Visualizing Nucleic Acid Metabolism using Non-Natural Nucleosides and Nucleotide Analogs

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Abstract. Nucleosides and their corresponding mono-, di-, and triphosphates play important roles in maintaining cellular homeostasis. In addition, perturbations in this homeostasis can result in dysfunctional cellular processes that cause pathological conditions such as cancer and autoimmune diseases. This review article discusses contemporary research areas applying nucleoside analogs to probe the mechanistic details underlying the complexities of nucleoside metabolism at the molecular and cellular level. The first area describes classic and contemporary approaches used to quantify the activity of nucleoside transporters, and important class of membrane proteins that mediate the influx and efflux of nucleosides and nucleobases. A focal point of this section is describing how biophotonic nucleosides are replacing conventional assays employing radiolabeled substrates to study the mechanism of these proteins. The second section describes approaches to understand the utilization of nucleoside triphosphates by cellular DNA polymerases during DNA synthesis. Emphasis here is placed on describing how novel nucleoside analogs such as 5-ethynyl-2'-deoxyuridine are being used to quantify DNA synthesis during normal synthesis as well as during the replication of damaged DNA. In both sections, seminal research articles relevant to these areas are described to highlight how these novel probes are improving our understanding of these biological processes.

The importance of nucleoside metabolism. Nucleosides and their corresponding nucleotides play important roles in cell physiology by functioning as both nutrients and modulators of cellular homeostasis (1). For example, nucleoside triphosphates such as ATP and GTP are used as energy sources in various biological reactions ranging from muscle contraction to processes involving cell signaling (2). In addition, nucleosides such as adenosine and guanosine as well as their corresponding cyclic nucleoside monophosphates (cAMP and cGMP) modulate a wide variety of cellular events by functioning as ligands for purinergic receptors and transducers of endocrine signals (3). For example, cAMP is a second messenger that mediates a wide range of intercellular effects including glucose, glycogen, and lipid metabolism (4). Likewise, adenosine participates in numerous processes ranging from the maintenance of cardiovascular tone to modulating immune responses (5). For instance, adenosine binding to the A₁ receptor leads to a reduction in cAMP levels (6) which increases K⁺ efflux in cardiac tissue to cause cell hyperpolarization. Another important role for adenosine is as an inflammatory modulator (7) in which increases in the extracellular levels of adenosine can activate the A_{2a} receptor on the surface of immune cells (8). Finally, pyrimidine and purine nucleosides comprise the monomeric building blocks of DNA and RNA and thus play central roles in the storage and expression of genetic information (9). Collectively, these examples highlight the importance of pyrimidine and purine nucleosides in many physiological processes ranging from cardiovascular activity to neurotransmission to cellular proliferation (10-12).

Nucleoside metabolism also plays important roles in the initiation and progression of several pathological conditions. Diseases such as cancer, autoimmune disorders, and viral infections, for example, require significantly high levels of DNA synthesis to sustain their hyperproliferative state. As such, these conditions require large amounts of nucleoside uptake

for cellular survival and propagation. The supply for this high demand is typically obtained through salvage pathways that rely on the activity of various nucleoside transporters. Equally important, the increased reliance on nucleoside uptake and metabolism provides an important focal point for therapeutic intervention against these conditions (13). For example, azidothymidine (AZT) is a pyrimidine analog in which the 3'-hydroxyl (-OH) moiety required for elongating DNA is replaced with an azide (N_3) group. This simple substitution makes AZT refractory to elongation by viral polymerases (14-16) and makes it an effective nucleoside reverse transcriptase inhibitor (NRTI) that targets viral replication in individuals infected with the human immunodeficiency virus (HIV) (17). In addition to AZT, other NRTIs such as didanosine, stavudine, and tenofovir are used to combat HIV replication. The basis for their pharmacological effects also relies heavily on modifications to the (deoxy)ribose moiety (Figures 1) (3). Modifications to the sugar moiety allow the corresponding deoxynucleoside triphosphates to function as non-obligate chain terminators to inhibit DNA synthesis (18-21). Nucleosides analogs are also important therapeutic agents against numerous types of cancers including leukemia, lymphoma, and certain solid tumors such as pancreatic and lung cancer. Indeed, approximately 20% of all anti-cancer drugs are classified as anti-metabolites which inhibit DNA synthesis (22). Nucleoside analogs including gemcitabine and fludarabine (Figure 1) target enzymes such as ribonucleotide reductase and DNA polymerases that play essential roles in DNA replication (23). As described above, the pharmacological activity these anti-cancer agents is also predicated on modifications to the (deoxy)ribose moiety.

