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Inhibition of pyrimidine and purine nucleoside phosphorylases by a 3,5-dichlorobenzoyl-substituted 2-deoxy-D-ribose-1-phosphate derivative

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ABSTRACT

The 3,5-dichlorobenzoyl-substituted 2-deoxy-D-ribose-1-phosphate derivative, designated Cf2891, was found to inhibit a variety of pyrimidine and purine nucleoside phosphorylases (NPs) with preference for uridine- and inosine-hydrolyzing enzymes [uridine phosphorylase (UP; EC 2.4.2.3), pyrimidine nucleoside phosphorylase (PyNP; EC 2.4.2.2) and purine nucleoside phosphorylase (PNP; EC 2.4.2.1)]. Kinetic analyses revealed that Cf2891 competes with inorganic phosphate (P_i) for binding to the NPs and, depending on the nature of the enzyme, acts as a competitive or non-competitive inhibitor with regard to the nucleoside binding site. Also, the compound prevents breakdown of pyrimidine analogues used in the treatment of viral infections and cancer. Since NPs are abundantly present in tumor tissue and may be overexpressed due to secondary bacterial infections in immunocompromised patients suffering viral infections, Cf2891 may serve as a lead molecule for the development of inhibitors to be used in nucleoside-based combination therapy.

1. Introduction

Nucleoside phosphorylases (NPs) catalyze the cleavage of the glycosidic bond in purine and pyrimidine nucleosides to yield the free base and the sugar-1-phosphate moiety. Salvage of nucleosides by enzymes such as NPs is a crucial process allowing the cell to circumvent the energy-costly *de novo* pathway in which a variety of amino acids and other precursors are used to produce nucleotides. Structural studies revealed two distinct families of NPs: the nucleoside phosphorylase-I (NP-I) family (containing trimeric or hexameric quarternary structures) and the nucleoside phosphorylase-II (NP-II) family (containing dimeric quaternary structures). Members of the NP-I family are the purine nucleoside phosphorylases (PNP; EC 2.4.2.1) that accept a wide range of purine nucleosides as substrate, and uridine (Urd) phosphorylases (UP; EC 2.4.2.3) that recognize the pyrimidine nucleoside uridine. Members of the NP-II family accept thymidine (dThd) and 2′-deoxyuridine (dUrd) in higher organisms (thymidine phosphorylase; TP; EC 2.4.2.4) and dThd, dUrd and Urd in certain lower organisms (pyrimidine nucleoside phosphorylase; PyNP; EC 2.4.2.2) [1].

NPs may affect the anticancer activity of nucleoside analogues that interfere with the synthesis of nucleic acids either by blocking crucial enzymes in nucleo(s)(t)ide metabolism or by incorporation into the DNA/RNA [2]. Due to the nature of these anticancer molecules, they are subject to activation (often phosphorylation) or degradation (often deamination, dephosphorylation or phosphorolysis) by cellular enzymes (e.g. cytidine/CMP or adenosine/AMP deaminases, 5′-nucleotidases, NPs). The dual role of NPs in cancer treatment has been subject of research for many years. *Escherichia coli* PNP has been studied for its use in suicide gene therapy of cancer as it catalyzes the release of the highly cytotoxic drug 6-methylpurine from its prodrug 9-(2-deoxy-beta-D-ribofuranosyl)-6-methylpurine [3,4]. Conversely, targeting PNP has been proposed as a promising treatment modality for T-cell malignancies [5,6] and a variety of (transition-state) analogue inhibitors of PNP have been described [7]. Similarly, TP and UP are key enzymes in the activation of fluoropyrimidines and their prodrugs such as capecitabine [8,9]. On the other hand TP and UP, which are often upregulated in tumor tissue [10–12] or abundantly expressed in the tumor microenvironment due to bacterial (i.e. mycoplasma) colonisation, are also known to either decrease or increase the cytostatic activity of fluoropyrimidines depending on the structural nature of these drugs [13,14].

Therefore different studies have been performed to investigate the potential of NP inhibitors for the treatment of cancer. BCX-1777 (Forodesine), the most potent PNP inhibitor, has been tested in phase I/II clinical trials for the treatment of hematologic malignancies [15,16]. TAS-102, a combination of TPI, the powerful specific TP

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inhibitor, and the cytostatic agent 5-trifluoromethyl-2′-deoxyuridine (TFT) is under investigation to protect TFT from metabolic breakdown by cellular TP and has entered clinical trials for the treatment of solid tumors [17–19]. Also specific UP inhibitors such as 5-benzylacyclouridine (BAU) have been studied and tested in the clinic: by elevating intracellular Urd levels, UP inhibitors may protect normal tissues from the toxicity caused by fluoropyrimidines and may thus increase their therapeutic index [20–22].

