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The cytostatic activity of pyrimidine nucleosides is strongly modulated by *Mycoplasma hyorhinis* infection: Implications for cancer therapy

*Annelies Bronckaers, Jan Balzarini *, Sandra Liekens **

Rega Institute for Medical Research, K.U. Leuven, B-3000 Leuven, Belgium

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ABSTRACT

Nucleoside analogues are widely used as chemotherapeutic agents in the treatment of cancer. Several cancers are reported to be associated with mycoplasmas (i.e. *Mycoplasma hyorhinis*), which contain a number of nucleoside-metabolizing enzymes. Pyrimidine nucleoside analogues, such as 5-fluoro-2′-deoxyuridine (FdUrd), 5-trifluorothymidine (TFT) and 5-halogenated 2′-deoxyuridines can be degraded by thymidine phosphorylase (TP) to their inactive bases. We found in *M. hyorhinis*-infected MCF-7 breast carcinoma cells (MCF-7/HYOR) a mycoplasma-encoded TP that dramatically (20–150-fold) reduces the cytostatic activity of these compounds. The reduction in cytostatic activity could be fully restored in the presence of TPI (5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride), a known inhibitor of human TP. This observation is in agreement with the markedly decreased formation of active metabolite (i.e. FdUMP for FdUrd) or diminished drug incorporation into nucleic acids (i.e. for TFT and 5-bromo-2′-deoxyuridine) in MCF-7/HYOR cells compared with uninfected MCF-7 cells. Antimetabolite formation is fully restored in the presence of TPI. In contrast, 5-fluoro-5′-deoxyuridine (5′DFUR), an intermediate metabolite of capecitabine, was markedly more cytostatic in MCF-7/HYOR cells than in uninfected cells, due to the activation of this prodrug by the mycoplasma-encoded TP. Thus, our data reveal that *M. hyorhinis* expresses a TP that activates 5′DFUR but inactivates FdUrd, TFT and 5-halogenated 2′-deoxyuridines, and that is highly sensitive to the inhibitory effect of the TP inhibitor TPI. Given the association of *M. hyorhinis* with several human cancers, our findings suggest that pyrimidine nucleoside-based but not 5FU-based anti-cancer therapy might be more effective when combined with a mycoplasmal TP inhibitor.

* *Corresponding authors*. Tel.: +32 16 337367; fax: +32 16 337340.
E-mail addresses: jan.balzarini@rega.kuleuven.be (J. Balzarini), Sandra.liekens@rega.kuleuven.be (S. Liekens).

Abbreviations: BrdUrd, 5-bromo-2′-deoxyuridine; CldUrd, 5-chloro-2′-deoxyuridine; 5′DFUR, 5-fluoro-5′-deoxyuridine; DPD, dihydropyrimidine dehydrogenase; dThd, thymidine; dUrd, 2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUrd, 5-fluoro-2′-deoxyuridine; 5FU, 5-fluorouracil; IC_{50}, 50% inhibitory concentration; IdUrd, 5-iodo-2′-deoxyuridine; MCF-7/HYOR, MCF-7 cells infected with *Mycoplasma hyorhinis*; PD-ECGF, platelet-derived endothelial cell growth factor; TFT, 5-trifluorothymidine; Thy, thymine; TK, thymidine kinase; TP, thymidine phosphorylase; TPI, 5-chloro-6-(1-[2-iminopyrrolidinyl]methyl)uracil hydrochloride; TS, thymidylate synthase; Ura, uracil.

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1. Introduction

The fluoropyrimidine 5-fluorouracil (5FU) is successfully used against a variety of solid tumors, including breast, oesophageal and colon carcinoma [1]. 5FU elicits its antitumor activity primarily by inhibiting thymidylate synthase (TS), a rate-limiting enzyme in DNA synthesis [2,3]. This requires conversion of 5FU to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which inhibits TS. However, the clinical efficacy of 5FU is limited by its rapid degradation [by dihydropyrimidine dehydrogenase (DPD)] and poor oral bioavailability [4]. Therefore, efforts have been made to develop oral 5FU-prodrugs. Doxifluridine (5-fluoro-5′-deoxyuridine, 5′DFUR) is a prodrug of 5FU that requires thymidine phosphorylase (TP) for its one-step conversion to 5FU. However, 5′DFUR therapy resulted in dose-limiting gastrointestinal toxicity [5,6]. Capecitabine (N4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine, Xeloda®) was designed to circumvent this toxicity by more selectively delivering 5FU to the tumor. Capecitabine is converted to 5FU in three distinct steps. It is first converted to 5′-deoxy-5-fluorocytidine by carboxylesterase in the liver, then to 5-fluoro-5′-deoxyuridine (5′-DFUR) by cytidine deaminase and finally to 5FU by TP [7]. Currently, capecitabine is being used for the treatment of metastatic breast and colorectal cancers [1,8,9].

TP is not only a key enzyme in the pyrimidine nucleoside salvage pathway [10] but is also identical to platelet-derived endothelial cell growth factor (PD-ECGF), an angiogenic factor with anti-apoptotic properties [11–13]. Increased TP levels are found in several solid tumors and are correlated with high neovascularisation, increased metastasis and poor prognosis. Nevertheless, high TP levels improve the effectiveness of 5FU prodrug-based chemotherapy [14].

In spite of good therapeutic results, a large number of patients eventually acquire resistance against 5FU-based chemotherapy. The fluoropyrimidine nucleoside 5-trifluorothymidine (TFT) has been shown to bypass this resistance. The mechanism of cytostatic action of TFT is based on inhibition of TS as its monophosphate and incorporation of the drug into the DNA after conversion to its triphosphate metabolite [15]. However, TFT is rapidly inactivated by TP, which converts TFT to its inactive base. Therefore, a new drug formulation containing TFT and a potent inhibitor of mammalian TP [5-chloro-6-(1-[2-iminopyrrolidinyl]methyl)uracil hydrochloride (TPI)], designated TAS-102, has been developed [16]. At present, TAS-102 is being evaluated in phase I clinical trials for the treatment of various solid tumors [17,18]. Thus, TP has an ambiguous role in fluoropyrimidine-based chemotherapy. It may enhance the anti-tumoral properties of 5FU prodrugs such as capecitabine on the one hand, but it may inactivate pyrimidine 2′-deoxyuridine derivatives, such as TFT, on the other hand.

