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Human Pyrimidine Nucleotide Biosynthesis as a Target for Antiviral Chemotherapy

Ayse Okesli, **Chaitan Khosla**, and **Michael Bassik**

Departments of Chemistry, Genetics, and Chemical Engineering, and Stanford ChEM-H, Stanford University, Stanford CA 94305

Abstract

The development of broad-spectrum, host-acting antiviral therapies remains an important but elusive goal in anti-infective drug discovery. To replicate efficiently, viruses not only depend on their hosts for an adequate supply of pyrimidine nucleotides, but also up-regulate pyrimidine nucleotide biosynthesis in infected cells. In this review, we outline our understanding of mammalian *de novo* and salvage metabolic pathways for pyrimidine nucleotide biosynthesis. The available spectrum of experimental and FDA-approved drugs that modulate individual steps in these metabolic pathways is also summarized. The logic of a host-acting combination antiviral therapy comprised of inhibitors of dihydroorotate dehydrogenase and uridine/cytidine kinase is discussed.

Introduction

Pyrimidine nucleosides are heterocyclic aromatic metabolites that include uridine, cytidine and thymidine. In addition to their fundamental role in nucleic acid biosynthesis, they are required for carbohydrate and lipid metabolism. For example, a number of glycosyltransferases utilize UDP-sugars, while CDP-diacylglycerol is an intermediate in the biosynthesis of glycerophospholipids. Although pyrimidine analogs such as azidothymidine (AZT), 5-fluorouracil (5-FU), and arabinosylcytosine (ara-C) have been used to target HIV reverse transcriptase or as anti-cancer chemotherapeutic drugs for decades, the potential for rationally targeting human pyrimidine nucleoside metabolism for antiviral chemotherapy has not been generally recognized. Here we review the rationale for such a chemotherapeutic strategy as well as the relevant features of mammalian pyrimidine nucleoside metabolism and its regulation.

Pyrimidine nucleotide biosynthesis through *de novo* and salvage pathways

Mammalian cells derive pyrimidine nucleotides through a combination of *de novo* biosynthesis and salvage [1]. *De novo* biosynthesis is initiated by a multifunctional enzyme

Address correspondence to: Michael Bassik: bassik@stanford.edu Chaitan Khosla: khosla@stanford.edu.

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(CAD) harboring carbamoyl phosphate synthase, aspartate transcarbamoylase, and dihydroorotase activities [2]. CAD uses an equivalent of L-glutamine, aspartate, and bicarbonate along with two equivalents of ATP to make dihydroorotate (DHO) (Figure 1). A mitochondrial membrane protein, dihydroorotate dehydrogenase (DHODH), then reduces DHO to orotic acid while transferring $2e^-$ to Coenzyme Q (CoQ, ubiquinone) [3]. Not only does DHODH catalyze the first committed step in *de novo* pyrimidine nucleoside biosynthesis, but it also links this pathway to the electron transport chain of aerobic respiration. Orotic acid is converted into uridine monophosphate (UMP) by a bifunctional protein, uridine monophosphate synthetase (UMPS). The N-terminal domain of UMPS transforms orotic acid into orotidylate (OMP) using phospho-α-Dribosyl-1-pyrophosphate (PRPP) as a cosubstrate, while its C-terminal OMP decarboxylase converts OMP into UMP [4]. UDP and UTP are synthesized by cytidine monophosphate kinase (CMPK) and nucleoside-diphosphate kinase (NDPK), respectively [5,6]. UTP is converted into CTP by CTP synthetase (CTPS) in an ATP dependent reaction that uses glutamine as an amine donor [7]. Alternatively, UDP and CDP are deoxygenated into deoxy-UDP (dUDP) and dCDP, respectively, by ribonucleotide reductase (RNR), and further phosphorylated by NDPK [8]. To avoid misincorporation into DNA, dUTP is rapidly broken down by dUTPase into dUMP. dUMP is a substrate of thymidylate synthase, yielding deoxy-TMP (dTMP) that can be phosphorylated into dTTP [9]. Thus, the *de novo* biosynthetic pathway in mammals is capable of supplying all pyrimidine ribonucleotides (CTP, UTP) and deoxyribonucleotides (dCTP, dTTP) for RNA and DNA biosynthesis, respectively.