In the present study we describe the 3,5-dichlorobenzoyl analogue of 2-deoxy-D-ribose-1-phosphate (2-dR-1-P) as a novel NP inhibitor. Interestingly, the compound inhibits human UP and mycoplasma PyNP related UP and TP activity as well as human and mycoplasma PNP, but showed markedly less, if any, activity against human TP. Therefore, to the best of our knowledge, the compound represents the first inhibitor combining activity towards the enzymes belonging to the NP-I and NP-II family and may serve as a lead molecule in the development of more powerful and/or broad spectrum NP inhibitors.

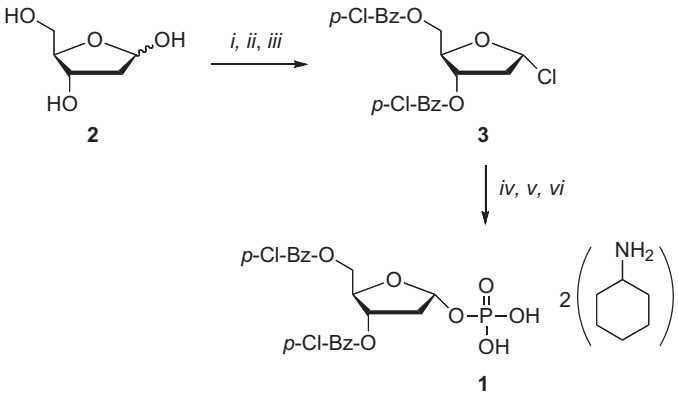

Fig. 1. Chemical synthesis of Cf2891. Reagents and conditions: (i) MeOH, acetyl chloride, 45 min at 25 °C and then pyridine. (ii) Pyridine, 4-dimethylaminopyridine, 4-chlorobenzoyl chloride, 1 h at 0 °C and then 12 h at 25 °C. (iii) HOAc, dry HCl gas, 0 °C, 7–10 min. (iv) 3.5 equiv. of o-H_3PO_4, 1.2 equiv. of $^nBu_3N$, 4 Å MS, acetonitrile, −5 °C. (v) 3.5 equiv. of $^nBu_3N$. (vi) cyclohexylamine.

2. Materials and methods

2.1. Chemicals and enzymes

Nucleosides [dThd, dUrd, Urd, Inosine (Ino)], nucleoside analogues [5-fluoro-2′-deoxyuridine (5-FdUrd); 5-iodo-2′-deoxyuridine (5-IdUrd)] and all inorganic compounds were purchased from Sigma-Aldrich (St. Louis, MO) unless stated differently. Human uridine phosphorylase (UPP1) was kindly provided by Dr. T. Roosild (Nevada Cancer Institute, USA) and human PNP was purchased from ProSpec (NJ, USA). The pMOAL-10T vector containing human *TP* was kindly provided by Dr. R. Bicknell [23]. Human TP and *E. coli* TP were expressed and purified as described [24,25]. A codon-optimized DNA sequence encoding the *M. hyorhinis pyrimidine nucleoside phosphorylase* (PyNP) was synthetically assembled between the *EcoRI* and *NotI* restriction sites of a pIDTsmart vector (Integrated DNA technologies, Coralville, IO). The *M. hyorhinis PNP* gene was isolated from genomic DNA of *M. hyorhinis* (ATCC 17981) and subcloned in the pGEM-T Easy vector (Promega, Madison, WI, USA). The forward (5′-GGATCCCCACACCACATATAAGTG) and reverse (5′-GCGGCCGCTTACTTTATTTCTAAAGC) primers (Invitrogen, Carlsbad, CA) were used to introduce a *BamHI* and *NotI* restriction site, respectively. The mycoplasma *PyNP* and *PNP* genes were subcloned in the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden) and both proteins were expressed as a GST fusion protein in *E. coli* as described by Liekens et al. [25].

2.2. Chemical synthesis of Cf2891

As visualized in Fig. 1, the α-chlorosugar **3** was prepared in three steps from 2-deoxy-D-ribose (Sigma–Aldrich) (**2**) according to a previously described procedure [26]. Reaction of **3** with orthophosphoric acid (3.5 equiv.), and tri(n-butyl)amine (Sigma–Aldrich) (1.2 equiv.) in acetonitrile (Sigma–Aldrich) produced the unstable mono(tributyl)amine phosphate, which was treated with 3.5 equiv. of tri(n-butyl)amine (Sigma–Aldrich) and then with cyclohexylamine (Sigma–Aldrich) to give 3,5-dichlorobenzoyl-α-D-ribose-1-phosphate **1** as a stable bis(cyclohexylamine) salt [27]. The reagents methanol (MeOH), acetyl chloride, pyridine, 4-dimethylaminopyridine and 4-chlorobenzoyl chloride were purchased from Sigma–Aldrich.

