Deoxyribonucleotide Metabolism, Mutagenesis, and Cancer

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Preface

Cancer was recognized as a genetic disease at least four decades ago, with the realization that the spontaneous mutation rate must increase early in tumorigenesis, to account for the many mutations in tumor cells as compared with their progenitor normal cells. The genetic basis for cancer was established also from the finding that viral oncogenes have cellular counterparts, expression of which could transform cells. Deoxyribonucleotide pool abnormalities have long been recognized as determinants of DNA replication fidelity, and hence, may contribute to mutagenic processes involved in carcinogenesis. In addition, many anticancer agents act as antagonists of deoxyribonucleotide metabolism. To what extent may aspects of deoxyribonucleotide metabolism contribute to our understanding of both carcinogenesis and the effective use of anticancer agents?

Introduction

Since the discovery of thymineless death in 1954[1], followed four years later by the identification of thymidylate synthase as the main target for the

anticancer action of fluorinated pyrimidines[2], it has been apparent that cancer can be treated by targeting enzymes of DNA precursor biosynthesis (Timeline in Figure 1). Even earlier, the synthesis and antitumor activities of 6-mercaptopurine and methotrexate identified nucleotide metabolism as a rich area in which to seek therapeutic targets[3]. However, the toxicity and lack of specificity of antimetabolites in cancer treatment have highlighted the importance of understanding the complexities of deoxyribonucleoside triphosphate (dNTP) enzymology, metabolism, and relationships to cellular control processes. This factor plus new understanding of dNTP metabolism *vis a vis* genomic stability[4,5,6] emphasize the need for a current treatment of dNTP metabolism and its relation to cancer and cancer therapy.

dNTP pools: metabolism and intracellular distribution

Cellular distribution of dNTPs. Eukaryotic cells must maintain two functionally distinct dNTP pools—one used for DNA replication and repair within the nucleus and available predominantly in S phase, and a smaller pool used for mitochondrial DNA replication and available throughout the cell cycle. Early results from our laboratory[7] showed substantial dNTP pools in mitochondria isolated from HeLa cells. Whereas mtDNA in these cells accounts for only about one percent of total DNA, the mitochondrial dNTP pools ranged from 1.4 to 10.4 percent of total dNTP pools. Rampazzo *et al* reported *c*omparable results for several mouse cell lines[8]. These workers found the mitochondrial and cytosolic pools to be distinct, but they also found considerable mixing to occur between

these pools[9]. Although identifying the enzymes and pathways for precursors to mtDNA replication is beyond the scope of this article, the reader is referred to a recent study of two human mitochondrial ribonucleotide and deoxyribonucleotide transporters[10], which suggests that mitochondrial nucleotide pools are derived substantially from nucleotides synthesized in the cytosol.

Curiously, the dNTP pools within the nucleus appear to be bound in a metastable state. Our laboratory used a rapid method to isolate nuclei from Chinese hamster ovary cells and found the nuclear-to-cytosolic pool ratios to correspond closely to the relative volumes of the two compartments[11]. The nuclear pools did not change with repeated cycles of washing the nuclei. Small molecules such as nucleosides and nucleotides should be capable of passive diffusion through pores in the nuclear membrane. What prevents their efflux during isolation and washing of nuclei is less clear, because the diameter of the pores is much larger than the size of the nucleotide molecules.

dNTP biosynthesis. Biosynthesis of deoxyribonucleotides begins with the reduction of ribonucleoside diphosphates (rNDPs) to deoxyribonucleoside diphosphates (dNDPs) through the action of ribonucleotide reductase (RNR; see Figure 2). In early studies the low activity of this enzyme in cell-free extracts suggested that RNR might be rate-limiting for DNA replication. Recent studies indicate that that may be true in yeast[12]. Whether that is generally true, its elegant allosteric control mechanisms establish RNR as a rate-limiting enzyme for dNTP synthesis. As reviewed in detail elsewhere[13], RNRs fall into three classes (I, II, and III) based on protein subunit structure, source of a catalytically

essential free radical, substrate specificity (rNDPs *vs.* rNTPs), and allosteric control processes. Human RNR is a class I enzyme; this heterotetrameric protein consists of two large α subunits, which form the R1 protein, and two small β subunits, which form the R2 protein. The β subunit contains an iron-oxygen center that stabilizes a catalytically essential tyrosyl radical, which communicates with the catalytic site on the α subunit *via* a chain of electron-transferring amino acid residues located on both subunits. Replacement of the 2'-hydroxyl group on the rNDP substrate occurs by a free radical mechanism that begins with abstraction of a hydrogen atom from C-3' of the rNDP substrate[14].

An alternative R2 protein is induced by expression of the tumor suppressor p53 [15,16,17,18], presumably as part of the genome protective action of p53. Originally thought to be expressed as part of a DNA damage response, it is evident that a heterodimer of R1 and p53R2 also plays an important role in mitochondrial DNA replication[18,19]. The relationship between this alternative RNR subunit and the tumor suppressive activity of p53 is not yet clear.

The α subunit also contains two allosteric control sites—the activity site, which responds to ATP or dATP and adjusts overall activity—and the specificity site, which responds to dATP, dGTP, and dTTP to adjust specificity for the four rNDP substrates, thereby ensuring that the four dNDPs are produced at rates commensurate with representation of each deoxyribonucleotide in genomic DNA. For example, as seen in Figure 2, dGTP bound in the specificity site activates ADP reduction and inhibits GDP reduction; hence, the enzyme coordinates purine dNTP synthesis by promoting dATP synthesis when dGTP accumulates.