This review article describes current research efforts applying small molecules as mechanistic probes to understand the complexities of nucleoside metabolism at the cellular level. A key focal point of this article is describing how biophotonic nucleosides are replacing

conventional assays using radiolabeled substrates to study the mechanisms by which intracellular concentrations of various nucleosides and deoxynucleosides are regulated and how defects in this regulation can perturb cellular homeostasis. Emphasis is placed in two important areas. The first section describes classic and contemporary approaches to monitor nucleoside transporter activity while the second section describes approaches to understand nucleotide utilization during the replication of normal and damaged DNA.

Nucleoside Transport. In general, the *de novo* biosynthesis of nucleosides and nucleotides is an energetically costly process that is restricted to selected cell types (24). In terms of cellular energy requirements, it is more efficient for cells to obtain nucleosides by salvage pathways which take advantage of nucleoside recycling (25, 26). The first step in this process is the transport of a nucleoside past the cell membrane (Figure 2). This seemingly simple task is in fact remarkably challenging as nucleosides and nucleotides are rather hydrophilic and thus show negligible permeability across hydrophobic cell membranes (27). To facilitate uptake, cells use specific proteins to catalyze movement of nucleosides from the extracellular milieu into their cytosol (28). There are two types of cellular process used in nucleoside transport. The first is an equilibrative transport system which allows for bi-directional movement of nucleoside, following a concentration gradient to achieve nucleoside influx or efflux. This system shows broad substrate specificity and relatively low affinity for nucleoside substrates. The second system is defined as concentrative as it catalyzes unidirectional movement of nucleosides against a concentration gradient using Na^+ as the co-substrate. This system exhibits some substrate specificity and displays considerable higher affinities for nucleoside substrates compared to the equilibrative system (29). These two types of nucleoside transport processes are catalyzed by classes of membrane-bound proteins designated as equilibrative nucleoside transporters (ENTs)

or concentrative nucleoside transporters (CNTs).

The human ENT family of membrane proteins has four members (designated hENT1-4) that contain a predicted protein topology of 11 transmembrane helices (30). The best characterized members of this family include hENT1, hENT2, and hENT3 which display similar and broad selectivities for purine and pyrimidine nucleosides (31-33). However, hENT2 and hENT3 can also transport nucleobases whereas hENT1 cannot (34). hENT4 is unique in its ability to selectively transport adenosine as well as various organic cations (35).

There are three hCNT isoforms that differ from hENT family members in several important ways. First, hCNTs contain 13 transmembrane domains (36) whereas hENTs contain only 11 transmembrane domains. More importantly, all three hCNTs transport nucleosides in a Na^+ -dependent manner whereas hENTs are Na^+ -independent (37). hENTs also differ in nucleoside specificity in which hCNT1 prefers pyrimidine nucleosides while hCNT2 prefers purines and hCNT3 shows broad substrate specificity. Finally, hCNT family members bind nucleosides with ~10-fold higher affinity than hENT family members, and this arguably results from the need for concomitant Na^+ transport.