2.3. Determination of the inhibitory effect of Cf2891 against nucleoside phosphorylases

A variety of inhibitor (Cf2891) concentrations were added to a reaction mixture that contained 100 μM substrate (dThd, Urd, Ino, 5-FdUrd or 5-IdUrd) in phosphorolysis buffer (10 mM Tris–HCl, pH 7.6; 1 mM EDTA; 150 mM NaCl and 2 mM potassium phosphate (all purchased from Sigma–Aldrich)). Next, the reaction mixture was exposed to different phosphorolytic enzymes (UPP1, mycoplasma PyNP, human TP, *E. coli* TP, human PNP and mycoplasma PNP) for 20 min at 37 °C ascertaining a linear substrate conversion within this incubation period. Samples were heated at 95 °C for 3 min to terminate the reaction by inactivating the enzyme and were rapidly cooled on ice for 15 min and cleared by centrifugation at 13,000 rpm 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). The separation was performed by a gradient from 100% buffer B (50 mM NaH_2PO_4 (Acros Organics, Geel, Belgium); 5 mM heptane sulfonic acid (Sigma–Aldrich); pH 3.2) to 50% buffer B and 50% acetonitrile (ACN) (BioSolve BV, Valkenswaard, the Netherlands) (10 min linear gradient of 100% buffer B to 98% buffer B + 2% ACN; 10 min linear gradient to 50% buffer B + 50% ACN; 10 min 50% buffer B + 50% ACN; 5 min linear gradient to 100% buffer B followed by equilibration at 100% buffer B for 10 min). UV-based detection of the nucleobases was performed at the specific wavelength, optimal for nucleobase absorption. IC_{50}-values, defined as the inhibitor concentration needed to reduce enzymatic phosphorolysis by 50%, were calculated.

2.4. Phosphate dependency of Cf2891

The inhibitory activity of Cf2891 was also studied in the presence of different concentrations of inorganic phosphate (Sigma–Aldrich). Urd (100 μM) phosphorolysis by *M. hyorhinis* PyNP and human UPP1, and dThd (100 μM) phosphorolysis by *M. hyorhinis* PyNP was studied in phosphorolysis-buffer containing varying concentrations of P_i (0.4–50 mM) in the presence or absence of the inhibitor. Nucleoside-to-nucleobase conversion was determined after 30 min incubation at 37 °C as described above.

2.5. Kinetic analyses

The inhibitory effect of different concentrations of Cf2891 (25–1000 μM) was evaluated against *M. hyorhinis* PyNP, human UPP1, *M. hyorhinis* PNP and human PNP at varying concentrations of nucleoside substrate (dThd: 100–10,000 μM; Urd: 50–2000 μM; Ino: 100–5000 μM) in the presence of a fixed concentration of P_i (2 mM), or at varying concentrations of P_i (HUPP1: 0.5–20 mM; *M. hyorhinis* PyNP and PNP: 2–200 mM; Human PNP: 0.1–10 mM) in

the presence of a saturating concentration of the natural nucleoside substrates. Determination of the nucleoside-to-nucleobase conversion was performed as described above. Kinetic parameters (K_m and V_{max}) were determined by means of non-linear regression analysis (using GraphPad Prism5) and data were visualized by double-reciprocal (Lineweaver–Burk) plots to determine the K_i values.