TP activity is not only upregulated in tumors, it is also expressed by several mycoplasma species, such as *Mycoplasma mycoides* and *Mycoplasma pirum* [19]. Mycoplasmas are the smallest self-replicating bacteria and are important human pathogens. They can cause severe respiratory and urogenital diseases [20]. Most mycoplasma infections, however, remain unidentified, because many people seem to be chronically infected with mycoplasmas without apparent clinical symptoms [21]. A possible association between mycoplasmas and leukaemia has already been suggested in the 1960s [22,23]. More recently, mycoplasmas were detected in tissues of ovarian and cervical cancer, by using sensitive PCR-ELISAs [24,25]. In addition, *Mycoplasma penetrans* was found to be associated with Kaposi's sarcoma [26]. Immunohistological analysis of carcinoma tissues, demonstrated a significant correlation between the presence of *Mycoplasma hyorhinis* and gastric and colon cancer [27].

Recently, a number of studies have highlighted the involvement of mycoplasmas in cancer progression. Chronic mycoplasma infections with *M. penetrans* and *Mycoplasma fermentans* induced chromosomal instability in C3H murine embryonic cells, prevented apoptosis and caused malignant transformation in 32D haematopoietic cells [28]. When injected into nude mice, these transformed 32D cells quickly developed tumors, while the control cells did not [29]. Infection with some strains of *M. fermentans* promoted immortalization of human peripheral blood mononuclear cells in culture [30]. *M. hyorhinis* was found to express p37, a protein that increases the invasiveness of prostate and melanoma cell lines *in vitro* [31]. This protein also altered gene expression, growth and migratory potential of the prostate cancer cell lines PC-3 and DU145 [32]. Recent data indicate that p37 promotes cancer cell invasiveness and metastasis by activation of MMP-2 and by phosphorylation of the epidermal growth factor receptor [33].

Since mycoplasmas have been associated with several cancers and often abundantly express TP, we investigated whether mycoplasma infection could influence the cytostatic properties of several fluoropyrimidine analogues. Our data reveal that *M. hyorhinis*-encoded TP significantly decreases the accumulation of cytostatic pyrimidine nucleoside metabolites into the tumor cells and markedly down-modulates the cytostatic activity of these compounds. Co-administration of a specific TP inhibitor with the nucleoside analogues can fully restore the cytostatic activity in the mycoplasma-infected cell cultures.

2. Materials and methods

2.1. Reagents

TPI, 5-chloro-6-(1-[2-iminopyrrolidinyl]methyl)uracil hydrochloride, a potent inhibitor of TP [34] was kindly provided by Prof. S. Akiyama (Kagoshima, Japan) and Dr. M. Fukushima (Taiho Pharmaceutical Co., Tokushima, Japan) [34]. 5-Fluoro-5′-deoxyuridine (5′DFUR), TFT, thymidine (dThd), 5-fluoro-2′deoxyuridine (FdUrd), 5-chloro-2′-deoxyuridine (CldUrd), 5-bromo-2′-deoxyuridine (BrdUrd), 5-iodo-2′-deoxyuridine (IdUrd), and 5FU were purchased from Sigma (St-Louis, MO). [CH_3-^{3}H]-Thymine, [6-^{3}H]-TFT, [2-^{14}C]-TF-thymine, [6-^{3}H]-BrdUrd, [6-^{3}H]-FdUrd, [6-^{3}H]-dUrd, [5-^{3}H]-uracil and [6-^{3}H]-5FU were obtained from Moravek Biochemicals (Brea, CA) and [CH_3-^{3}H]-dThd from MP Biomedicals (Solon, OH). Plasmocin was purchased from Invivogen (San Diego, CA). The antibody against β-actin was obtained from Sigma, the polyclonal antibody against TP (clone G-19) from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

TP-negative MCF-7 breast carcinoma cells were kindly provided by Prof. G.J. Peters (Amsterdam, The Netherlands) [35]. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% foetal bovine serum (FBS) (Harlan, Sera-Lab Ltd., Loughborough, UK) and 10 mM Hepes (Invitrogen). Cells were grown at 37 °C in a humidified incubator with a gas phase of 5% CO_2. MCF-7 cells overexpressing human TP were obtained by transfection of MCF-7 cells with the TP/PD-ECGF full-length cDNA expression vector that was kindly provided by Prof. S. Akiyama [36].

2.3. Culture of M. hyorhinis

M. hyorhinis (ATCC 17981) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The freeze-dried bacteria were reconstituted by adding 1 ml of DMEM. MCF-7 cells were seeded at 20,000 cells/cm^2 in DMEM containing 10% FBS (mycoplasma-screened). Two days later, the MCF-7 cell cultures were infected with *M. hyorhinis* by adding 500 μl of the freshly reconstituted mycoplasmas. The co-culture of MCF-7 cells and *M. hyorhinis* was maintained under the same conditions as the uninfected MCF-7 cells.

2.4. Identification of M. hyorhinis by PCR

To confirm the infection of MCF-7 cells by *M. hyorhinis*, a species-specific PCR for *M. hyorhinis* was performed as described by Kong et al. [37]. All PCR reactions were performed using Taq Polymerase (Sphaero Q, Leiden, The Netherlands). The primers used for the PCR were HYR+ (5′catgatgagtaatagaaaggagcttcacagcttc-3′) and UNI− (5′-ccagggtatctaatcctgtttgctccc-3′), which produce a PCR-fragment of 616 bp long [36]. PCR amplification consisted of 40 cycles of denaturation at 96 °C for 1 s, annealing at 68 °C for 1 s and extension at 74 °C for 10 s.