With the exception of hENT4, all nucleoside transporters can tolerate diverse, large structural perturbations to the nucleobase portion of the nucleoside. In fact, the major recognition element in defining a substrate is the presence of a (deoxy)sugar moiety. For example, hENT1 and hENT2 have strong interactions with the hydroxyl group at the 3'-position of the sugar while nucleosides containing substitutions or configurational inversion at this position are not transported by hCNT1(38).

Conventional Approaches to Study Nucleoside Transport. Historically, studying the cellular transport of nucleosides has relied extensively on the use of radiolabelled nucleosides. As

outlined in Figure 3, cells are treated with variable concentrations of a nucleoside cocktail containing both radiolabeled and unlabeled substrate. Radiolabeled nucleosides typically contain ^3H and/or ^{14}C at non-reactive positions which increases their overall stability in cell culture. In general, the radiolabeled nucleoside is used as a tracer and thus maintained at less than 5% of the total amount of nucleoside. At variable times, cells are harvested and fixed to terminate nucleoside movement into or out of cells. Cell pellets are obtained via centrifugation and normalized by standard cell counting techniques and/or by measuring total protein content. Liquid scintillation counting techniques are used to measure the amount of radioactive nucleoside inside the cells, and the total amount of uptake is calculated by dividing the measured CPMs in each sample by the specific activity of the nucleoside cocktail. The amount of nucleoside uptake is then plotted as a function of time to define the rate of transport at that fixed concentration of nucleoside substrate. This experiment is then repeated at several different concentrations of nucleoside to define the dose-dependency on the rate of uptake. Measured rates are then plotted as a function of nucleoside concentration, and the resulting plot is of diagnostic importance in determining whether nucleoside uptake occurs by active transport or by simple passive diffusion. A linear plot is consistent with a mechanism in which the nucleoside enters a cell via passive transport. In contrast, the observation of a non-linear curve resembling a rectangular hyperbola, i.e., “saturation” kinetics, is indicative of an active transport mechanism catalyzed by one or more nucleoside transporters. In this latter case, the Michaelis-Menten plot provides a way to empirically measure kinetic parameters that help define the overall efficiency of nucleoside transport. As illustrated, V_{max} defines the maximal rate of nucleoside transport at saturating concentrations of nucleoside. While K_m technically defines the concentration of nucleoside

required to achieve one-half $V_{\max}$, under certain conditions this value can define the true binding affinity of a nucleoside for a transporter (39). However, the most important parameter to consider is $V_{\max}/K_m$ as this reflects the specificity constant for a substrate (40). This value represents transport efficiency under cellular conditions and is a good estimate of the “speed” at which a nucleoside is transported into the cell at concentrations below its K_m value.

There are numerous literature reports of using radiolabeled substrates to define the kinetic parameters for natural and modified nucleosides (41-48). One insightful report is from the Cass laboratory that has extensively studied the mechanism of nucleoside transport using different model systems (41). In this particular study, Visser *et al.* examined the effects of a specific point mutation on the efficiency of nucleoside transport catalyzed by hENT1 and hENT2. This analysis is important as hENT2 differs from hENT1 in several ways such as possessing lower affinities for transportable substrates as well as displaying less sensitivity to inhibitors such as dilazep and dipyridamole. The concentration dependence of ^3H -labeled uridine, cytidine, adenosine, inosine, and guanosine on the rate of transport was determined using recombinant hENT1 and hENT2 overexpressed in yeast. Michaelis-Menten kinetics were observed for all nucleosides that behaved as functional substrates for both wild type and mutant transporters. With hENT1, the three purines (adenosine, inosine, and guanosine) displayed lower K_m values compared to pyrimidines. Specifically, adenosine displayed the lowest K_m value (18 μM) followed by inosine (30 μM) and guanosine (48 μM). Surprisingly, the K_m value of 44 μM measured for uridine is similar to that measured for guanosine while both cytidine and thymine are poor substrates for hENT1. Since the $V_{\max}$ values for all nucleosides were similar to each, the measured differences in their catalytic efficiencies ($V_{\max}/K_m$) appear to be controlled at the level of nucleoside binding.