Analysis of rNDP reduction by an assay that monitors all four activities simultaneously[20] shows that competition among the four rNDP substrates at the catalytic site also contributes toward regulation of RNR specificity. dATP and ATP are the prime regulators affecting the activity site. For the human RNR dATP converts an active $\alpha_2\beta_2$ tetramer to an inactive $\alpha_6\beta_2$ heterooctamer[21]. ATP binding at the activity site stimulates all four activities.

Figure 2 shows additional enzymes that are subject to allosteric regulation—CTP synthetase and dCMP deaminase. Mutations affecting allosteric control of each of these enzymes have been shown to affect dNTP pool sizes and to express a mutator phenotype[5]. Hence, the ability to maintain dNTP pools within defined limits is critical to supporting accurate DNA replication. Although flux rates for the reactions of dNTP synthesis *in vivo* must be correlated with the nucleotide composition of the genome, the relative dNTP pool sizes bear no relationship to representation of the four deoxyribonucleotides in DNA. For example, in nearly all cells studied, dGTP is underrepresented, comprising five per cent or less of the total dNTP pool[22]. dATP and dTTP are usually the most abundant dNTPs. In mitochondria, on the other hand, dGTP is over-represented[23].

Once rNDPs have been reduced to dNDPs, their conversion to dNTPs involves nucleoside diphosphate kinase (NDPK). The pathways to dTTP are more involved, as shown in Figure 2, reflecting the need to exclude uracil from DNA. NDPK is an enzyme of low specificity, both for phosphate donor and acceptor. It has not been considered to play a role in regulating dNTP pool sizes.

However, one of the human isoforms of NDPK has been identified as the transcription factor PuF, which is also a suppressor of tumor metastasis[24,25]. NDPK is evidently a “moonlighting protein,” because the tumor-suppressing activity does not depend upon the enzymatic activity.

The ribonucleotides shown in Figure 2 arise *via de novo* pathways, involving the stepwise assembly of purine and pyrimidine rings. Salvage pathways, on the other hand, involve reutilization of nucleosides and nucleobases that arise through nucleic acid degradation. Nucleobases are salvaged by reaction with 5-phosphoribosyl-1-pyrophosphate (PRPP), catalyzed by phosphoribosyltransferases, while nucleosides are salvaged by nucleoside kinases. The principal salvage enzymes in human cells are shown in Figure 3. Many anticancer drugs are analogs of normal nucleosides or nucleobases and depend on salvage pathways for conversion to active nucleotide analogs (nucleotides themselves cannot traverse the plasma membrane). Some examples are presented later in this article.

Intracellular sites of dNTP synthesis. DNA replication, of course, occurs in the nucleus. Most studies show enzymes of dNTP synthesis to be located primarily in the cytosol. For example, immunolocalization of RNR subunits through the mammalian cell cycle[26] showed both subunits to be localized to the cytosol, and more recent evidence[27] also supports a cytosolic location for ribonucleotide reduction. These findings, plus the existence of substantial cytosolic dNTP pools, mentioned earlier[11], suggest that dNTPs are synthesized primarily in cytosol and pass into the nucleus through pores in the nuclear

membrane. However, recent findings require re-evaluation of this picture. Niida *et al*[28] found that RNR relocates to sites of DNA damage, within the nucleus. Following ionizing radiation, both R1 and R2 subunits were found in chromatin, associated with the Tip60 histone acetyltransferase. Thymidylate kinase behaves similarly[29]. The importance of the latter finding is that the conversion of dTMP to dTDP, catalyzed by thymidylate kinase, is essential for dTTP synthesis (see Figure 2). In its absence dUTP can accumulate and be incorporated into DNA, leading to DNA breakdown *via* DNA-uracil glycosylase and base excision repair. This finding points to thymidylate kinase as a possible target for cancer therapy. Indeed, Hu *et al*[29] screened a library of low-molecular-weight compounds and discovered a cell-permeable nontoxic inhibitor of thymidylate kinase, which sensitizes cells to the DNA-damaging anticancer drug doxorubicin.

The enzymes of thymidylate metabolism play a special role in DNA synthesis, acting to prevent incorporation of uracil into DNA. As seen in Figure 4, dTMP is formed in a “thymidylate synthesis cycle,” in which a single-carbon group is transferred from serine to tetrahydrofolate by serine hydroxymethyltransferase. Thymidylate synthase then catalyzes the reductive transfer of a methylene group to dUMP, which becomes the methyl group of dTMP. The resultant dihydrofolate must be reduced to tetrahydrofolate to sustain the cycle. These reactions are targets for the actions of important anticancer drugs. 5-Fluorouracil and 5-fluorodeoxyuridine are converted to 5-fluoro-dUMP, an irreversible inhibitor of thymidylate synthase, while folate analogs such as methotrexate exert their actions through inhibition of dihydrofolate reductase.

Both classes of inhibitors cause dUTP levels to rise, leading to its incorporation into DNA and subsequent DNA breakdown *via* uracil-*N*-glycosylase and base excision repair.

The importance of the thymidylate synthesis cycle can be seen in a series of papers from Patrick Stover's laboratory[30,31,32,33,34]. The three enzymes involved form a multienzyme complex that is transferred from cytosol to nucleus at the beginning of S-phase. One isoform of SHMT undergoes reaction with a small ubiquitin-like modifier (SUMO), which is essential for its transport and anchoring to the nuclear lamina, along with the two associated enzymes. Whether this occurs by the same process as relocation of thymidylate kinase to DNA damage sites remains to be seen.

