, Geel, Belgium); 5 mM heptane sulfonic acid; pH 3.2] to 98% buffer A + 2% acetonitrile (BioSolve BV, Valkenswaard, the Netherlands); 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 10 min equilibration at 100% buffer A. Fractions of 1 mL were collected, transferred to 9 mL OptiPhase HiSafe 3 and radioactivity was counted in a liquid scintillation analyzer.

2.5. Purification of M. hyorhinis CDA (CDA_{Hyor})

A codon-optimized DNA sequence encoding the *M. hyorhinis* cytidine deaminase (CDA_{Hyor}) was synthetically assembled between the EcoRI and NotI restriction sites of a pIDTsmart vector (Integrated DNA technologies, Coralville, IO). The fragment was subsequently subcloned between the EcoRI and NotI sites of the pGEX-5X-1 bacterial expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden) and CDA_{Hyor} was expressed in *Escherichia coli* as a GST-fusion protein (hereafter referred to as CDA_{Hyor}) according to a procedure previously described by Liekens et al. [27]. SDS–PAGE revealed that the protein was of expected size (∼38–40 kDa) and purity (⩾95%) (Fig. 2). Since *E. coli* CDA consists of 294 amino acids, it would be characterized by a molecular weight of around 31 kDa [28]. Therefore, the contaminating protein bands shown in Fig. 2 are not likely related to *E. coli*-encoded CDA.

2.6. Enzyme assays

2.6.1. Determination of the substrate specificity of CDA_{Hyor} and CDA_{Human}

To study the deamination of different nucleosides and nucleoside analogues by CDA_{Hyor} and CDA_{Human} (ProSpec, Rehovot, Israel) different potential substrates (100 μM) were exposed to both enzymes (80 nM CDA_{Hyor} or 27 nM CDA_{Human}) and incubated at 37 °C in PBS in a total volume of 300 μL. At different time points, 100 μL-fractions were withdrawn and 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. Nucleosides were separated on a reverse phase RP-8 column (Merck) and quantified by HPLC analysis. For each product UV-based detection was performed at the specific wavelength of optimal absorption.

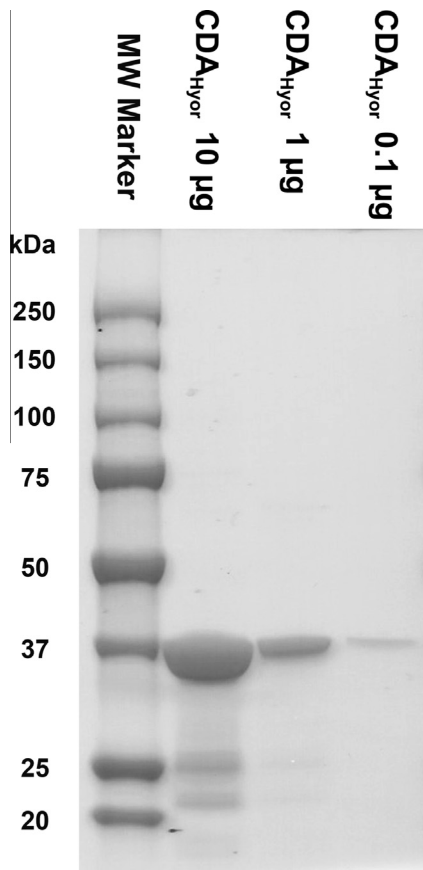

Fig. 2. Purity evaluation of the CDA_{Hyor}-GST fusion protein. Three different concentrations (i.e. 10, 1 and 0.1 µg) of the purified enzyme preparation were analyzed using SDS–PAGE. Proteins were stained using Bio-Safe™ Coomassie G-250 Stain (Bio-Rad Laboratories, CA, USA).