As mentioned earlier, the kinetic properties of hENT2 are different than those measured for hENT1. In particular, cytidine, thymine, and guanosine show extremely low transport activities with wild-type hENT2. While adenosine, inosine, and uridine function as hENT2 substrates, their catalytic efficiencies are ~2-fold lower compared to values obtained using hENT1. Surprisingly, this reduced efficiency is caused by 5-fold higher K_m values that are slightly offset by faster V_{max} values. The I33M mutant of hENT2 displayed K_m values that were more similar to those measured for hENT1 than wild-type hENT2. In fact, the K_m values for uridine and cytidine are 4-fold lower than those obtained using wild-type hENT2. This mutation had a stronger effect on the recognition of pyrimidines as the K_m values of adenosine, inosine, and guanosine were lower than those of wild-type hENT2 yet still higher than those of hENT1. The V_{max} values of hENT2-I33M for the purine nucleosides, particularly adenosine, were significantly higher than those of either hENT1 or hENT2.

Collectively, the application of radiolabeled substrates to measure the kinetics of nucleoside uptake has provided useful information into the mechanism and regulation of nucleoside homeostasis. In addition, this methodology has been applied in studies using site-directed mutants of various nucleoside transporters to generate structure-activity relationships (SAR) that define structural features required for efficient nucleoside transport (43-45). These kinetic studies have also found utility in other research areas. For example, kinetic studies have aided in developing diagnostic indications to identify which transporter is responsible for the uptake of nucleoside analogs such as gemcitabine and fludarabine which are used as therapeutic agents for treating certain types of cancer (46-48).

Other model systems have been developed to measure nucleoside transport. One powerful alternative to using human cell lines is to transfect overexpression plasmids containing

various CNT and ENT transporters into *Xenopus* oocytes. This represents a useful model due to the ease in performing experiments as well as the close evolutionary relationship of *Xenopus* and humans. Based upon these features, *Xenopus* oocytes are a leading system for studies of ion transport and channel physiology (49). Indeed, this last feature provides a convenient way to measure the activity of a specific nucleoside transporter without complications associated with competing activities of other nucleoside transporters. This is important since the vast majority of nucleoside transporters are promiscuous and can catalyze the uptake of several natural nucleosides. In addition, standard electrophysiology techniques can be used to easily measure Na^+ -dependent nucleoside transport catalyzed by CNT family members (50). After transfection, nucleoside uptake is measured using radioactive substrates and techniques described above. One interesting study reported with this model organism was conducted by Damraju *et al.* in which the uptake of benzamide riboside and tiazofurin, two novel nucleosides that produce cytostatic effects by inhibiting IMP dehydrogenase activity, were directly compared with each other (51). In this study, the transport of tritiated labeled tiazofurin and benzamide riboside were measured in oocytes transfected with several recombinant human nucleoside transporters including hENT1, hENT2, hCNT1, hCNT2, and hCNT3. Quantitative uptake measurements demonstrated that tiazofurin is a better substrate compared to benzamide riboside, displaying a rank order of transportability in oocytes of hCNT3 >> hENT1 > hENT2 > hCNT2 >> hCNT1 (51). In addition, cell-based experiments demonstrated that transport-competent human cells were more sensitive to both drugs compared to transport-deficient cells (51). Collectively, these results validate using *Xenopus* oocytes as a model system to study the mechanism of nucleoside transport and to correlate the activity of nucleoside transporters with the anti-cancer effects of various nucleoside analogs.