Cell cycle regulation of dNTP biosynthesis. dNTP pool sizes fluctuate during the cell cycle, with the largest pools seen during S phase. Much of the basis for S-phase elevation of dNTP pools relates to control of the expression of genes encoding enzymes of dNTP synthesis, particularly ribonucleotide reductase [35,36,37]. Curiously, levels of the RNR R2 protein rise and fall, with highest levels during S-phase, while R1 expression is constitutive, and the R1 protein is metabolically stable[26,38]. For enzymes such as NDP kinase, which participates also in ribonucleoside triphosphate synthesis, expression is constitutive through the cell cycle.

Among the enzymes of salvage pathways, thymidine kinase is cell cycle-regulated, but deoxycytidine kinase is not[39]. Factors such as this may need to be taken into account in scheduling the administration of antineoplastic nucleoside

analogues, such as 5-fluorodeoxyuridine, which is anabolized by thymidine kinase, and arabinosylcytosine, which depends upon deoxycytidine kinase for its conversion to active nucleotides.

dNTP turnover and pool regulation

Until recently most attention to regulation of dNTP pools focused on control of deoxyribonucleotide *synthesis*. However, cell culture experiments with radiolabeled nucleosides suggested that nucleotide *degradation* also contributes—that nucleotide pools are controlled in part by "substrate cycles," involving interconversion between nucleosides and nucleotides[40]. *Nucleotide* kinase reactions have equilibrium constants near unity, so they are controlled largely by mass action. However, both *nucleoside* kinase and 5'-nucleotidase reactions are exergonic and represent potential control points, albeit indirect, for regulation of dNTP pools. In fact, evidence summarized by Rampazzo *et al*[40] indicates that substrate cycles between 5'-nucleotidases and nucleoside kinases do represent sites of control of dNTP pools. A direct involvement of 5'-nucleotidases with cancer was realized in 2013, with the discovery that a number of patients with acute lymphocytic leukemia became resistant to therapy with purine analogs because of overproduction of one such nucleotidase, cN-II, encoded by gene *NT5C2*[41].

An additional mode of dNTP pool control at the level of degradation appeared in 2011, when two laboratories reported that a protein called SAMHD1 (for sterile α-motif and histidine-aspartate domain-containing protein) could

cleave dNTPs to the respective deoxyribonucleoside plus tripolyphosphate[42,43]. SAMHD1 had originally been recognized as a protein that restricts lentivirus infection. Genetic deficiency of *SAMHD1* in humans is associated with Aicardi-Goutières syndrome, a rare hereditary neuroinflammatory disorder. Deficiency of SAMHD1 is seen also in a small proportion of chronic cases of lymphocytic leukemia[44,45]. The discovery of a nucleoside triphosphatase activity associated with SAMHD1 suggested that its antiviral activity resulted from depletion of dNTP pools, which are needed for reverse transcription.

Early studies of human SAMHD1 showed its activity to be stimulated by dGTP, raising the interesting possibility that dGTP is both a substrate and an allosteric modifier of the enzyme. However, the enzyme is also stimulated by GTP, a nucleotide present at much higher concentration in most cells, and it is generally accepted, based on this and on structural studies, that GTP is the prime allosteric activator[46]. The protein is an inactive monomer-dimer mixture with two regulatory sites and one catalytic site per monomer. Binding of either GTP or dGTP at regulatory site 1 of SAMHD1 induces tetramerization, and binding at site 2 by any dNTP adjusts the specificity of the active tetrameric enzyme[47]. Ji *et al*[48] determined the crystal structures of the 26 possible tetramers (containing GTP or dGTP in site 1 and any one of the four dNTPs in site 2), showing how binding of substrates and modifiers influence the structure of the enzyme.

Franzolin *et al* used RNA interference to establish that SAMHD1 functions in controlling dNTP pool sizes[49]. Inhibiting expression of *SAMHD1* caused dNTPs

to accumulate and blocked cell growth in G1. For the human enzyme Miazzi *et al*[50] reported half-maximal stimulating concentrations of dATP and dTTP to be 1 μM and 2 μM, respectively. These values are higher than intracellular concentrations of dATP and dTTP, but possibly close enough to suggest that these nucleotides function as physiological regulators of SAMHD1 activity. Hansen *et al*[51] reported that the activated form of the enzyme persists for hours, even after pools of the stimulatory dNTPs are depleted. If an important function of the enzyme is to control retrovirus reverse transcription by depriving the infected cell of dNTP substrates, it is reasonable to expect the enzyme to maintain this activity for some time after the pools are effectively depleted.

Recent studies[52] describe a ribonuclease activity associated with SAMHD1 and point to this activity, not the dNTPase, as the key antiviral activity associated with this protein. However, the function of SAMHD1 in regulating dNTP pool sizes in cycling cells seems well established.

dNTP pools, mutagenesis, and cancer

As noted earlier, cancer results in large part from the accumulation of spontaneous or induced mutations. Loeb *et al*[53] predicted that the mutation rate must increase during carcinogenesis to account for the large number of genetic differences between tumors and the normal tissue from which they originated. The large number of mutations in cancer cell genomes[54] is in accord with this prediction. Using an ultrasensitive method for measuring single-nucleotide substitution mutations, Bielas *et al*[55] reported the spontaneous mutation

frequency in tumor tissues to be at least 200-fold higher than that of the normal tissue from which they were derived. Relatively few of the accumulated mutations participate directly in oncogenic transformation. Indeed, for lung and colon adenocarcinomas Tomasetti *et al*[56] concluded that only three sequentially acting "driver" mutations suffice to convert a normal cell to a cancer cell, no matter the total number of mutations. Presumably, a general increase in the spontaneous mutation rate stimulates mutations at all loci, including those that drive tumorigenesis. More recent experiment at the single-cell level with yeast[57] show considerable variation among different cells in mutation frequencies, suggesting that a mutator cell can adopt one of two mutation rates that differ by an order of magnitude.