3. Results

3.1. Cf2891 inhibits the activity of a broad range of human and bacterial phosphorolytic enzymes

The inhibitory capacity of Cf2891 towards enzymatic phosphorolysis of different natural nucleosides and nucleoside analogues was determined. Table 1 displays the IC_{50} values (defined as the inhibitor concentration needed to reduce enzymatic phosphorolysis by 50%) of Cf2891 towards different human and bacterial NPs. In the presence of 100 μM of the natural substrate Urd, Cf2891 efficiently inhibited uridine phosphorolysis by human UPP1 (IC_{50} = 63 μM). Also the thymidine and uridine phosphorylase activity of mycoplasma (*M. hyorhinis*) PyNP was inhibited by the compound in the presence of 100 μM dThd or Urd (IC_{50} = 188 μM and 113 μM, respectively). The mycoplasma PyNP-mediated phosphorolysis of 5-FdUrd and 5-IdUrd, both clinically approved nucleoside analogues for the treatment of cancer and viral (herpes) infections, respectively, was inhibited to a similar extent (IC_{50} = 128 μM and 159 μM, respectively). The phosphorolysis of dThd and 5-IdUrd by *E. coli* TP was inhibited to a minor extent (IC_{50} = 748 μM and 937 μM, respectively) whereas the activity of human TP was not affected by the inhibitor at the highest concentration tested (no significant inhibition observed at Cf2891 = 1000 μM). When examined against purine nucleoside phosphorylases, inhibition of inosine (100 μM) phosphorolysis by human and mycoplasma PNP was also observed (IC_{50} = 372 and 270 μM, respectively).

3.2. The inhibitory activity of Cf2891 is highly dependent on the levels of inorganic phosphate (P_i)

M. hyorhinis PyNP-mediated dThd and Urd phosphorolysis and human UPP1-mediated Urd phosphorolysis were monitored in the presence of different concentrations of P_i. Inhibition of *M. hyorhinis* PyNP-mediated dThd (Fig. 2A) and Urd (Fig. 2B) phosphorolysis by Cf2891 (250 μM) dose-dependently decreased with increasing P_i levels. When compared with a control condition, thymine and uracil formation was inhibited by ~90% and 70%, respectively, at the lowest P_i concentration tested (0.4 mM) whereas phosphorolysis was only inhibited by 15% under saturating P_i levels (50 mM). Likewise, uridine phosphorolysis catalyzed by human UPP1 was inhibited by ~70% in the presence of 100 μM Cf2891 at P_i 0.4 mM but the inhibition dose-dependently decreased to <5% when P_i levels increased to saturating concentrations (50 mM) (Fig. 2C).

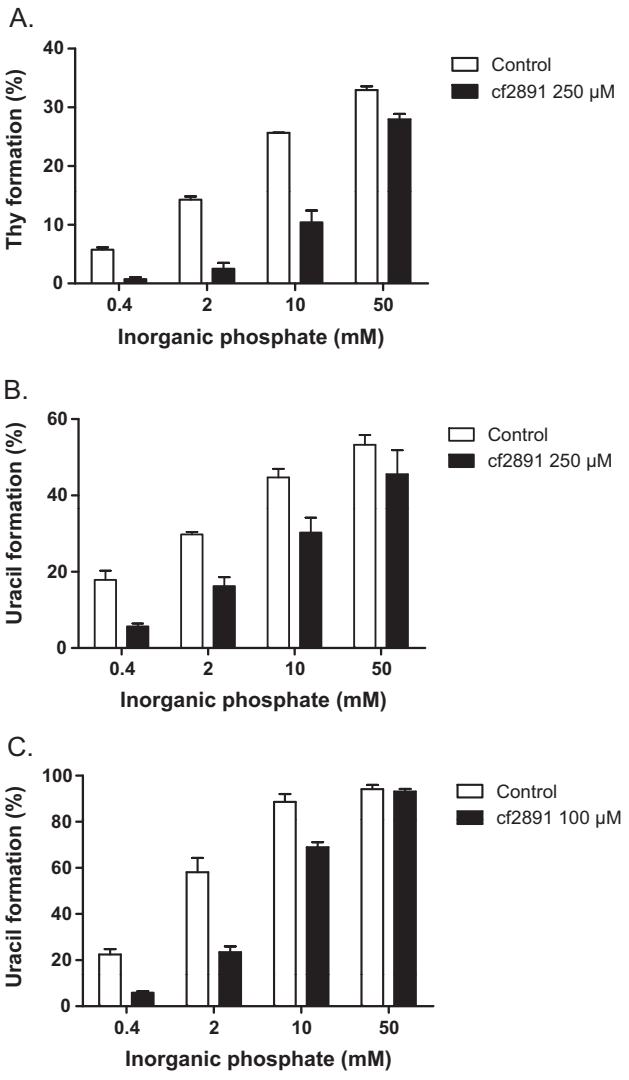

Fig. 2. Inorganic phosphate dependent inhibition of NPs by Cf2891. Inhibition of *M. hyorhinis* PyNP-mediated dThd (A) and Urd (B) phosphorolysis and human UPP1-mediated Urd phosphorolysis (C) by Cf2891 in the presence of different P_i concentrations. Data are the mean of at least 2 independent experiments (±SD).

These data point to a direct competition of the inhibitor with (endogenous) P_i levels.