In addition to *de novo* biosynthesis, pyrimidine nucleotides can also be salvaged from intracellular nucleic acid degradation or from extracellular nucleosides, which circulate in the bloodstream. The latter pathway depends on several nucleoside transport channels and pumps in mammalian cells. The relative importance of *de novo* biosynthesis and salvage varies from organ to organ and is also highly dependent on the physiological state of cells. RNA catabolism yields UMP and CMP, which can be converted into the corresponding NTPs via the successive action of CMPK1 and NDPK. With a plasma concentration of ~5 μM, uridine is the dominant circulatory nucleoside in mammals [10]; the plasma concentrations of all other pyrimidine nucleosides are at least an order of magnitude lower [11], and are therefore insufficient to support cellular demands of the corresponding nucleotides via direct salvage. Uridine/cytidine kinase (UCK) converts transported pyrimidine nucleosides into the corresponding NMPs, which can be further phosphorylated and modified as discussed above. Since both *de novo* biosynthesis as well as intracellular and extracellular salvage require CMPK1 activity, this enzyme is essential for pyrimidine utilization in all cells.

As an alternative to salvage, pyrimidine nucleosides can also be irreversibly degraded. Uridine and cytidine catabolism is initiated by the action of uridine phosphorylase (UPase) and cytidine deaminase, respectively, giving rise to uracil, while thymidine phosphorylase releases thymine from thymidine. In principle, these phosphorylases can also catalyze the reverse reactions to convert circulatory bases into nucleosides (as in OMP biosynthesis), although mammals appear to predominantly utilize these enzymes in the catabolic direction [12].

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Intracellular regulation of pyrimidine nucleotide biosynthesis

The multifunctional CAD protein is the primary site for regulation of *de novo* pyrimidine biosynthesis. Transcription factors such as Myc are known to induce its gene expression [13]. The enzyme is activated by MAP kinase-catalyzed phosphorylation before the S-phase of the cell cycle, and is inhibited by protein kinase A-catalyzed phosphorylation at a distinct site at the end of S-phase [14,15]. CAD is also activated by phosphorylation at a third site by the mammalian target of rapamycin complex 1 (mTORC1) or the ribosomal protein S6 kinase 1 (p70S6K), thus enabling post-translational control in response to increased anabolic activity in the cell [16,17].

The importance of coordinately regulating intracellular pyrimidine nucleotide biosynthesis at multiple sites is underscored by our recent observation that genetic knockout of a negative regulator of mTORC1 activity sensitizes cells to pharmacological inhibition of DHODH with the small molecule GSK983 [3]. Similarly, the activities of mTORC1 and p70S6K are post-translationally regulated in response to the extracellular availability of uridine [18].

The activity of UCK, which plays a pivotal role in pyrimidine nucleoside salvage, is also subject to both negative regulation by CTP and UTP (i.e., the ultimate pathway products) and positive regulation by ATP. Such dual control is achieved through changes in the quaternary structure of UCK; CTP and UTP are competitive inhibitors (K_i ~ 6 μM [19]) that stabilize its inactive monomeric state, whereas ATP allosterically stabilizes UCK as an active tetramer [20].

Finally, CMPK1, which sits at the crossroads between *de novo* biosynthesis and intracellular/extracellular salvage is subject to feedback regulation of its activity by CTP, UTP and dCTP, but not dTTP [5]. Moreover, *in vitro* analysis has revealed a need for reducing agents to maintain its catalytic activity, suggesting that the intracellular redox potential may also play a significant role in metabolic flux control at this step [5].