Related to the above, Tomasetti and Vogelstein[58] estimated for many cancers that two thirds of the variation among different tissues in frequency of mutations leading toward cancer is accounted for not by exogenous carcinogens or genetic predisposition but by random processes—underlying the importance of understanding spontaneous mutagenesis.

Unbalanced dNTP pools and mutagenesis. Abnormalities of DNA precursor metabolism, particularly unbalanced dNTP pools, contribute toward spontaneous mutagenesis. Early investigations, using *in vitro* systems or purified DNA polymerases, indicated that dNTP imbalances could drive substitution mutagenesis either by competition between correctly and incorrectly base-paired nucleotides at the nucleotide insertion step, or by a next-nucleotide effect, in which correct base pairing past the site of a mismatch prevents proofreading of

an upstream mismatched nucleotide (reviewed in 4, 5, and 6). Short insertions and deletions occurred *in vitro* also, through stabilization of misaligned intermediates (Figure 5).

All of the effects shown in Figure 5 have now been demonstrated in living cells, both in yeast[59] and *E. coli*[60]. In the yeast investigation Kumar *et al*[59] studied three mutations affecting the RNR specificity site, each of which had a distinct effect on dNTP pools. Sequence analysis of spontaneous mutant sites in the CAN1 locus, conferring canavanine resistance, confirmed that the mutagenic consequences of specific dNTP imbalances seen in living cells are identical to those earlier described from investigations using *in vitro* systems. In a more recent analysis this group[61] has shown, for a yeast mutant containing both dCTP and dTTP in excess, that mutation frequencies and mutant spectra are essentially the same, whether the initial replication error occurs during leading- or lagging-strand replication. This finding was unexpected, because leading- and lagging-strand replication are carried out by different DNA polymerases (Pol ε and Pol δ, respectively), using some different accessory proteins[62,63]. The naive expectation would have been that the two polymerases differ sufficiently in kinetic properties to respond differently to the same dNTP perturbation.

In a more recent study St. Charles *et al*[64] have been able to determine the relative contributions of polymerase nucleotide selectivity, proofreading, and mismatch repair to overall fidelity in yeast. In this study base selectivities of Pol ε and Pol δ were seen to differ. Also proofreading in general was seen to contribute more toward overall fidelity than mismatch repair. However, in human

cells, a recent study by Supek and Lehner[65] identifies differential mismatch repair efficiency as the basis for variation of mutation rates across the genome.

In a different approach with *E. coli* Ahluwalia *et al*[60] developed a powerful technique for selecting RNR mutations that lead to unbalanced dNTP pools. Analysis of dNTP pools, spontaneous mutation frequencies, and mutant sites indicate that the mutagenic mechanisms demonstrated with *in vitro* systems—insertion errors, next-nucleotide effects, and misaligned intermediates (Figure 5)—also occur in living cells. For the studies of pool-directed mutagenesis with both yeast and *E. coli*[59,60] it was essential to carry out the *in vivo* analyses of mutation frequencies and spectra in strains lacking mismatch repair; otherwise many or most of the premutagenic intermediates would be corrected before they could lead to detectable mutations.

Balanced dNTP accumulation and mutagenesis. Mutagenesis is stimulated not only by unbalanced dNTP pools, but by balanced accumulation of all four dNTPs[66,67,68,69]. This was seen whether the pool accumulations occurred as a result of DNA damage or of overexpression of RNR. Presumably this enhanced mutagenesis occurred as a result of next-nucleotide effects at all loci. To be sure, these studies were carried out with *E. coli* or yeast, and comparable data for mammalian cells are sparse. However, tumor cells are characterized by generally uniform accumulation of the four dNTPs[22] (Table 1), and it is interesting to consider whether increases in dNTP pools, whether unbalanced or not, contribute toward the enhanced mutagenesis that is part of the carcinogenic

process. Proportional *depletion* of dNTP pools also causes genomic instability, possibly resulting from replication stress[70].

Relations between error-prone DNA polymerases, dNTP pools, and mutator phenotypes. The high accuracy of genome replication depends on base selectivity of replicative polymerases, proofreading of replication errors, and DNA mismatch repair. It has been known for some time that human genetic defects in mismatch repair create mutator phenotypes that predispose toward colorectal cancer[71]. More recently, mutations affecting human replicative polymerases ε and δ have been associated with colorectal cancer and endometrial cancer[72,73]. Two recent studies[74,75] have modeled these mutations in the corresponding polymerases of yeast, yielding fresh insight into relationships between dNTP metabolism, mutator phenotypes, and cancer.