The separation of (2′-deoxy)cytidine [(d)Cyd] from (2′-deoxy)uridine [(d)Urd] was performed by HPLC using linear gradient B [from 100% buffer A and 2% acetonitrile to 80% buffer A and 20% acetonitrile] as follows: 5 min 100% buffer A; 5 min linear gradient to 80% buffer A + 20% acetonitrile; 5 min linear gradient to 100% buffer A followed by 5 min equilibration at 100% buffer A. Samples containing dFdC, ara-Cyd, 5-aza-2′-deoxycytidine, 5-aza-cytidine, 2′,3′-dideoxycytidine (ddC) or 2′,3′-dideoxy-3′-thiacytidine (3TC) were analyzed by HPLC using linear gradient A as described above.

2.6.2. Kinetic assays

Kinetic studies were performed for the deamination of different substrates [Cyd, dCyd, dFdC and ara-Cyd] by CDA_{Hyor} and CDA_{Human}. Deamination was studied at varying substrate concentrations ranging from 100 µM to 45 mM in a reaction containing 4 nM CDA_{Hyor} or 11 nM CDA_{Human} incubated in PBS at 37 °C for 10 min. Samples were processed and analyzed by HPLC as described above. Kinetic parameters (K_M and k_{cat}) were determined by means of non-linear regression analysis (using GraphPad Prism5) and the ratio k_{cat}/K_M (catalytic efficiency) was calculated.

3. Results

3.1. Deamination of gemcitabine by mycoplasma CDA can be prevented in the presence of exogenous natural purine and pyrimidine nucleosides

The cytostatic activity of gemcitabine (dFdC) was decreased by ~36 fold in *M. hyorhinis*-infected MDA-MB-231 breast cancer cell cultures compared with uninfected control tumor cells (Table 1). This could be prevented by co-administration of 250 µM of the cytidine deaminase inhibitor tetrahydrouridine (THU), but also by natural purine nucleosides (i.e. Ado, Ino or Guo) as well as by Immucillin-H (Imm-H), a potent inhibitor of mycoplasma PNP (Table 1).

Since THU could efficiently restore the cytostatic activity of dFdC in the presence of *M. hyorhinis* in the tumor cell culture medium, the stability of radiolabeled dFdC ([5-^{3}H]dFdC) was studied in the tumor cell-free (but mycoplasma-containing) culture medium of *M. hyorhinis*-infected MDA-MB-231 (Fig. 3A) and MCF-7 (Fig. 3B) breast cancer cells. A pronounced inhibition of [5-^{3}H]-dFdC deamination (i.e. decreased formation of the inactive metabolite [5-^{3}H]-dFdU) by 1 mM THU could be observed [10], but also, a dose-dependent inhibition of [5-^{3}H]dFdC deamination by the exogenous supply of natural pyrimidine (i.e. dThd and Urd) or purine (i.e. Ado and Ino) nucleosides was observed in the spent tumor cell culture medium (Fig. 3). Also, [5-^{3}H]-dFdU formation could be inhibited by exogenous administration of other natural nucleosides such as Guo, dAdo, dIno or dGuo (data not shown).

3.2. Substrate specificity of human- and mycoplasma-encoded CDA

The substrate specificity of recombinant *M. hyorhinis* CDA (CDA_{Hyor}) was studied and compared with CDA_{Human}. Both enzymes catalyzed the deamination of the natural pyrimidine nucleosides Cyd and dCyd, and the well-known anticancer drugs dFdC and ara-Cyd. Deamination of 5-aza-2′-deoxycytidine (decitabine) and 5-aza-cytidine (vidaza), both used for the treatment of myelodysplastic syndromes [29], was also observed in the presence of CDA_{Hyor} but could not be demonstrated for CDA_{Human}. The antiviral (i.e. HIV) drugs 2′,3′-dideoxycytidine (ddC; zalcitabine) and 2′,3′-dideoxy-3′-thiacytidine (3TC; lamivudine) were found to be insensitive to deamination by both human and mycoplasma CDA.