3.3. Kinetic analysis of the inhibition of nucleoside phosphorolysis

M. hyorhinis PyNP and PNP activity and human UPP1 and PNP activity were determined in the presence of varying concentrations of inhibitor, nucleoside substrate and co-substrate P_i. Using linear regression analysis of the data points, Cf2891 was found to behave

Table 1
Inhibition of different phosphorolytic enzymes by Cf2891.

	IC_{50}[a] (μM)					
Substrate	Human UPP1	*M. hyorhinis* PyNP	Human TP	*E. coli* TP	Human PNP	*M. hyorhinis* PNP
Urd	63 ± 15	188 ± 52	–	–	–	–
dThd	–	113 ± 37	>1000	748 ± 45	–	–
5-FdUrd	–	128 ± 13	>1000	>1000	–	–
5-IdUrd	–	159 ± 18	>1000	937 ± 55	–	–
Ino	–	–	–	–	372 ± 46	270 ± 33

–: no substrate.
[a] Inhibitor concentration needed to reduce the enzymatic phosphorolysis by 50%.

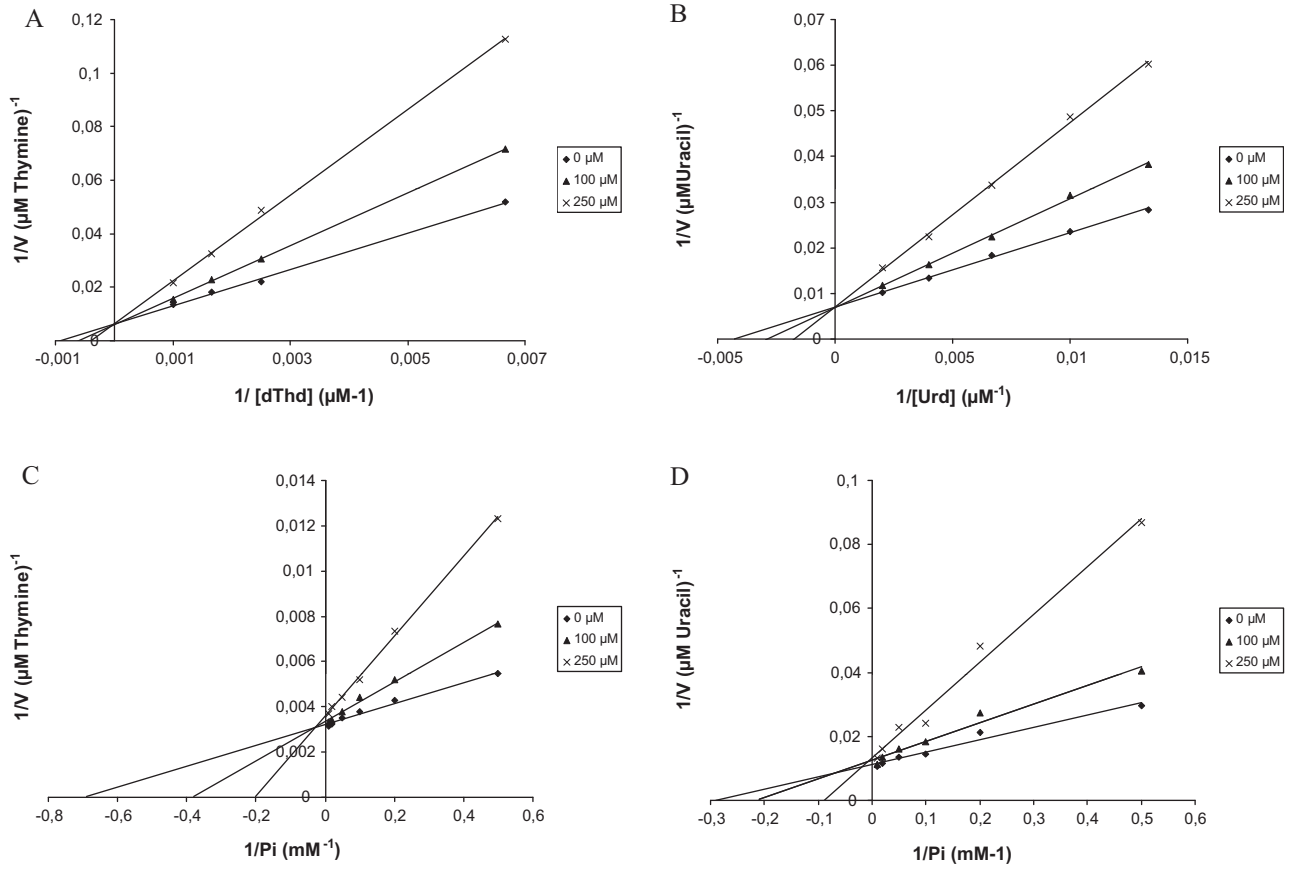

Fig. 3. Kinetic analysis of mycoplasma PyNP inhibition by Cf2891. Double-reciprocal (Lineweaver–Burk) plots for the inhibition of *M. hyorhinis* PyNP-mediated dThd (A) and Urd (B) phosphorolysis in the presence of a fixed P_i concentration and dThd (C) and Urd (D) phosphorolysis in the presence of varying P_i concentrations (but fixed dThd and Urd concentrations). Plots represent the data obtained in a representative experiment.

as a competitive inhibitor of *M. hyorhinis* PyNP with regard to the natural substrates Urd and dThd (Fig. 3A and B). The compound also competitively inhibited PyNP-mediated dThd and Urd phosphorolysis in the presence of varying concentrations of P_i (Fig. 3C and D). K_i/K_m ratios were calculated as a measurement for the inhibitory potential of the compound (Table 2). *M. hyorhinis* PyNP showed a ~13- and ~2-fold higher affinity for the inhibitor when compared with the natural substrates dThd (K_i/K_m = 0.076) and Urd (K_i/K_m = 0.56), respectively (Table 2A). The affinity of the enzyme for the inhibitor was found to be ~20- to 25-fold higher than for P_i (Table 2B).