In yeast the *pol3-R696W* mutation affecting lagging-strand Pol δ models the human cancer-associated variant POLD1-R689W. Mertz *et al*[74] found that expression of the *pol3-R696W* mutant Pol δ in yeast leads to a checkpoint-dependent dNTP pool expansion. Action of the *pol3-R696W* mutant polymerase *in vitro*, in the presence of dNTPs at concentrations reflecting those determined from pool measurements *in vivo*, generated a mutation spectrum closely resembling the spectrum observed *in vivo*. The results suggest operation of a "vicious cycle," in which Pol δ replication errors lead to a checkpoint-dependent dNTP pool expansion, which further stimulates replication errors by facilitating DNA chain extension past mismatched primer termini.

A relationship with checkpoint activation was seen also by Williams *et al*[75], in a study of mutations affecting leading-strand polymerase ε. As noted above, mutations affecting Pol ε are associated with human colorectal and endometrial cancer. The yeast *pol2-M664G* mutation affects polymerase ε dNTP selectivity. The mutator effect of this mutation was shown to depend upon Dun1, an S phase-dependent protein kinase that up-regulates dNTP synthesis upon checkpoint activation[76,77]. *Dun1pol2-M664G* double mutants displayed elevated dNTP pools and a mutator phenotype. The results highlight the need to define cell cycle-dependent checkpoint mechanisms in human cells, which do not contain the Dun1 protein but do contain some parts of this regulatory system. Such insight could allow investigators to minimize genomic instability resulting from dNTP pool expansion.

RNR and cancer

The role of RNR in catalyzing the first reaction committed to dNTP synthesis gives this enzyme special importance in controlling rates and fidelity of DNA replication. A recent survey[78] identified *RRM2*, the structural gene for the β polypeptide, as being among the top 10% of overexpressed genes in 73 of 168 cancer analyses, involving multiple organs. The comparable figure for *RRM1*, which encodes α, was top 10% in 30 of 170 analyses, again involving multiple organs. Transgenic mice engineered to overexpress *RRM2* developed neoplasms of the lung only[79]; the basis for this tissue specificity is unknown.

As expected from its central metabolic role, RNR interacts with numerous proteins. Most of our current understanding of these interactions comes from model systems, particularly yeast. Experiments in fission yeast demonstrated an interaction between RNR and PCNA—the “sliding clamp” in DNA replication[37]. Such an interaction, if seen also in metazoa, could provide a direct link between the machineries of dNTP synthesis and DNA replication. Because dNTPs have no known metabolic roles except as DNA precursors, it seems logical that dNTPs be synthesized at sites connected with their greatest utilization. As suggested in the previous section, another important interaction is with CHK1[80], an interaction related to the cell cycle-dependent control of dNTP synthesis.

RNR has long been recognized as an attractive target for cancer treatment. The classic RNR inhibitor, hydroxyurea, is not heavily used for cancer treatment, but its use as a free radical scavenger helped to reveal the free radical mechanism used by RNR. Evidently hydroxyurea donates a single electron to the tyrosine radical in the R2 protein, inactivating the enzyme by converting the essential tyrosine radical cation to a normal tyrosine residue. Mammalian cells that become resistant to hydroxyurea do so by overexpressing the hRRM2 gene[81].

Because of its key role in dNTP biosynthesis, RNR remains an attractive target for anticancer drugs. As mentioned later in this article, nucleoside analogs, such as gemcitabine and clofarabine, are useful drugs that act as RNR inhibitors following their intracellular conversion to nucleoside diphosphates[82,83,84]. A more recent paper[85] describes a newly synthesized RNR inhibitor called COH29, which

shows a preferential inhibition of BRCA1-dependent DNA repair, specifically, the repair of double-strand DNA breaks. These results highlight the importance of identifying specific DNA synthesis targets—for example, replication or repair—of agents that interfere with dNTP metabolism.

dNTPs, oncogenes, and oncogene-induced senescence

Correlating actions of enzyme inhibitors with downstream metabolic targets, as just mentioned, leads naturally to exploring relationships between dNTP enzymology and oncogenes. This is an evolving and still somewhat fragmented area of investigation. In some ways the budding yeast, *Saccharomyces cerevisiae*, has led the way in identifying relationships between deoxyribonucleotide metabolism and the actions of oncogenes. Yeast has a genome integrity checkpoint that senses stress, possibly leading to DNA damage, and acting to relieve that stress. One part of this checkpoint involves actions of the previously mentioned Dun1 and its downstream targets Mec1 and Rad53, to ensure that balanced and adequate dNTP pools are available[77,86,87,88]. Because the human homologs of Mec1 and Rad53 (ATR and CHK2, respectively) are upstream regulators of p53, this system may yield insight into the tumor-suppressing actions of p53. Dun1 is a protein kinase that phosphorylates Sml1, a protein inhibitor of RNR activity, targeting it for proteolysis and relieving inhibition of RNR[77,87]. Dun1 phosphorylation of another protein, Dif1, causes release of RNR small subunits from the nucleus, where they are sequestered, to the cytoplasm, where they can combine with the large subunit and stimulate

deoxyribonucleotide synthesis, leading to severalfold increase in dNTP pools. Whether p53 plays a similar function in mammalian cells remains to be seen. However, preactivation of the yeast genome integrity checkpoint increases tolerance to several DNA-damaging reagents, including cisplatin[89,90], a finding that may help explain the development in humans of resistance to certain anticancer drugs.

The Rb tumor suppressor also acts in part at the level of dNTP metabolism[91]. Rb action in culture of mouse embryo fibroblasts attenuated transcription of genes for RNR, thymidylate synthase, and dihydrofolate reductase, with concomitant depletion of dNTP pools. Targeted disruption of the *Rb* gene in these cultured cells had the opposite effect[91].