2.5. Staining of DNA with Hoechst 33342

10,000 cells/cm^2 (MCF-7 and MCF-7/HYOR) were seeded in 8-well chamber slides (Nunc, Roskilde, Denmark). After 24 h, 10 μM TPI was added and the cells were incubated for an additional 72 h. Next, the cells were fixed with Carnoy's fixative (1 part glacial acetic acid to 3 parts absolute methanol) for 10 min, air-dried and exposed to the DNA-binding dye Hoechst 33342 (Sigma) at a concentration of 0.5 μg/ml for 15 min at room temperature. Next, the cells were washed twice with de-ionised water and covered with mounting medium ('*glycergel*', Dako, Glostrup, Denmark) and a cover slip. Fluorescence was visualised with a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany).

2.6. Western blot assay

MCF-7 and MCF-7/HYOR cells were seeded at 8000 cells/cm^2. Forty-eight hours later, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed as described previously [38]. Lysates were cleared by centrifugation, and the protein concentration of the supernatants was determined. One ml of the culture medium was centrifuged at 1200 rpm for 5 min. The supernatant was sonicated and concentrated 10 times by using a vivaspin concentrator with a cut-off size of 5000 Da (Sartorius AG, Goettingen, Germany). SDS-polyacrylamide gel electrophoresis of 40 μg of the cell lysates and 20 μl of the concentrated medium was performed after which the proteins were transferred to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in PBS containing 0.1% Tween 20) and subsequently for 1 h in blocking buffer with primary antibodies raised against β-actin (1/5000) or TP (1/1000). After washing, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (anti-mouse, 1/2000; Dako) in blocking buffer for 25 min at room temperature. Next, the membranes were washed extensively. Immunoreactive proteins were detected by chemiluminescence (ECLplus; GE Healthcare). As a positive control a cell lysate from MCF-7 cells transfected with the human TP gene (MCF-7/TP) was loaded on the gel.

2.7. Enzyme activity assays

The TP activity of *M. hyorhinis* and the conversions of dThd, FdUrd, 5′DFUR and TFT to thymine, 5FU, 5FU or TF-thymine, respectively were measured by high-pressure liquid chromatography (HPLC) analysis. MCF-7 and MCF-7/HYOR cells were seeded at a density of 20,000 cells/cm^2 in DMEM with 10% FBS. Four days later, the medium was collected and cleared by centrifugation at 1400 rpm. For some experiments, the medium of MCF-7/HYOR cells was filtered using a 0.1 μm micro filter (Acrodisc syringe filter, PALL Corporation, East Hills, NY) to remove the mycoplasmas from the medium. 600 μl of the medium was incubated with 200 μM of substrate (dThd, 5′DFUR, TFT or FdUrd) at 37 °C in the presence or absence of 10 μM TPI. At different time points (i.e. 0, 15, 30, 60, 120 min and 16 h), 100 μl aliquots were withdrawn, transferred to Eppendorf tubes and heated at 95 °C for 3 min. Next, the samples were rapidly cooled on ice, exposed for 20 min to 200 μl ice-cold methanol and cleared by centrifugation at 15,000 rpm for 15 min. As a positive control, an enzyme activity assay with *E. coli* TP (Sigma) was performed. For this reaction, 0.025 U of TP were incubated with 200 μM of substrate in TP-buffer (10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 2 mM potassium phosphate and 150 mM NaCl) in a total volume of 600 μl. Aliquots of 100 μl were withdrawn from the reaction mixture at several time points and treated as described above. The nucleosides were separated from their nucleobases on a reversed-phase RP-8 column (Merck, Darmstadt, Germany) and quantified by HPLC analysis (Aliance 2690, Waters, Milford, MA). The separation was performed by a linear gradient from 100% buffer B (50 mM NaH_2PO_4 and 5 mM heptane sulfonic acid, pH 3.2), to 20% buffer B and 80% acetonitrile. Retention times of thymine and thymidine were respectively 5.1 and 10.8 min. UV-based detection of all nucleosides was performed at 267 nm.

2.8. Tumor cell proliferation assays

MCF-7 and MCF-7/HYOR cells were seeded in 48-well plates at 10,000 cells/cm^2. After 24 h, different concentrations of the test compounds (5FU, 5′DFUR, CldUrd, BrdUrd, FdUrd, IdUrd and TFT) with or without 10 μM TPI were added. The cells were incubated for another 4 days, trypsinized and counted by a Coulter counter (Analis, Suarlée, Belgium). In some experiments, the antibiotic plasmocin was added 1 or 3 days before addition of the test compounds.

2.9. Nucleotide incorporation assay

MCF-7 and MCF-7/HYOR cells were seeded at 10,000 cells/cm. After 48 h, cells were treated with 1 μCi of ^{3}H-labeled nucleoside with or without 10 μM TPI. 16 h later, the medium was removed and the cells were washed twice with PBS. Next, the cells were trypsinized, transferred to Eppendorf tubes and centrifuged for 10 min at 1400 rpm. The pellet was resuspended in 1 ml absolute ice-cold methanol and kept on ice for 20 min. After centrifugation for 20 min at 13,000 rpm the pellet was washed twice with methanol, resuspended in methanol and transferred to scintillation vials containing 9 ml of Ready safe liquid scintillation reagent ('*Hisafe* 3', PerkinElmer, Waltham, MA). The radioactivity was measured by a liquid scintillation analyzer (2300 TR, Packard, Canberra, Australia).