Table 2
Inhibitor kinetics.

Enzyme	Substrate[a]	K_m (µM)	K_i (µM)	K_i/K_m
(A)				
M. hyorhinis PyNP	dThd (P_i)	1093 ± 244	83 ± 1.8	0.076
	Urd (P_i)	310 ± 89	173 ± 46	0.56
Human UPP1	Urd (P_i)	239 ± 14	194 ± 57	0.81
Human PNP	Ino (P_i)	491 ± 37	441 ± 97	0.90
M. hyorhinis PNP	Ino (P_i)	1796 ± 44	280 ± 14	0.16
(B)				
M. hyorhinis PyNP	P_i (dThd)	1900 ± 450	91 ± 37	0.048
	P_i (Urd)	3300 ± 1500	128 ± 31	0.039
Human UPP1	P_i (Urd)	1300 ± 170	105 ± 24	0.081
Human PNP	P_i (Ino)	742 ± 175	115 ± 0.7	0.16
M. hyorhinis PNP	P_i (Ino)	6660 ± 1013	N.D.[b]	–

[a] Natural substrate (saturating co-substrate between parentheses).
[b] Value was not determined due to insolubility of the compound at the highest required concentration.

Cf2891 was found to be a non-competitive inhibitor of human UPP1 with regard to the natural nucleoside substrate Urd (Fig. 4A). The enzyme displayed comparable affinity for the nucleoside and inhibitor (K_i/K_m = 0.81). However competitive inhibition with regard to P_i (Fig. 4B) was observed with a ~12-fold higher affinity of UPP1 for Cf2891 when compared with P_i (K_i/K_m = 0.081). Similar to human UPP1, Cf2891 showed non-competitive inhibition of human PNP-mediated inosine phosphorolysis (Table 2A; Fig. 5A) with regard to the nucleoside (K_i/K_m = 0.9) and competitive inhibition with regard to P_i (K_i/K_m = 0.16) (Table 2B; Fig. 5B).

For mycoplasma PNP, competitive inhibition with regard to inosine was observed (K_i/K_m = 0.16) (Table 2A; Fig. 6). Since the compound was not soluble at the highest concentrations required to determine a reliable K_i value for mycoplasma PNP-mediated inosine phosphorolysis at varying P_i concentrations, no kinetic analysis could be performed for competition of Cf2891 with inorganic phosphate (Table 2B).