Studies on the actions of the MYC oncoprotein on nucleotide metabolism have led in two interesting directions. Mannava *et al*[92] found that downregulation of MYC led to specific repression of the genes for thymidylate synthase, inosinate dehydrogenase, and PRPP synthetase 2, with corresponding depletion of dNTP pools and cell cycle retardation. Overexpression of MYC had opposite effects, suggesting a major action of MYC at the level of gene expression in regulating dNTP synthesis.

Cunningham *et al*[93] also observed an effect of MYC on PRPP synthetase 2, and their further data led them to identify PRPS2 as “a single rate-limiting enzyme to drive cancer.” PRPP synthetase catalyzes reactions in the *de novo* pathways to both purine and pyrimidine nucleotides, so it is involved in both DNA and RNA synthesis. Cunningham *et al* identified a *cis*-regulatory region within the

5' untranslated region of the *PRPS2* gene, controlled by the oncogene and translation initiation factor eIF4E, but not in the 5' untranslated region in the gene encoding the functionally related PRPP synthetase 1. The data indicate the importance of an overall balance between protein and nucleic acid synthesis—perhaps more so than stimulation of dNTP synthesis—in the metabolic decision between controlled and abnormal growth.

The laboratories of M. Nikiforov[94,95] and R. Zhang[96,97] have independently reported important relationships between deoxyribonucleotide metabolism and oncogene-induced senescence (OIS). This is a stable growth arrest that occurs when an activated oncogene is expressed in a normal cell. Mannava *et al*[94,95] found that human fibroblasts undergoing OIS, induced by $HRAS^{G12V}$ or MYC expression underexpressed both RNR and thymidylate synthase and contained low dNTP pools. Ectopic expression of the two enzymes restored dNTP pools and suppressed senescence-associated phenotypes. Aird *et al*[96] also reported that either addition of exogenous nucleosides or stimulation of RNR β subunit expression suppresses the DNA damage response. Marusyk *et* al[98] in an earlier study had described a function for p53 in leading toward senescence as a response to replication stress. Stimulating cancer cells to undergo OIS is a promising approach to cancer treatment[97,99], so the importance of understanding this phenomenon is evident.

Two other oncoproteins, FHIT[100] and BCL-2[101], have recently been reported to influence deoxyribonucleotide metabolism. In several cell lines DNA defects resulting from loss of *FHIT* expression could be traced to decreased

expression of thymidine kinase 1 (the cytosolic isoform), with a consequent specific decrease in dTTP pools[100]. The effect could be reversed by exogenous thymidine.

BCL2[101] is an antiapoptotic protein that mediates inhibition of the G1-to-S phase transition. Overexpression of this protein in transgenic mice promotes development of lymphomas. Xie *et al*[101] found one effect of BCL2 to be disruption of the interaction between the RNR R1 and R2 proteins, with consequent RNR inhibition and depletion of dNTP pools. The authors proposed that the resultant DNA replication stress enhances genome instability and contributes toward carcinogenesis, although the specific relationships are not yet clear.

dNTP metabolism and telomeres

The linear DNA of eukaryotic and some prokaryotic chromosomes is protected at each end by telomeres—tandemly repeated short DNA sequences. Telomeric DNA is bound by proteins that protect chromosome ends from being recognized as double-strand breaks in need of repair[102]. Telomerase is a reverse transcriptase that uses a bound RNA as template for one of the two telomere strands[103]. In human telomerase the bound RNA has the sequence 3'-CAAUCCCAAUC-5', which encodes in tandem the telomere repeat sequence 5'-GTTAG. A shortening of telomeres due to decreased telomerase activity has long been associated with aging[104]. By contrast, cancer cells have robust telomerase activity, and this is thought to be related to the immortalization of tumor cells in culture[105].

Because telomeres are G-rich, and dGTP usually comprises ten percent or less of total dNTP pools[22], Maine *et al* investigated the effects of dNTP variations *in vitro* on telomerase activity and the length of telomere chains[106]. Both were sensitive to dGTP concentrations in the physiological range. Working with budding yeast, Gupta *et al*[107] studied the effects of dNTP pool variation *in vivo* on telomere homeostasis. Varying the ratios of dNTP pools had major effects, with telomere length most sensitive to relative changes in dGTP concentration, suggesting that telomerase activity is controlled *in vivo* by dNTP pools. Because dNTP pools are severalfold higher in tumor cells in culture than in normal cells (Table 1), this finding may help explain the persistence of telomerase activity in cancer cells. It may also spur a search for ways to control dGTP pool sizes in tumor cells, to promote senescence by lowering telomerase activity.

Sanitation of damaged dNTP pools

Oxidation of guanine residues in DNA to 8-oxoguanine has long been recognized as one of the most important mutagenic and cytotoxic effects of reactive oxygen species (ROS)[108]. 8-Oxoguanine in DNA readily base-pairs with adenine, initiating a transversion mutagenesis pathway. The idea that the damage might actually occur at the nucleotide level originated with the demonstration that free intracellular nucleotides are orders of magnitude more reactive with modifying reagents than are the corresponding nucleotides in DNA[109]. The idea gained impetus with the finding that the *mutT* gene in *E. coli*,

which counteracts ROS-stimulated mutagenesis, encodes a nucleoside triphosphatase, which preferentially cleaves the oxidized nucleotide, 8-oxodGTP, to the corresponding deoxyribonucleoside monophosphate, 8-oxo-dGMP, plus pyrophosphate[110]. The enzyme acts similarly on oxidized derivatives of dATP[111]. A human homolog, hMTH1, has similar activities[112,113,114]. Two other human nucleotidases, MTH2 and MTH3, have related substrate specificities, including abilities to cleave oxidized ribonucleotides and nucleoside diphosphates[115].