3.3. Kinetics of human- and mycoplasma-encoded CDA

The kinetic parameters (K_M and k_{cat}) for CDA_{Hyor}- and CDA_{Human}-catalyzed deamination of Cyd, dCyd, dFdC and ara-Cyd were determined. Relatively high K_M values (ranging from high micromolar to low millimolar concentrations) were observed for both enzymes. CDA_{Hyor} typically displayed higher K_M values compared with CDA_{Human} (Tables 2 and 3 and Fig. 4). However, the catalytic efficiency (calculated as k_{cat}/K_M) of CDA_{Hyor}-catalyzed reactions was ~2–4 fold higher compared with CDA_{Human} (Tables 2 and 3).

Interestingly, deamination of both dFdC and ara-Cyd by CDA_{Hyor}, but not by CDA_{Human}, was characterized by a second K_M at very high (and presumably biologically irrelevant) concentrations. However, precise values could not be determined due to insolubility of the highest drug concentrations (~45 mM in the reaction mixture) tested. The calculated K_M for dFdC and ara-Cyd as represented in Table 2 was therefore based on the measurements obtained when using up to 30 mM substrate. Measurements obtained using higher concentrations were excluded from the non-linear regression analysis but are still displayed in Fig. 4E (for dFdC) and Fig. 4G (for ara-Cyd). For these above-mentioned reasons, we preferred not to draw a curve line fitting the experimental data points in Fig. 4E and G.

4. Discussion

Recent studies have focused on the role of commensal prokaryotes in the efficiency of chemotherapeutic cancer treatment. For example, an intact intestinal microbiome seems to be essential

Table 1
Cytostatic activity of gemcitabine (dFdC) in MDA-MB-231 and MDA-MB-231. Hyor cells in the absence/presence of the selective CDA inhibitor tetrahydrouridine, natural purine nucleosides or the selective purine nucleoside phosphorylase inhibitor Immucillin-H (Imm-H). Results are the mean ± S.D. of at least two independent experiments.

	IC_{50}[a] value of dFdC (μM)					
	As such	+THU (250 μM)	+Ado (100 μM)	+Ino (100 μM)	+Guo (100 μM)	+Imm-H (10 μM)
MDA-MB-231	0.0042 ± 0.00041	0.004 ± 0.0003	0.0031 ± 0.0014	0.0037 ± 0.00076	0.0042 ± 0.0012	0.0037 ± 0.00076
MDA-MB-231.Hyor	0.15 ± 0.016	0.004 ± 0.0001	0.0096 ± 0.0049	0.0099 ± 0.0042	0.025 ± 0.0089	0.0051 ± 0.0021
Fold difference	~36	1	~3	~3	~6	~1.5

[a] 50% Inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.