4. Discussion

Many studies have reported the design and evaluation of nucleoside phosphorylase (NP) inhibitors. The vast majority of such molecules are nucleoside analogues containing chemical modifications in the base part, sometimes resulting in highly potent enzyme inhibitors such as TPI, BAU and forodesine. These molecules are highly specific for well-defined nucleoside phosphorylases (i.e. TPI for TPs, BAU for UPs and forodesine for PNPs) and their use in anticancer therapy is currently under investigation. In an attempt to generate a broad spectrum NP inhibitor, we designed a molecule that lacks a base part but solely represents a

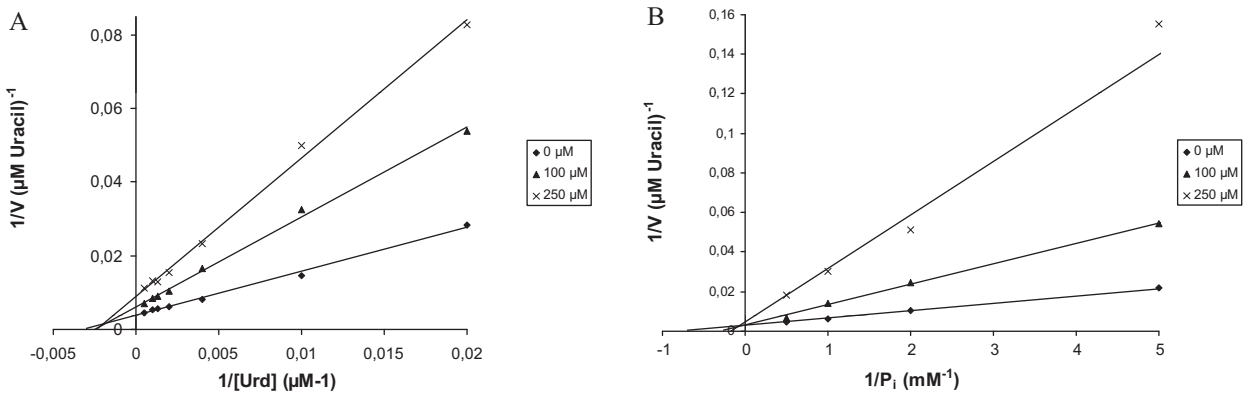

Fig. 4. Kinetic analysis of human UPP1 inhibition by Cf2891. Double-reciprocal (Lineweaver–Burk) plots for the inhibition of human UPP1-mediated Urd phosphorolysis by Cf2891 in the presence of a fixed P_i concentration (A) and in the presence of a fixed Urd concentration (varying P_i concentration) (B). Plots represent the data obtained in a representative experiment.

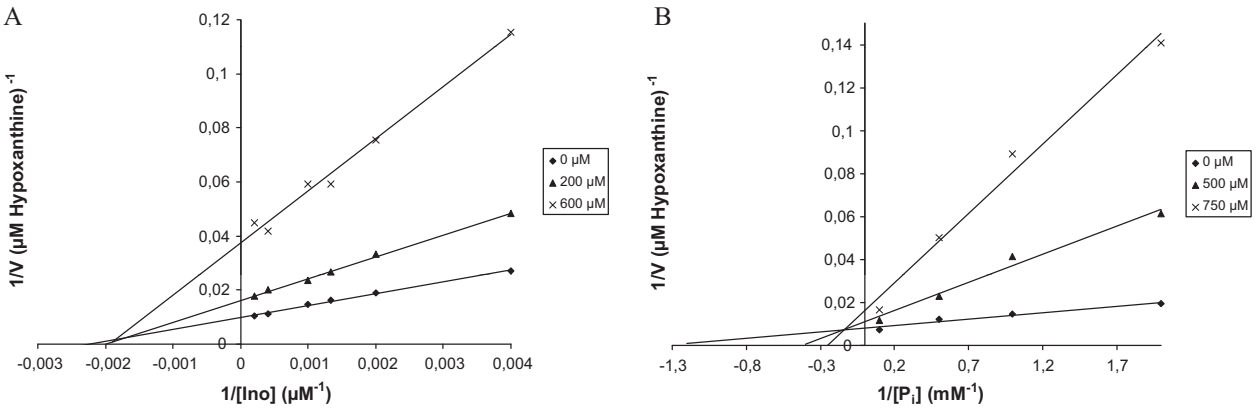

Fig. 5. Kinetic analysis of human PNP inhibition by Cf2891. Double-reciprocal (Lineweaver–Burk) plots for the inhibition of human PNP-mediated Ino phosphorolysis by Cf2891 in the presence of a fixed P_i concentration (A) and in the presence of a fixed Ino concentration (varying P_i concentration) (B). Plots represent the data obtained in a representative experiment.