MTH1 knockout mice show spontaneous tumorigenesis[116], providing an additional link between oxidized nucleotides and mutagenesis leading to cancer. However, a double knockout, inactivating both MTH and OGG—a DNA glycosylase that initiates a base excision repair pathway for DNA 8-oxo-dGMP residues—showed no more tumorigenesis than the mice with the single OGG knockout[117]. This finding led the authors to suggest that the base excision repair pathway is more efficient than nucleotide pool sanitation as a route for minimizing oxidized DNA nucleotide residues.

Attempts to detect 8-oxo-dGTP in *mutT*$^{-}$ bacteria were unsuccessful, suggesting that the pool size was quite low[118]. However, substantial amounts of 8-oxo-dGTP were detected in mammalian mitochondria, at levels sufficient to drive replication errors, as seen in an *in vitro* system[119]. Despite the paucity of attempts to detect and quantitate damaged dNTPs *in vivo*, a robust literature now supports the idea that incorporation of oxidized nucleotides into DNA is an important contributor to mutagenesis and carcinogenesis[110,120,121].

Accordingly, it was a surprise in 2014 when two laboratories, using different approaches, identified hMTH1 as a target for inhibition by potential anticancer agents[122,123]. Based on previous observations that MTH1 overexpression prevented DNA damage and genome instability induced by H-RAS, Gad *et al*[122] deliberately targeted MTH1 with small interfering RNAs. Positive results led them to screen a library of low-molecular-weight compounds and to identify two that bound in the active site of MTH1, increased incorporation of oxidized nucleotides in cancer cells, and showed therapeutic responses in patient-derived mouse xenografts. By contrast, Huber *et al*[123] used an unbiased approach, involving a compound known to suppress the anchorage-independent growth of KRAS-transformed fibroblasts. Analysis of proteins bound to this drug identified MTH1 as the likely target. Screening existing drugs that might be MTH1 inhibitors, these authors identified crizotinib, a known protein kinase inhibitor currently approved for treatment of some non-small-cell lung carcinomas. Surprisingly, the *S*-stereoisomer was far more active as an MTH1 inhibitor and as an antitumor agent in their system than was the *R*-isomer, which is in present clinical use. Both author teams acknowledged the possible oncogenicity of prolonged MTH1 inhibition[122,123], but suggested the likelihood of an appropriate therapeutic window for MTH1 inhibitors.

Cancer chemotherapy, fluorinated pyrimidines, and thymineless death

Many cancer drugs are antimetabolites, analogs of DNA precursors—purines and pyrimidines—or of folate, that act on enzymes of nucleotide

metabolism. Table 2 lists several commonly used antimetabolites, with indications of their clinical applications. For most of these drugs a “target enzyme” is identified. However, this is an oversimplification, because most of these agents interact with multiple enzymes, usually after metabolic conversions. Some, such as arabinosylcytosine, function less as enzyme inhibitors than as substrates, whose incorporation into DNA as a nucleotide prevents chain elongation because of the unnatural configuration of the nucleotide sugar.

To illustrate the complex relationships among analog metabolism, interactions with target enzymes, and pharmacological actions, a good example is the fluorinated pyrimidines—5-fluorouracil (FU), a pyrimidine base analog, and 5-fluorodeoxyuridine (FUdR), a deoxyribonucleoside. Both compounds were synthesized by Charles Heidelberger[124], following observations that tumor cells take up and metabolize uracil more rapidly than do normal cells. Heidelberger predicted that FU or FUdR could interfere with tumor growth by inhibiting this process. Cohen *et al*[2] found that the corresponding dNMP, 5-fluorodeoxyuridine 5'-monophosphate (F-dUMP) is a potent inhibitor of thymidylate synthase. In the presence of the folate cofactor, 5,10-methylene-THF, inhibition of TS by F-dUMP was shown to be stoichiometric and irreversible. This finding not only pointed directly to TS as the chief target for fluorinated pyrimidines, it provided a tool for elucidating the mechanism of TS catalysis[125].

The kinetics of cell killing by FU or FUdR were reminiscent of thymineless death[1], in which bacterial mutants defective in TS were found to rapidly lose viability when deprived of thymidine in otherwise complete medium. Accordingly,

much effort has been expended in trying to learn why these cells die instead of simply undergoing reversible growth arrest. Much evidence supports the premise that TS inhibition or genetic deficiency causes accumulation of the substrate, dUMP, which undergoes phosphorylation to dUTP and incorporation into DNA as dUMP[126]. Enzymes of base excision repair then cleave DNA at sites occupied by dUMP, causing lethal double-strand breaks if dUMP is present at nearby sites on both strands. In fact, dUTP pools and DNA dUMP residues accumulate in cells treated with fluorinated pyrimidines[127]. Consistent with this idea is the finding that inhibiting dUTPase activity potentiates the action of thymidylate synthase inhibitors[128]. Accumulation of 5-fluoro-dUTP occurs as well, with its incorporation into DNA as F-dUMP[126,127].