2.10. Nucleoside metabolism experiments

MCF-7 and MCF-7/HYOR cells were seeded and treated with 1 μCi of nucleoside with or without TPI as described above. 16 h later, medium was collected and the cells were washed twice with PBS. Next, the cells were incubated in 0.5 ml absolute ice-cold methanol and kept on ice for 20 min. After centrifugation for 20 min at 13,000 rpm, the supernatant was subjected to HPLC analysis. The nucleobases, nucleosides and nucleotides in the supernatant were separated by a Partisphere 10 SAX anion exchange column (Whatmann International Ltd., Maidstone, England) as described earlier [39], while the nucleobases and nucleosides present in the collected medium were separated using an RP-8 column. The amount of compound incorporated into nucleic acids was measured as described above.

3. Results

3.1. Identification of M. hyorhinis infection in MCF-7/HYOR cell cultures

Productive infection of MCF-7 cells with *M. hyorhinis* was confirmed by a species-specific PCR, which detected a PCR-band of 616 bp in the MCF-7/HYOR cell extracts (Fig. 1(A)). No PCR-bands were found in the uninfected MCF-7 cell extract or in the non-template control. Infection of MCF-7 cells with *M. hyorhinis* was also evaluated by staining the cellular and bacterial DNA with the Hoechst 33342 dye (Fig. 1(B)). Nucleic acid-rich particles can be visualized in the cytosol of the MCF-7/HYOR cells and MCF-7/HYOR cells that were treated for 3 days with TPI (10 μM) indicating that TPI is not inhibitory to the growth of *M. hyorhinis* in MCF-7 cell cultures.

3.2. Detection of human TP in MCF-7 and MCF-7/HYOR cell extracts and cell culture medium

Western blot analysis using a polyclonal antibody against human TP did not detect the protein in extracts of MCF-7 and MCF-7/HYOR cells (Fig. 2). However, human TP could be abundantly detected in extracts from MCF-7 cells that were transfected with the human TP gene. This confirms that MCF-7 cells do not express human TP and indicates that *M. hyorhinis* infection does not induce the expression of human TP in MCF-7 cells. Also, human TP was not detected in the medium of uninfected MCF-7 and *M. hyorhinis*-infected MCF-7/HYOR cells

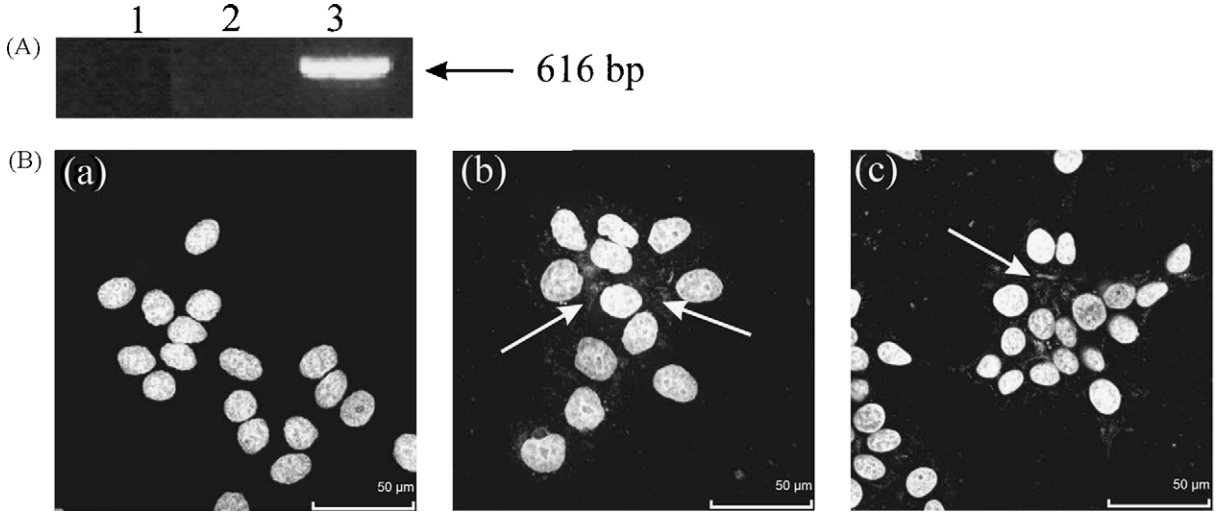

Fig. 1 – (A) PCR analysis for *Mycoplasma hyorhinis* in cell extracts of MCF-7 and MCF-7/HYOR. Lane 1 shows the non-template control; lane 2 shows the uninfected MCF-7 extract; lane 3 shows the infected MCF-7/HYOR extract. (B) DNA staining with Hoechst 33342 in control MCF-7 (a), MCF-7/HYOR (b) and MCF-7/HYOR cells treated with 10 μM TPI (c). Arrows indicate the presence of nucleic acid-rich particles in the cytosol.

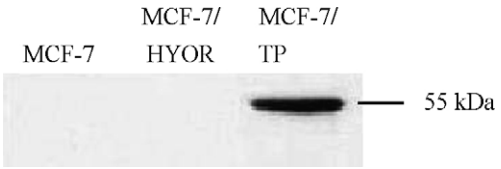

Fig. 2 – Western blot analysis using a polyclonal antibody against human TP. A band of 55 kDa could be detected in cell lysates of MCF-7 that were transfected with the human TP gene. No human TP was detected in cell extracts of MCF-7 or mycoplasma-infected MCF-7/HYOR cells.

(data not shown). The polyclonal antibody used in this assay, did not show any cross-reactivity with the mycoplasmal TP present in the culture medium of MCF-7/HYOR cells.