Regulation of uridine concentration in the bloodstream

As discussed above, uridine plays a unique role as a reservoir of circulating pyrimidine nucleosides in mammals. Its plasma concentration is therefore subject to tight regulation. Indeed, plasma uridine levels are maintained within a narrow range in healthy humans even after fasting [21] or uridine administration [22]. The role of plasma uridine as a system-wide control variable is further underscored by two observations. First, oral administration of large doses of CDP-choline, a bioavailable form of cytidine, increased plasma uridine levels without significantly altering those of either cytidine or choline [22]. Second, a sharp increase in systemic uridine demand has a relatively modest effect on the concentration of plasma uridine, presumably due to its replenishment from reservoir organ(s). For example, blocking *de novo* pyrimidine synthesis by DHODH inhibition (in mice [23]) or CAD inhibition (in humans [24]) results in markedly higher use of the salvage pathway but only modest perturbation of plasma uridine levels.

The liver has been suggested as a potential site for regulating plasma uridine based on the observation that uridine is cleared in a single pass through the liver and is replaced by *de*

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novo synthesized uridine also from the liver [25], but the mechanistic logic of this unusual exchange process is unknown. Oral administration of glucose also increases the levels of uridine in the bloodstream [26], presumably due to the acute need for UDP-glucose during glycogen synthesis in the liver and muscle [27].

Plasma uridine levels are also regulated by the degradative activity of UPase, as well as by cellular uptake mechanisms that involve both facilitated diffusion and Na^+-dependent active transport. Genetic and pharmacological inhibition of UPase in mice led to a major elevation of uridine concentrations in the blood (6-fold), lung and gut (5 to 6-fold), and liver and kidney (2 to 3-fold) [12]. Cellular uptake of uridine can be promoted via several nucleoside transporters [28].

Pharmacological tools to modulate pyrimidine nucleotide biosynthesis in humans

Due to their diverse metabolic roles, pyrimidine nucleotide biosynthesis inhibitors have been used to treat a variety of diseases. Many such drugs are nucleoside analogs. Once transported into the cells by facilitated diffusion, they are phosphorylated and either incorporated into DNA or RNA, or they can inhibit host or pathogen enzymes such as polymerases. Table 1 lists approved or experimental drugs that modulate *de novo* biosynthetic or salvage pathways in humans.

Modulators of the *de novo* pathway

Although there are no FDA-approved inhibitors of CAD, N-phosphonacetyl-L-aspartate (PALA) is a bisubstrate analog inhibitor of aspartyl transcarbamoylase that has been introduced into human clinical trials. It failed to show efficacy as monotherapy or in combination with other agents in Phase II clinical trials on cancer patients [29–31]. In contrast, DHODH inhibitors such as teriflunomide (or its prodrug, leflunomide) and brequinar have been successfully used as immunosuppressive agents in rheumatoid arthritis and multiple sclerosis patients. The clinical benefit of DHODH inhibitors is thought to arise from reduced proliferation of activated T and B lymphocytes by decreasing pyrimidine pools in both cell types [32]. Prolonged administration of both leflunomide and brequinar causes hepatic microvesicular steatosis (a.k.a., lipid accumulation in the liver), a condition that can also be induced by artificially manipulating plasma uridine levels and is reversed by exogenous uridine administration [33,34]. DHODH inhibitors were also recently shown to initiate differentiation in multiple acute myeloid leukemia (AML) subtypes [35].

Inhibitors of CAD and DHODH have broad-range anticancer and antiviral effects *in vitro* [3,36], and have been tested in a range of clinical trials [37,38]. However, *in vitro* efficacy has not, for the most part, been translated *in vivo*, presumably due to the ability of the body to maintain a robust and relatively constant uridine supply for cellular salvage of pyrimidine nucleotides. The reason why uridine salvage is unable to neutralize the immunosuppressant effects of DHODH inhibitors is unknown. It is possible that the salvage pathway is less dominant in lymphocytes compared to the *de novo* synthesis pathway.