The above observations suggest that FUdR should be more effective clinically than FU, because its conversion to FdUMP involves but one metabolic reaction, while FU undergoes a complex metabolism[129] en route to several fluorinated metabolites—including its incorporation into RNA, which has several metabolic consequences[130,131]. However, FU is used far more widely than FUdR in the clinic despite its more complex metabolism[132]. These observations suggest that fluorinated pyrimidines have several modes of action.

Indeed, concepts regarding the mechanism of thymineless death may need revision in light of recent findings by Itsko and Schaaper[133]. These investigators devised conditions whereby *E. coli* could undergo specific limitation of dGTP pools. Cells deprived of dGTP underwent lethality with kinetics similar to those of cells undergoing thymineless death—"guanineless death," so to speak.

dUTP incorporation into DNA obviously cannot be a factor here. Rather, these results suggest that starving cells for any DNA precursor leads to cell death, rather than quiescence, by a still unknown mechanism.

Concluding remarks

These final observations underscore the fact that many useful cancer treatments can arise through manipulation of deoxyribonucleotide metabolism, but that effective use of nucleotide antagonists requires deep understanding of uptake, metabolic conversions, interactions with enzyme systems, and effects on DNA precursor pools. Alternative approaches may target the same enzymes, without the complications of pool effects and secondary targets. As an example, Cannaza *et al*[134] have used the crystal structure of human TS to design peptides that inhibit the essential dimerization of the enzyme protein, and the same laboratory is working toward nonpeptide analogs that bind to the same target but that resist degradation and can be taken up into tumor cells[135]. A related approach[136] involves identification of amino acid residues essential to dimerization, leading toward the design of dimerization inhibitors targeting these sites. These studies suggest that the future of cancer chemotherapy involving enzymes of deoxyribonucleotide metabolism may focus less on metabolic complexities and more on protein structure and enzyme function. New insights into cancer treatment may come as well from the exploitation of nucleotide sanitizing enzymes as drug targets[122,123], analysis of relationships between dNTP pools and telomerase activity in human cells[107], elucidation of oncogene-induced

senescence[94,95,96,97], exploitation of thymidylate kinase as a drug target[29], and further understanding of cell cycle checkpoints in human cells and their influence on dNTP pools and genomic stability[89,90].

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Figure legends

Figure 1. Timeline of significant events in developing our concepts of relationships among deoxyribonucleotide metabolism, mutagenesis, and cancer.

Figure 2. Pathways of *de novo* dNTP biosynthesis in mammalian cells. Salvage pathways are shown in Figure 3. Each enzyme name is in italics. Allosteric effectors are shown with upward and downward arrows, denoting activators and inhibitors, respectively. A myokinase-type reaction converts CTP and CMP to 2 CDP, and 2 dCDP to dCTP and dCMP. Reprinted with permission from reference 6.

Figure 3. Principal reactions in salvage routes to deoxyribonucleotides in human cells. Thymidine kinase 2 and deoxyguanosine kinase are localized in mitochondria. The other enzymes are located primarily in the cytosol.

Figure 4. The thymidylate synthesis cycle.

Figure 5. Replication errors leading to mutations, induced by dNTP pool imbalances. Realignment can occur on either the template or product DNA strand, to maximize the number of hydrogen-bonded base pairs. Realignment mutations include +1 and +2 insertions or deletions. The example shown is a –1 deletion.

Table 1

dNTP pools in cultured normal and transformed human cell nuclei

Cell line	dNTP pool pmol/10^6 nuclei			
	dATP	dTTP	dCTP	dGTP
Normal human fibroblasts	15	15	10	3
HeLa cells	32	63	25	12

Data are from Martomo and Mathews[22].

Table 2

Anticancer Agents that Act by Antagonizing Deoxyribonucleotide Metabolism

Agent	Target Enzyme(s)	Clinical Uses[132]
Folate analogs		
Methotrexate	Dihydrofolate reductase	Acute lymphocytic leukemia; breast, head, neck, and lung cancers; osteosarcoma; bladder cancer; choriocarcinoma
Pemetrexed	Thymidylate synthase, Glycinamide ribonucleotide transformylase (as polyglutamate)	Mesothelioma, lung cancer
Pyrimidine analogs		
5-Fluorouracil	Thymidylate synthase (as FdUMP)	Breast, colon, esophageal, stomach, pancreas, head and neck, premalignant skin (topical)
Arabinosylcytosine (araC, cytarabine)	DNA polymerase (as araCTP)	Acute myelogenous and acute lymphocytic leukemia; non-Hodgkins lymphoma
Gemcitabine (2',2'-difluoro-deoxycytidine)	Ribonucleotide reductase (as gemcitabine diphosphate)	Pancreatic, ovarian, lung cancer
5-Azacytidine, (2'-deoxy-5-aza-cytidine)	DNA-cytosine methyltransferase	Myelodysplasia
Purine analogs		
6-Mercaptopurine	*De novo* purine nucleotide synthesis	Acute lymphocytic and acute myelogenous leukemias; small-cell non-Hodgkins lymphoma
Fludarabine (fluoroarabinosyl-adenine)	DNA polymerase (as the 5'-triphosphate), Ribonucleotide reductase	Chronic lymphocytic leukemia; (2-low-grade lymphomas
Cladribine (2-chloro-deoxyadenosine)	(Causes strand breaks after incorporation into DNA) Ribonucleotide reductase	Hairy cell leukemia; low-grade lymphomas
Clofarabine (2-chloro-2'-fluoro-araA)	Ribonucleotide reductase	Pediatric acute leukemias