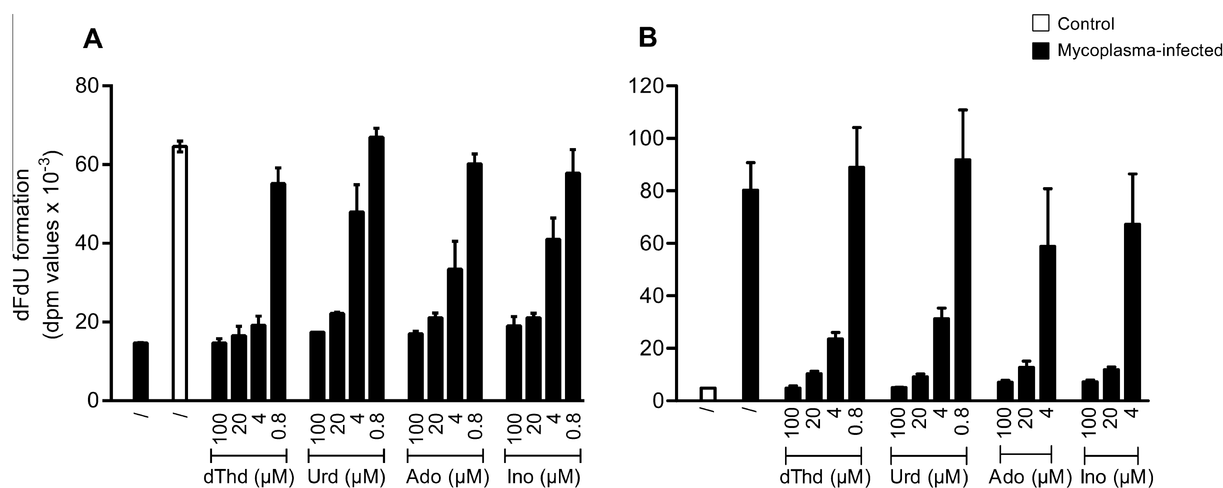

Fig. 3. Inhibition of mycoplasma-associated [5-^{3}H]dFdU formation by natural nucleosides. Formation of [5-^{3}H]dFdU from [5-^{3}H]dFdC in the tumor cell-free supernatant of mycoplasma-infected and control MDA.MB.231 (A) and MCF-7 (B) breast cancer cell cultures in the presence/absence of different concentrations of pyrimidine (dThd and Urd) and purine (Ado and Ino) nucleosides. The data are the mean of at least two independent experiments (±S.E.M.).

Table 2
Kinetic parameters of CDA_{Hyor}. The K_M and k_{cat} values ± S.E.M. for the natural substrates of CDA_{Hyor} and for gemcitabine and cytarabine were determined using nonlinear regression analysis (using GraphPad Prism 5) from data obtained in at least two independent experiments.

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s μM)$^{-1}$
Cytidine	1898 ± 163	171 ± 5	0.090
2′-Deoxycytidine	2586 ± 114	127 ± 2	0.049
dFdC	9064 ± 1487	105 ± 7	0.012
ara-Cyd	6172 ± 2336	119 ± 13	0.019

Table 3
Kinetic parameters of CDA_{Human}. The K_M and k_{cat} values ± S.E.M. for the natural substrates of CDA_{Human} and for gemcitabine and cytarabine were determined using nonlinear regression analysis (using GraphPad Prism 5) from data obtained in at least two independent experiments.

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s μM)$^{-1}$
Cytidine	811 ± 181	17 ± 1	0.021
2′-Deoxycytidine	373 ± 138	7.8 ± 0.64	0.021
dFdC	3080 ± 598	14 ± 0.91	0.004
ara-Cyd	2853 ± 741	15 ± 1.3	0.005

for optimal response to immune therapy and platinum-, doxorubicin- or cyclophosphamide-based cancer chemotherapy [30,31]. Conversely, the presence of some prokaryotes (e.g. mycoplasmas) in the tumor microenvironment may negatively influence the outcome of nucleoside-based treatment of cancer [23]. A thorough understanding of the implications of the (tumor) microbiome on cancer chemotherapy may ultimately lead to a more optimal treatment strategy (e.g. by combination therapy with antibiotics or specific prokaryotic enzyme inhibitors) and may also attenuate adverse side-effects.

In this report, we investigated the catabolic action of mycoplasma-encoded cytidine deaminase against the anticancer nucleoside analogues gemcitabine and cytarabine. To the best of our knowledge, this study is the first to describe the biochemical properties of the CDA encoded by *M. hyorhinis*, a commensal that has been repeatedly reported to preferentially associate with tumor tissue in patients. We found that CDA_{Hyor} does not only catalyze the deamination of natural cytidine and 2′-deoxycytidine but also of several clinical nucleoside antimetabolites, including gemcitabine and cytarabine. The efficiency of substrate conversion catalyzed by CDA_{Hyor} was consistently higher compared with the human equivalent enzyme. In addition, we observed deamination of 5-aza-2′-deoxycytidine and 5-aza-cytidine by CDA_{Hyor} but not by CDA_{Human} under similar experimental conditions. Based on these observations it could be expected that pronounced inactivation of different anticancer (2′deoxy)cytidine analogues may occur in mycoplasma-infected tumors leading to reduced efficacy.

