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

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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.

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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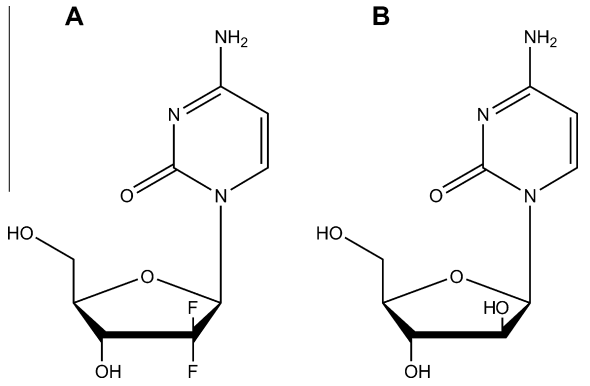

Fig. 1. Molecular structure of the (2′-deoxy)cytidine analogues gemcitabine (A) and cytarabine (B).

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.