3.3. *TP enzyme activity assays in the supernatant of MCF-7/HYOR cell cultures*

The TP enzyme activity and time-course of the enzymatic reaction were determined in the medium of 4-day-old MCF-7/HYOR cell cultures (Table 1, Fig. 3). Seventy-one percent of dThd (200 μM) was converted into thymine within 2 h. All dThd had disappeared from the reaction mixture after 16 h. The pyrimidine nucleoside analogues FdUrd, 5′DFUR and TFT were also converted to their respective pyrimidine bases, although to a lesser extent than the natural substrate dThd (Table 1). In the MCF-7/HYOR culture medium, the conversion of all compounds (200 μM dThd, TFT, FdUrd and 5′DFUR) to their respective free bases could be completely inhibited in the presence of 10 μM TPI (a potent inhibitor of human and *E. coli* TP). In contrast, no conversion of dThd, TFT, FdUrd or 5′DFUR was observed in the medium of uninfected MCF-7 cells, even after 24 h of incubation (data not shown). Interestingly, no TP activity was found in the filtered (0.1 μm) supernatant of MCF-7/HYOR cell cultures. Thus, by removing the mycoplasmas from the medium, the TP activity in the cell culture medium is lost, indicating that the measured TP activity is bacteria-associated and not extracellularly secreted by the mycoplasmas.

The time-course curve of the TP activity shows an initial lag-phase (Fig. 3). This may indicate that dThd first has to be taken up by the intact mycoplasmas present in the medium before it can be converted into thymine.

Table 1 – TP activity in the medium of MCF-7/HYOR cell cultures (% conversion of nucleoside to the free pyrimidine base) or in the presence of 0.025 U of recombinant *E. coli* TP

Time (h)	dThd	FdUrd	TFT	5′DFUR
Percent conversion of nucleoside in MCF-7/HYOR medium				
2	71 ± 2.0	43 ± 6	8 ± 4	5 ± 1.0
16	97 ± 3.1	77 ± 6	55 ± 11	22 ± 3.3
Recombinant *E. coli* TP				
2	82 ± 5.6	57 ± 3.7	48 ± 1.8	26 ± 2.2
16	93 ± 4.0	63 ± 2.0	85 ± 2.1	64 ± 6.9

Values are presented as means ± S.E.M. of at least three independent experiments.

3.4. *Cytostatic activity of pyrimidine nucleoside analogues*

The cytostatic activity of 5′DFUR, TFT, FdUrd, CldUrd, BrdUrd and IdUrd was determined in both MCF-7 and MCF-7/HYOR cell cultures in the absence or presence of TPI (Table 2). With the exception of 5′DFUR, the cytostatic activity of the nucleoside analogues was 20–150-fold lower in the infected MCF-7/HYOR cell cultures compared to control MCF-7 cells. The decreased cytostatic activity of the nucleoside analogues observed in the MCF-7/HYOR cell cultures could be completely restored by co-administration of TPI (10 μM) (Table 2). These results indicate that *M. hyorhinis*-encoded TP converts the pyrimidine nucleoside analogues into their respective pyrimidine bases, resulting in a decreased cytostatic activity of these compounds. In contrast, 5′DFUR was markedly more cytostatic in infected MCF-7/HYOR cells, indicating that the mycoplasma-encoded TP efficiently converted this prodrug into 5FU. The IC_{50} values of the parent compound 5FU were not significantly different in MCF-7 and MCF-7/HYOR cell cultures. This is obviously due to the TP-independent conversion of 5FU to its active metabolite (FdUMP).

The cytostatic activity of TFT, FdUrd, BrdUrd, 5′DFUR, and 5FU was also investigated in the presence of the antibiotic plasmocin (25 μg/ml), which was added to the MCF-7 and MCF-7/HYOR cells 1 day or 3 days before addition of the test compounds (Table 3). Addition of plasmocin to the MCF-7 cells did not alter the IC_{50} values of the test compounds (data not shown). However, pre-incubation of the MCF-7/HYOR cell cultures with the antibiotic for 1 day partially restored the decreased cytostatic activity of the test compounds, while 3 days pre-incubation with plasmocin completely restored the anti-proliferative activity of TFT, FdUrd and BrdUrd. Whereas plasmocin did not affect the activity of 5FU, 5′DFUR lost its cytostatic activity in MCF-7/HYOR cell cultures pre-treated with plasmocin.

3.5. *Metabolism and incorporation of pyrimidine nucleoside analogues into nucleic acids*

Most pyrimidine nucleoside analogues are cytostatic because they inhibit DNA and/or RNA synthesis by inhibiting thymidylate synthase and/or by being incorporated into the nucleic acids of tumor cells. The incorporation of dThd, BrdUrd, TFT and dUrd into nucleic acids was respectively 85-, 45-, 40- and 3-fold reduced in infected MCF-7/HYOR cells in comparison with uninfected MCF-7 cells (Fig. 4). Addition of TPI to the radiolabeled drug-exposed MCF-7/HYOR cell cultures fully restored the impaired incorporation to normal levels. These results show that *M. hyorhinis*-encoded TP markedly prevents the conversion of the drugs to their active metabolites, presumably by releasing the free pyrimidine base and thus by preventing proper anabolism of the pyrimidine nucleoside analogues to their phosphorylated metabolites. There was no difference in the incorporation of the free pyrimidine bases thymine, uracil, 5FU and TF-thymine into nucleic acids between the infected and uninfected MCF-7 cells. Interestingly, the incorporation of these pyrimidine bases was very