Indeed, expression of CDA_{Hyor} explains the predominantly high levels of (the poorly cytostatic) [5-^{3}H]dFdU derived from radiolabeled gemcitabine in the culture medium of mycoplasma-infected tumor cell cultures and, as a result, the consequently decreased cytostatic activity of this drug in these tumor cell cultures (Table 1) and xenografts in mice [10]. Somewhat surprisingly, we found that the stability and biological (cytostatic) activity of gemcitabine in mycoplasma-infected tumor cell cultures could be restored by (i) the co-administration of natural pyrimidine (i.e. dThd and Urd) and purine (i.e. Ado, Ino and Guo) nucleosides and (ii) administration of a specific PNP inhibitor. Interestingly, inhibition of *Escherichia coli* and human CDA by different nucleosides [i.e. dThd, (d)Urd, (d)Ado and (d)Guo] has been

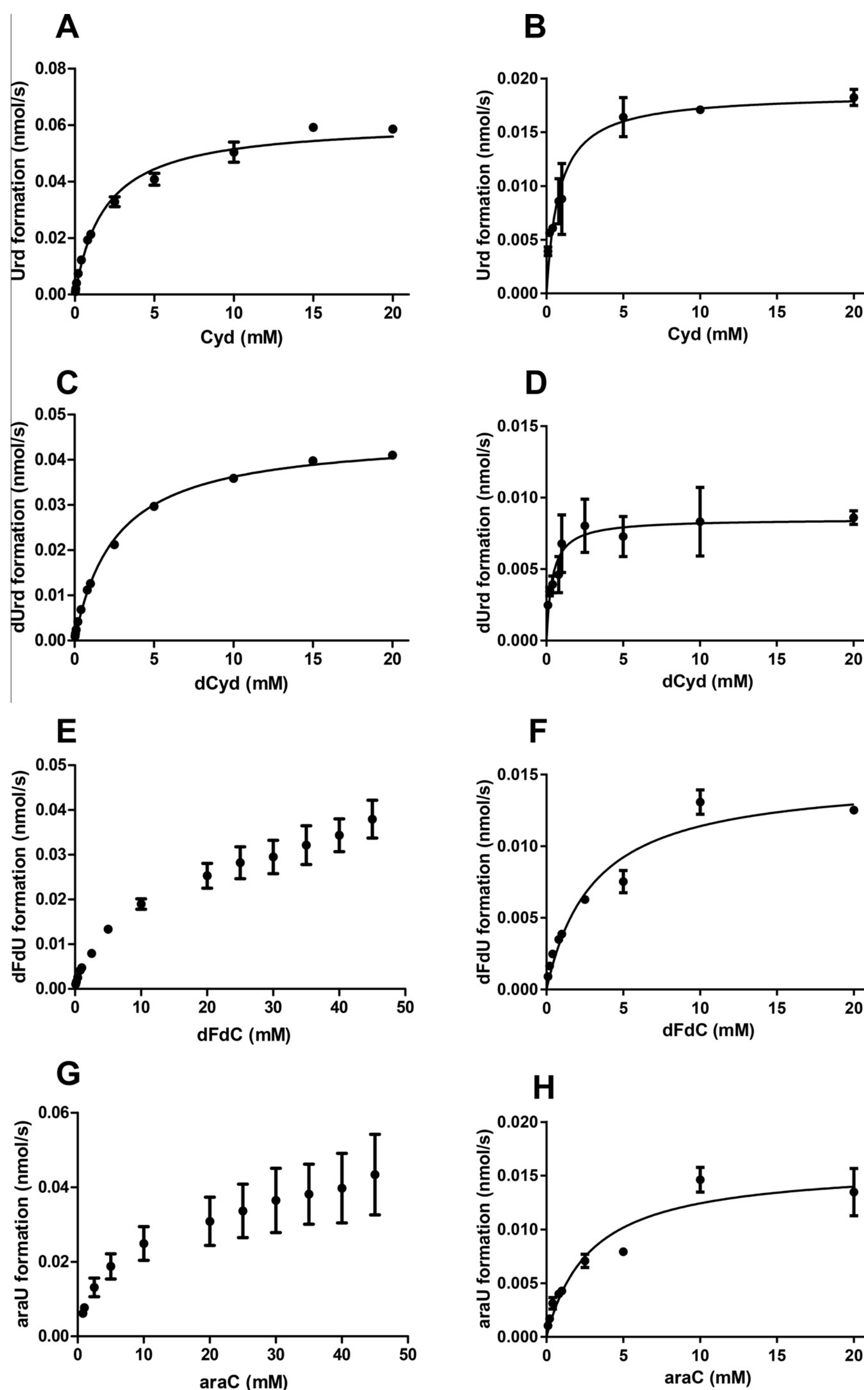

Fig. 4. Kinetic analysis of CDA_{Hyor}- and CDA_{Human}-catalyzed deamination of natural nucleosides and nucleoside analogues Deamination of different concentrations of Cyd (A and B), dCyd (C and D), dFdC (E and F) and ara-Cyd (G and H) by CDA_{Hyor} (A, C, E and G) or CDA_{Human} (B, D, F and H). The data are the mean of at least two independent experiments (±S.E.M.).

reported earlier [32–34]. Previously, we have shown that *M. hyorhinis* PNP is responsible for the catabolism of different purine nucleoside analogues, including cladribine and fludarabine [9]. It can be hypothesized that *M. hyorhinis*-related PNP activity may also indirectly contribute to the deamination of cytidine analogues by depletion of those intracellular purine nucleosides that seem to act as natural inhibitors of CDA_{Hyor}. Exogenous administration of these natural nucleosides may then restore the depleted purine nucleoside pools and, as a result, also the cytostatic potential of gemcitabine. This hypothesis would then also explain the rescuing cytostatic activity of gemcitabine by Immucillin-H, a potent and selective PNP inhibitor. Earlier, we reported a similar concerted action between