substituted pentose sugar phosphate. The 5-iodinated analogue of ribose-1-phosphate was previously shown to inhibit different enzymes that use 5-methylthio-ribose-1-P, Rib-1-P, Glc-1-P or P-Rib-1-PP as a substrate, including PNP and hypoxanthine-guanine phosporibosyltransferase [28]. In the present study, the 3,5-dichlorobenzoyl-substituted analogue of ribose-1-phosphate (Cf2891) was found to inhibit the phosphorolysis of pyrimidine and purine nucleosides by a wide variety of NPs derived from both human and bacterial origin. Cf2891 was found to compete with inorganic phosphate for binding to all NPs tested. Competitive inhibition of mycoplasma PyNP and PNP with regard to the nucleosides Urd, dThd and Ino was observed and non-competitive inhibition of human UPP1 and PNP with regard to the natural substrates Urd and Ino. In fact, Cf2891 can be considered as a 3,5-modified product of the NP reaction. Nucleobase recognition is no longer necessary but the C-1-phosphate of the inhibitor most likely makes use of the free phosphate binding site in the enzyme. This explains the competitive inhibition of all susceptible NPs in the presence of variable P_i concentrations. Kinetic studies revealed that mycoplasma PyNP and PNP as well as human UPP1 and PNP show equal or more affinity for Cf2891 when compared with the natural nucleoside substrate but 10–25 fold higher affinity for the inhibitor when compared with inorganic phosphate. Since intracellular P_i levels are known to range from 0.5 to 2 mM [29], NPs most likely are never saturated with P_i *in vivo*, making them susceptible to competitive inhibitors such as Cf2891.

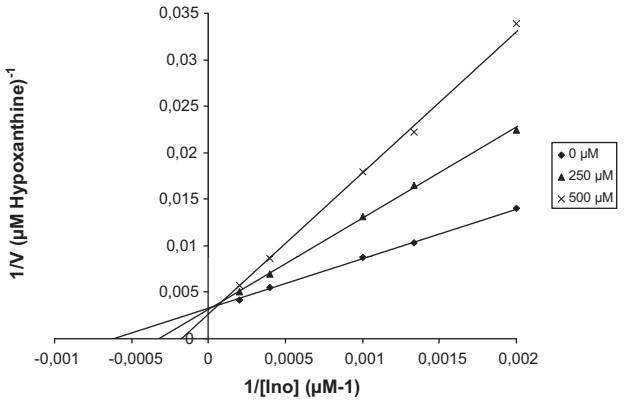

Fig. 6. Kinetic analysis of mycoplasma PNP inhibition by Cf2891. Double-reciprocal (Lineweaver–Burk) plot for the inhibition of *M. hyorhinis* PNP-mediated Ino phosphorolysis by Cf2891 in the presence of a fixed P_i concentration. Plot represents the data obtained in a representative experiment.

Suprisingly Cf2891, which has a 2′-deoxyribose configuration, shows less, if any, inhibitory potential against the NPs that exclusively recognize dThd (human and *E. coli* TP). It would therefore be of particular interest to synthesize the ribose derivative of Cf2891 to search for more potent and selective inhibitors of ribonucleoside phosphorylases such as UPs, PNPs and PyNPs. Also other and additional substituents on the 3,5-aryl moieties should be designed and considered for further modifications of the lead molecule.

The treatment of cancer and many viral infections [caused by e.g. human immunodeficiency virus, herpes simplex virus, varicella-zoster virus, cytomegalovirus, hepatitis C virus, hepatitis B virus] is largely based on the use of nucleoside-derived therapeutics [2,30]. Due to the nature of these drugs they may be subject to enzymatic inactivation (e.g. deamination, dephosphorylation or phosphorolysis) by enzymes involved in nucleo(s)(-t)ide catabolism. Several studies show that the expression of NPs is upregulated in tumor cells [10–12] and bacterial NPs may be abundantly present in the tumor microenvironment due to the preferential colonisation of malignant tissues by mycoplasmas [31–37]. Also immunocompromised patients (e.g. patients suffering from AIDS) are known to be prone to mycoplasma infections [38,39]. Mycoplasma encoded NPs such as PyNP from *M. hyorhinis* were found to severely compromise the cytostatic and antiviral activity of therapeutic pyrimidine analogues *in vitro* [40]. Therefore we hypothesized that treatment with nucleoside analogues that are subject to NP-mediated phosphorolysis may be suboptimal in patients suffering cancer or viral infections [14]. We show here that Cf2891 efficiently inhibits the enzymatic breakdown of therapeutic nucleoside analogues such as 5-FdUrd, used in the treatment of colorectal cancer, and 5-IdUrd, used as a topical antiherpetic agent. Nucleoside analogue-based chemotherapy may thus be optimized by the addition of a NP inhibitor. Since the target enzymes showed superior affinity for Cf2891 when compared with their natural substrates, the inhibitor may represent a lead compound for inhibiting a much broader spectrum of NPs than the currently available NP inhibitors.

Acknowledgements

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