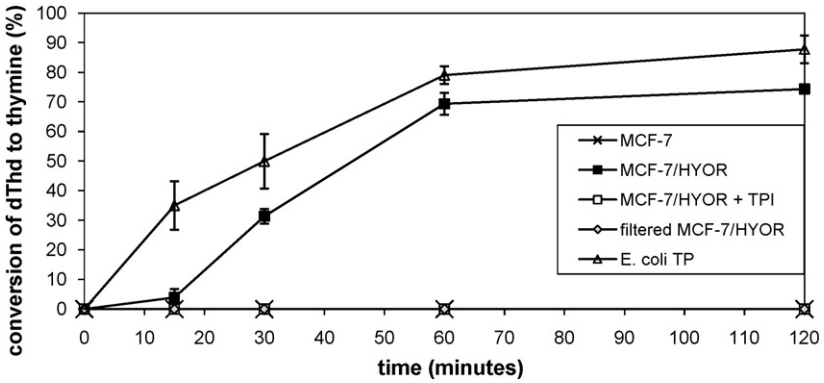

Fig. 3 – Time-course of the conversion of dThd to thymine by *M. hyorhinis*-infected MCF-7 cell culture supernatants. The medium of MCF-7 and MCF-7/HYOR cells was incubated with 200 μM dThd at 37 °C. At different time points, aliquots were withdrawn and the conversion of dThd into thymine was quantified by HPLC analysis. As a positive control 0.025 U of recombinant *E. coli* TP were used. In one assay, the medium of MCF-7/HYOR cells was filtered through a 0.1 μm syringe filter. In contrast to MCF-7/HYOR cells, no conversion of dThd was observed in the medium of MCF-7 cells, MCF-7/HYOR cell cultures treated with TPI or filtered medium of MCF-7/HYOR cells. Values are the means of three separate experiments ± S.E.M.

small, presumably by poor, if any, TP-induced conversion to their respective nucleoside derivatives.

Unlike what may have been expected from the cell proliferation data, *M. hyorhinis* infection did not affect the incorporation of FdUrd into nucleic acids. FdUrd elicits its cytostatic activity by inhibition of thymidylate synthase as its 5′-monophosphate derivative FdUMP. The formation of phosphorylated FdUrd metabolites was therefore investigated and compared with the metabolites of dThd, BrdUrd and TFT (Table 4). In MCF-7/HYOR cells, low, if any significant levels of di- and triphosphate derivatives of dThd, BrdUrd, FdUrd and TFT were detected. However, in the presence of TPI, the levels of TFT-5′-monophosphate were increased by 2.7-fold, whereas FdUrd 5′-monophosphate levels were increased by 18-fold. These data are strongly suggestive for TS as the main mechanism of cytostatic action of FdUrd whereas the other drugs, including TFT, may predominantly exert their cytostatic activity upon incorporation into nucleic acids. In the presence of TPI, almost all dThd or BrdUrd was incorporated into nucleic acids while 66% of the TFT but almost no FdUrd was incorporated into the nucleic acids. This is obviously due to the fact that dThd and BrdUrd are much better substrates for cellular TK than TFT and FdUrd [40]. The data in Table 4 again confirm the degradation of all nucleosides to their inactive bases in MCF-7/HYOR cells, whereas administration of TPI to the cell cultures inhibits this catabolic activity.

4. Discussion

TP is an enzyme of the pyrimidine nucleoside salvage pathway that catalyzes the reversible conversion of thymidine and phosphate into thymine and 2-deoxy-D-ribose-1-phosphate. Previously, TP activity has been detected in the mycoplasma species *M. pirum* and *M. mycoides* [19,41]. Others have reported that [^{3}H]-thymidine incorporation into DNA was impaired in cell cultures contaminated with mycoplasmas, suggesting an enzymatic cleavage of thymidine by TP activity originating

Table 2 – Cytostatic activity of pyrimidine nucleoside analogues against *Mycoplasma hyorhinis*-infected and uninfected MCF-7 cells in the presence or absence of TPI

Compound	IC_{50}[a] (μM)					
	MCF-7			MCF-7/HYOR		
	As such (1)	+TPI (10 μM) (2)	Ratio[b] (1)/(2)	As such (1)	+TPI (10 μM) (2)	Ratio[b] (1)/(2)
FdUrd	0.003 ± 0.002	0.003 ± 0.002	1.0	0.42 ± 0.18	0.003 ± 0.001	140
TFT	0.39 ± 0.12	0.21 ± 0.11	1.8	6.0 ± 3.19	0.18 ± 0.07	33
CldUrd	0.76 ± 0.19	0.64 ± 0.15	1.2	13 ± 2.87	1.4 ± 0.70	9.3
BrdUrd	0.59 ± 0.10	0.36 ± 0.01	1.6	8.6 ± 1.17	0.84 ± 0.24	10
IdUrd	1.1 ± 0.26	0.98 ± 0.39	1.1	12 ± 0.5	0.31 ± 0.05	39
5FU	0.81 ± 0.24	0.62 ± 0.29	1.3	0.75 ± 0.24	0.53 ± 0.25	1.4
5′DFUR	>100	>100	>1<	3.5 ± 0.53	>100	<0.035

Values are presented as means ± S.E.M. of at least three independent experiments.
[a] 50% inhibitory concentration, or compound concentration required to inhibit tumor cell proliferation by 50%.
[b] The ratio (1)/(2) represent the ratio of IC_{50} in the absence of TPI to the IC_{50} in the presence of TPI.

Table 3 – Cytostatic activity of pyrimidine nucleoside analogues against *M. hyorhinis*-infected MCF-7 and uninfected MCF-7 cells, pretreated with plasmocin for 1 day or 3 days prior to addition of the test compounds

Compound	IC_{50}[a] (μM)			
	MCF-7/HYOR			MCF-7
	As such	+Plasmocin (25 μg/ml) 1 day prior to addition of test compounds	+Plasmocin (25 μg/ml) 3 days prior to addition of test compounds	As such
TFT	6.0 ± 3.19	0.45 ± 0.09	0.19 ± 0.06	0.39 ± 0.12
BrdUrd	8.6 ± 1.17	2.22 ± 1.1	0.74 ± 0.2	0.59 ± 0.10
FdUrd	0.42 ± 0.18	0.018 ± 0.0022	0.003 ± 0.001	0.003 ± 0.002
5FU	0.75 ± 0.24	0.74 ± 0.11	0.67 ± 0.11	0.81 ± 0.24
5′DFUR	3.5 ± 0.53	>100	>100	>100