CDA_{Hyor} and the *M. hyorhinis*-encoded pyrimidine nucleoside phosphorylase which catabolizes dThd, Urd and dUrd [10]. However, when exposing purified CDA_{Hyor} to different substrates (Cyd, dCyd or dFdC) in the presence of natural purine nucleosides or dThd, we could not observe decreased deamination (data not shown). It is therefore unlikely that the observed rescue of gemcitabine is due to a direct interaction of CDA_{Hyor} with purine nucleosides or dThd. Although inhibition of CDA by (d)Urd may be explained by end-product feedback inhibition, the mechanism of CDA_{Hyor}-inhibition by dThd and the purine nucleosides remains unclear. In this respect, it cannot be excluded that interaction (i.e. inhibition) of CDA can

be attributed to the mono-, di- or triphosphorylated derivatives of the natural nucleosides that were found inhibitory to CDA_{Hyor}-catalyzed deamination of gemcitabine. Alternatively, the natural nucleosides may compete for uptake with dFdC by *M. hyorhinis* and therefore lower the amount of drug to be deaminated intracellularly by the mycoplasma.

In conclusion, we have kinetically characterized the *M. hyorhinis*-encoded CDA and found it more catalytically efficient than human CDA. It deaminates (inactivates) anticancer drugs such as gemcitabine and cytarabine, but also 5-aza-(2′-deoxy)cytidine. Its deaminating action may negatively affect the cytostatic activity of anti-cancer drugs such as gemcitabine, but could be annihilated by co-administration of natural nucleosides or a specific PNP inhibitor.

Acknowledgments

We thank Christiane Callebaut, Eef Meyen, Lizette van Berckelaer, and Ria Van Berwaer for their excellent technical assistance. We are also grateful to Dr. Vern Schramm (Albert Einstein College of Medicine, Bronx, NY) for providing Imm-H. This work was supported in part by KU Leuven Grant GOA/15/019/TBA. JVV and PV performed the experiments; JVV, PV, SL and JB analyzed and interpreted the data; JVV, SL and JB designed and wrote the manuscript.

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