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Pyrazofurin (PZF) is a nucleoside analog that inhibits OMP decarboxylase in the *de novo* pathway. It has been tested in clinical trials for the treatment of various cancers but failed to proceed beyond phase II trials due to toxicity and lack of efficacy [39].

Modulators of shared (d)NTP salvage steps

Nucleoside transport through membrane channels and pumps is the first step in pyrimidine salvage. Because these transporters recognize all four bases, their inhibition disturbs both dNTP and NTP salvage. FDA-approved nucleoside channel inhibitors such as dipyridamole (DP) [40] and dilazep [41] have been used to treat stroke due to their ability to block adenosine uptake by platelets, endothelial cells, and erythrocytes [42].

Modulators of the NTP-specific steps

Cyclopentenyl cytosine (CPE-C), a pyrimidine analogue, is an inhibitor of CTP synthetase, and has both antiviral and anticancer activity. Because CTP synthetase activity is upregulated in many cancers [43], CPE-C was tested in a Phase I clinical trial for solid tumors [44], where 5 out of 26 patients experienced unexplained cardiotoxicity. CPE-C has also shown significant activity against both DNA and RNA viruses *in vitro* including HSV and influenza virus (Hong Kong flu) [45].

Diazo-5-oxo-L-norleucine (DON) is a glutamine mimic that inhibits several enzymes involved in nucleotide biosynthesis including CAD, CTP synthetase, and guanosine monophosphate synthetase. Although DON was tested in phase I/II clinical trials for the treatment of cancer [46,47], its therapeutic index was inadequate for further development.

Notwithstanding the potential to modulate pyrimidine nucleotide biosynthesis by targeting the kinases in the salvage pathway, to our knowledge none of these enzymes (UCK, CMPK, NDPK) have inhibitors that have entered human clinical trials.

Modulators of the dNTP specific steps

Ribonucleotide reductase (RNR) catalyzes a crucial step of *de novo* DNA synthesis by converting ribonucleoside diphosphates (ADP, GDP, UDP, CDP) into their deoxyribonucleoside counterparts. Because tight control of the dNTP pool is essential for cellular homeostasis, RNR inhibitors have been widely used to treat cancers. They include fludarabine [48–50], cladribine [51], gemcitabine [52–54], and clofarabine [55], although these compounds also block other steps in DNA synthesis in addition to RNR activity (Table 1).

Thymidylate synthase, followed by nucleoside diphosphate kinase and UTPase [56], has been targeted by the widely used nucleoside analog 5-fluorouracil (5-FU) and its prodrug, capecitabine [57]. Inside the cell 5-FU is processed into 5-fluoro-2′-deoxyuridine monophosphate (FdUMP), a covalent inhibitor of thymidylate synthase. Drug toxicity is mitigated with a recently FDA-approved uridine prodrug, uridine triacetate [58]. Alternatively, clinical trials have also been conducted to mitigate the toxicity of 5-FU by co-administration with dipyridamole [59]. A related molecule, trifluorothymidine, is also believed to inhibit thymidylate synthase [60] in addition to blocking viral replication or cell

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growth by incorporation into viral or host DNA, respectively [61]. It is used in eye drops for the treatment of herpes virus, and is also undergoing clinical trials for metastatic colorectal, colon cancers, and solid tumors.

Thymidine kinase, which converts dTMP into dTDP, is also an important therapeutic target, because it facilitates the incorporation of unnatural thymidine analogs into DNA. Examples of clinically useful thymidine kinase inhibitors include AZT and stavudine (anti-HIV), and idouridine (anti-herpes).