Values are presented as means ± S.E.M. of at least three independent experiments.
[a] 50% inhibitory concentration or compound concentration required to inhibit tumor cell proliferation by 50%.

from mycoplasmas [42,43]. In the present study, we report that also *M. hyorhinis* contains TP activity (Fig. 3). Moreover, we show that the TP encoded by this mycoplasma species not only catalyzes the conversion of thymidine to thymine, but also efficiently recognizes FdUrd, TFT and 5′DFUR, which are known substrates of *E. coli* and mammalian TPs [44,45] (Table 1). Although the enzymatic activity of TP is reversible, the equilibrium of this reaction is towards the nucleobase and not towards the pyrimidine nucleoside. Within 60 min almost all thymidine is degraded into thymine (Fig. 3). These results are in line with the previously reported pronounced phosphorolysis of thymidine by *E. coli* TP or TP extracted from human platelets [44,46,47]. Infection of TP-negative MCF-7 cells by *M. hyorhinis* did not induce the expression of human TP as was demonstrated by Western blot analysis on cell lysates of MCF-7/HYOR cells (Fig. 2). Thus, the effects observed in the *M. hyorhinis*-infected MCF-7 cell cultures were due to the expression of mycoplasma-specific TP and not to upregulated or induced human TP.

TP produced by *M. hyorhinis* significantly decreased the sensitivity of MCF-7 cells to the antiproliferative activity of FdUrd, TFT and other 5-halogen-substituted dUrd analogues (Table 2). The reduced antiproliferative activities of these cytostatic compounds in MCF-7/HYOR cell cultures could be fully restored by adding TPI, a well-known human TP inhibitor [34], but also by adding the anti-mycoplasmal antibiotic plasmocin (25 μg/ml) to the cells 3 days prior to addition of the drugs (Table 3). Plasmocin efficiently inhibits DNA replication and protein synthesis of mycoplasma (www.plasmocin.com). These observations again demonstrate that

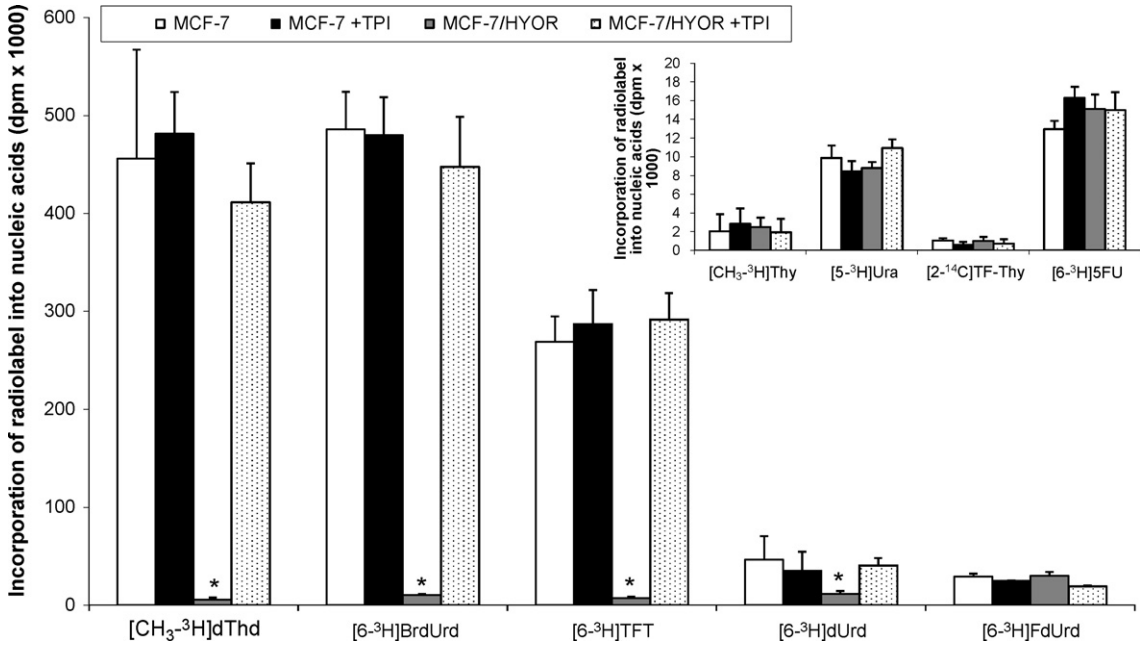

Fig. 4 – Incorporation of dThd, thymine, 2′-deoxyuridine and fluoropyrimidine nucleoside analogues into nucleic acids in the presence or absence of 10 μM TPI. MCF-7 and MCF-7/HYOR cells were incubated overnight with 1 μCi of radiolabeled compound. The next day, the amount of radioactive compound that was incorporated into the nucleic acids was measured. Values are presented as means ± S.E.M. of at least three independent experiments; *$p < 0.01$ compared to control MCF-7 cells.

Table 4 – Percent of drug-derived radiolabel (i.e. from TFT, FdUrd, BrdUrd and dThd) added to MCF-7/HYOR cell cultures

Compound	In medium		In the cytosol of the cells				Incorporation into DNA/RNA
	Base	Nucleoside	Nucleoside /nucleobase	5′-Mono-phosphate	5′-Di-phosphate	5′-Tri-phosphate	
TFT	56.7	23.3	11.0	6.1	0.6	0.6	1.8
TFT + TPI	0.0	6.4	8.6	16.7	2.1	0.8	65.4
FdUrd	49.5	33.2	11.6	3.5	0.9	0.3	1.0
FdUrd + TPI	5.3	11.4	18.1	62.9	0.8	0.6	0.9
BrdUrd	49.5	42.0	6.4	0.4	0.2	0.2	1.4
BrdUrd + TPI	0.1	0.9	2.7	1.1	0.4	0.4	94.5
dThd	74.6	11.5	7.5	0.6	0.3	0.2	5.3
dThd + TPI	0.7	0.5	1.3	0.3	0.1	0.2	97.0