The enzyme dUTPase, which converts dUTP into dUMP and pyrophosphate, is inhibited by TAS-114, a first-in-class oral fluoropyrimidine that prevents the degradation of another fluoropyrimidine used in combination [62]. TAS-114 also moderately inhibits dihydropyrimidine dehydrogenase, the initial step in pyrimidine catabolism. Analogously, tipiracil, a thymidine phosphorylase inhibitor, is also clinically used to prevent the catabolism of other fluoropyrimidine nucleoside drugs including trifluorothymidine [63]. Curiously, analogous drugs that block the catabolism of therapeutic cytidine analogs have not yet been developed [64].

Implications for antiviral chemotherapy

Whereas most drugs that block pyrimidine nucleotide biosynthesis are targeted at cancer chemotherapy or immunosuppression, a deeper understanding of these metabolic pathways in humans could also be the foundation for the design of novel antiviral therapies. When viruses infect host cells, they up-regulate nucleotide biosynthetic flux [65]. Therefore, not only would inhibitors of nucleotide biosynthesis have the potential to neutralize a wide range of viruses, but their likelihood of eliciting drug-resistant mutants may also be lower than drugs targeted at viral proteins.

Although inhibitors of *de novo* pyrimidine nucleotide synthesis are known to exhibit broad-spectrum antiviral activity *in vitro* [66,67], they are ineffective *in vivo* due to efficient salvage of exogenous uridine. In this regard, our recent discovery that blocking the UCK isozyme, UCK2, sharply sensitizes cells toward DHODH inhibitors in the presence of a non-limiting uridine supply opens a new door for designing a combination antiviral agent comprised of a DHODH and a UCK2 inhibitor antiviral [3]. Inhibition of both the *de novo* and the salvage pyrimidine synthesis could be particularly effective at limiting the fast proliferation of RNA viruses. In fact, a combination regimen containing PALA and dipyridamole has been tested in clinical trials for the treatment of cancer, albeit with limited efficacy [68]. Weak activity could be due to inefficient inhibition of *de novo* pyrimidine synthesis by a CAD inhibitor as opposed to a DHODH inhibitor.

Since CMPK1 inhibition was also shown to sensitize cells to DHODH inhibitors [3], a similar outcome might also be achieved with a CMPK1 inhibitor. Indeed, given the location of CMPK1 at the convergence point of *de novo* biosynthesis and salvage, a sufficiently potent CMPK1 inhibitor could also be an effective form of monotherapy. However, unlike a DHODH/UCK2 combination agent, a CMPK1 inhibitor would also be expected to block the salvage of CMP and UMP derived from RNA degradation, and may therefore have a

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narrower therapeutic window. Future studies along either direction must await the development of medicinally appropriate small molecule inhibitors of UCK2 and CMPK1.

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Highlights

- Human pyrimidine nucleotide biosynthesis has been targeted for the treatment of many diseases.
- Chemotherapy combining DHODH and UCK inhibitors can be a broad-spectrum antiviral.
- Targeting the host cell, such an antiviral therapy could mitigate resistant viruses.

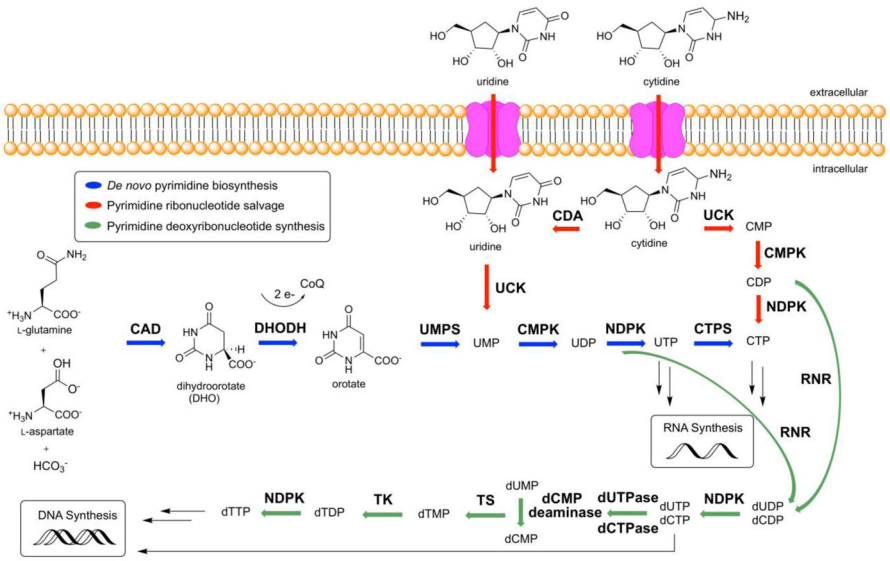

Figure 1. De novo and salvage biosynthesis of pyrimidine nucleotides in humans
For details, see text

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Table 1
Modulators of Pyrimidine Nucleotide Biosynthesis

Acronyms are defined in the text.

Drug name	Mode of action	Status	On- & off-target effects	Clinical use
***De novo* pathway**				
N-phosphonacetyl-L-aspartate (PALA)	Inhibits CAD	Not approved	Inhibits DNA synthesis	-
Leflunomide	Inhibits DHODH	FDA-approved	Inhibits DNA synthesis, liver problems, flu symptoms, diarrhea	Rheumatoid arthritis and multiple sclerosis
Brequinar	Inhibits DHODH	FDA-approved	Inhibits DNA synthesis, leukocytopenia, thrombocytopenia	Rheumatoid arthritis and multiple sclerosis
Pyrazofurin	Inhibits OMP decarboxylase	Not approved	Inhibits DNA synthesis, myelosuppression, stomatitis	Phase I/II clinical trials for various cancers
Shared salvage				
Dipyridamole	Inhibits nucleoside transporters (ENT1–4) & phosphodiesterase	FDA-approved	Increases cAMP and cGMP levels in platelets, vasodilation	Anti-platelet
Dilazep	Inhibits nucleotide transporter (ENT1)		Disturbances, allergic reactions, mouth ulcers, headache	Stroke
NTP-specific				
Cyclopentenyl cytosine (CPE-C)	Inhibitor of CTP synthetase	Not approved (Phase I)	The depletion of CTP and dCTP pools	Anticancer, antiviral
Diazo-5-oxo-L-norleucine (DON)	Inhibitor of CAD, CTP synetthase, GMP synthetase	Not approved (Phase I/II)	Inhibits glutaminolysis, uric acid synthesis	Anticancer
dNTP-specific				
Fludarabine	Inhibits RNR, DNA polymerase, primase	FDA-approved	Inhibits DNA synthesis, causes lymphopenia	Acute leukemias, lymphoproliferative disorders
Cladribine	Inhibits RNR, DNA polymerase	FDA-approved for cancer	Inhibits DNA synthesis, myelosuppression, rashes, and nausea	Acute leukemia, lymphoproliferative disorders
Gemcitabine	Inhibits RNR, DNA synthesis	FDA-approved	Inhibits DNA synthesis, bone marrow suppression, nausea, fever, hair loss	Ovarian, breast, non-small cell lung, pancreatic cancer
Clofarabine	Inhibits RNR, DNA polymerases	FDA-approved	Tumor lysis, inflammation, dehydration, low blood pressure	Acute lymphoblastic leukemia
Fluorouracil (5-FU, prodrug capecitabine & floxuridine)	Inhibits thymidylate synthase	FDA-approved	Toxicity in patients with DPD deficiency, nausea, vomiting & diarrhea	Colon, esophageal, gastric, pancreatic, breast, & cervical cancers
Trifluridine	Inhibits thymidylate synthase (TS) and DNA synthesis	FDA-approved as eye-drops,	Transient burning, stinging, local irritation of the eyelids	Herpes simplex virus, vaccinia virus in eye
TAS-114	dUTPase inhibitor, DPD inhibitor	Not approved (Phase I/II)	Enhancer of fluoropyrimidines	Non-small cell lung cancer