Values are presented as means of at least three independent experiments. The S.E.M. are not shown but are less than 5% of the values.

mycoplasma-encoded enzyme(s) (i.e. TP) may markedly compromise the cytostatic action of the nucleoside analogues. Thus, *M. hyorhinis* TP efficiently converts FdUrd, TFT and other 5-halogen-substituted dUrd, to their respective free pyrimidine bases. However, previously it has been reported that transfection of MCF-7 and KB cells with human TP does not significantly alter the cytotoxic activity of FdUrd [36,48]. The markedly reduced sensitivity of MCF-7/HYOR cell cultures to the cytostatic activity of FdUrd (and TFT) may therefore suggest that *M. hyorhinis* TP has a better substrate affinity for FdUrd and/or a higher catalytic activity than human TP in the transduced MCF-7/TP cells. Alternatively, our data may also point to a much faster inactivation of the drugs by *M. hyorhinis* TP in the extracellular medium than uptake and activation by the anabolic cellular thymidine kinase in MCF-7 cells. Further studies are needed to clarify the issues.

The markedly decreased incorporation of dThd, TFT and BrdUrd in MCF-7/HYOR nucleic acids and the decreased formation of FdUrd 5′-monophosphate in MCF-7/HYOR cells are in line with our findings that *M. hyorhinis*-encoded TP prevents the cytostatic activity of these drugs (Fig. 4, Table 4). Thus, mycoplasma-infected tumor tissue, a phenomenon seen in a variety of tumors [23–27], may render pyrimidine nucleoside-based anticancer therapy markedly less efficient. Instead, the TP-dependent fluoropyrimidine prodrug capecitabine is efficiently activated by mycoplasmal TP in MCF-7/HYOR tumor cells (Table 2). Indeed, 5′DFUR, which is an intermediate metabolite of capecitabine, was markedly more cytostatic in mycoplasma-infected MCF-7/HYOR cells. The increased cytostatic activity of 5′DFUR in MCF-7/HYOR cell cultures was efficiently annihilated by TPI. Transfection of the human TP gene into cancer cell lines such as MCF-7, KB, HT-29 and PC-9 was also shown to increase the sensitivity to 5′DFUR in comparison to the parental cell lines, providing direct evidence for the role of TP in 5′DFUR sensitivity [49,50]. Thus, successful outcome of capecitabine treatment highly depends on the TP activity of the tumors. Therefore, clinical therapies that upregulate TP expression, such as taxanes and X-ray irradiation, have been shown to improve the effectiveness of capecitabine [8]. Since mycoplasmas such as *M. hyorhinis* abundantly express TP, capecitabine sensitivity may be further increased in tumor tissue containing mycoplasmas.

Taken together, we revealed in this study that mycoplasma species such as *M. hyorhinis* may play a far underestimated detrimental role in compromising the cytostatic activity of certain pyrimidine nucleoside drugs such as FdUrd and TFT, but also in improving the cytostatic activity of TP-dependent prodrugs of 5FU such as capecitabine. In addition, we showed that a highly specific human TP inhibitor (i.e. TPI) is able to efficiently inhibit this mycoplasma-encoded enzyme, and restore the impaired active metabolite formation and cytostatic potential of the pyrimidine nucleoside analogues. TAS-102, a combination of TFT and TPI is currently subject of phase I clinical trials for the treatment of various solid tumors. This therapy seems to enhance the anti-tumor properties and to decrease the toxicity of TFT [18]. An additional advantage of this combination therapy would be that it can also inhibit TP of mycoplasmas that may be associated with the treated cancer, thus preventing a premature breakdown of TFT in human plasma and/or tumor tissue of mycoplasma-infected cancer patients.

Mycoplasmal contaminations are a recurrent problem in the use of cell cultures. Studies pointed out that 10–80% of cell cultures are infected by mycoplasmas [20]. *M. hyorhinis* but also *Mycoplasma orale*, *Mycoplasma arginini*, *M. fermentans* and *Acholeplasma laidlawii* are commonly found in such cell cultures. The sources of mycoplasma contaminations in cell cultures are usually culture reagents (FBS), cross-contamination from infected cell cultures and infections that originate from the laboratory staff [51]. Numerous reports have stated that mycoplasma infections of cell cultures can lead to unreliable experimental results [37,51]. For example, they can alter cell metabolism, protein synthesis, RNA and DNA synthesis, cell membrane composition and cell morphology, and they can trigger cell death [51]. Our data demonstrate that mycoplasma infections may also interfere with the eventual cytostatic activity of a variety of nucleoside analogues. Therefore, laboratories that investigate antitumoral properties of nucleoside analogue drugs should remove mycoplasmas from their cell cultures and establish an effective routine mycoplasma screening program.

Since mycoplasmas are implicated in many diseases and are also associated with cancer [23], we believe that our findings have high relevance for cancer treatment with fluoropyrimidine nucleosides such as FdUrd and TFT. *M. hyorhinis* is frequently found in tissues of gastric, colon, oesophageal, lung and breast

cancer, but not in analogous non-tumorigenic tissue [27]. Our data reveal that the presence of this mycoplasma species markedly compromises the cytostatic efficacy of several fluoropyrimidine nucleoside-based chemotherapeutic agents. Therefore, we believe that pyrimidine nucleoside-based anticancer chemotherapy should be combined with a TP inhibitor and/or a specific antibiotic directed against mycoplasmas to prevent premature inactivation of the drug in the plasma and at the site of the tumor. FdUrd and other pyrimidine nucleoside analogues should be revisited as potential anticancer agents in combination with a TP inhibitor or a mycoplasma-specific antibiotic. Finally, our findings also stress the importance of investigating anticancer drugs in mycoplasma-free cell cultures.

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