Although using radiolabeled nucleosides to measure uptake is the most direct method for evaluating transport activity, there are several experimental variations that can be employed to quantify the uptake of other non-radioactive nucleoside substrates and analogs. For example, classical inhibition studies are widely used to provide indirect proof that a nucleoside analog can interact with a specific nucleoside transporter. The basis for this technique is to quantify the ability of a nucleoside analog to block the uptake of a radiolabelled nucleoside that functions as a transportable substrate. For example, the inhibitory effects of a nucleoside analog can be accurately determined by measuring its dose-dependency toward blocking the cellular uptake of ^3H or ^{14}C -labeled adenosine or uridine. Plotting the percent transport activity as a function of nucleoside analog generates a dose-response curve that defines its IC_{50} value, the concentration required to inhibit 50% transporter activity. The K_d value for the nucleoside, which represents its true binding affinity for a transporter, can be obtained by using the Cheng-Prusoff relationship to normalize the measured IC_{50} value by the concentration of radiolabeled nucleoside substrate and its reported K_m value (52). In general, K_d values are important as they reflect the potency of a nucleoside analog, and this information is again useful in generating structure-activity relationships (SARs) that define structural elements required for binding to specific nucleoside transporters.

Limitations with using radiolabeled nucleosides. There are several experimental limitations with using radiolabeled nucleosides to study the mechanism of nucleoside transport. One issue is that these assays are performed in a discontinuous manner in which several steps must be completed sequentially to properly process samples. The use of multiple steps is time consuming and can introduce systematic errors at each stage of the assay. Both features can compromise the validity of experimental results. Another problem is the need for special requirements to produce and properly

handle radiolabeled nucleosides. These include employing special safety precautions while performing experiments and additional safeguards for storing materials and generated waste products. While conventional analogs such as ^3H -uridine and ^3H -adenosine are commercially available, the vast majority of nucleoside analogs used as anti-cancer or anti-viral agents are not. Thus, the corresponding radioactive nucleosides must be synthesized. Collectively, complications associated with using radioactive nucleosides have illustrated a pressing need to produce alternative substrates that function as efficient surrogates for natural nucleosides.

Alternative approaches using biophotonic probes to visualize nucleoside uptake. Attempts to overcome these deficiencies have focused on developing fluorescent nucleoside analogs that have spectroscopic properties to allow researchers to monitor their movements using non-invasive techniques such as fluorescence microscopy. There are several obvious advantages toward using fluorogenic nucleosides as biophotonic probes to monitor transport. First, the uptake of a fluorescent analog can be monitored in a continuous fashion to provide a more direct and simple approach to quantify transport activity and specificity. A continuous assay also reduces the number of experimental manipulations and thus saves time in addition to reducing the risk of introducing systematic errors. Fluorescence based assays also eliminate the need for specialized equipment to synthesize and handle radiolabeled nucleosides. Unfortunately, naturally occurring nucleosides such as adenosine and guanosine are fluorescently silent and thus cannot be measured using this approach. As a consequence, novel analogs must be developed which possess intrinsic fluorogenic properties and that function as transportable substrates. These two criteria would allow transport of these nucleosides inside a cell should generate an increase in fluorescence intensity which provides an accurate way to monitor the kinetics of nucleoside transport. Provided below are highlights

describing recent developments using biophotonic nucleosides to measure the activity of nucleoside transporters as well as to define their cellular distribution.

One report by Zhang *et al.* tested the ability of two fluorescent nucleoside analogs designated FuPmR and dFuPmR (Figure 4) to function as biophotonic substrates for several nucleoside transporters (53). The utilization of both analogs was tested against five recombinant human nucleoside transporters including hENT1, hENT2, hCNT1, hCNT2, and hCNT3 (53). In these studies, overexpression plasmids containing each transporter were transfected into *Saccharomyces cerevisiae* or *Xenopus* oocytes. Real-time analysis of the transport of FuPmR into live cells was performed using fluorescent confocal microscopy and employing the autofluorescence of FuPmR to directly visualize its movement into cells (53). This group demonstrated that the transport of FuPmR is catalyzed by hENT1 as demonstrated by the ability of nitrobenzylmercaptapurine ribonucleoside (NBMPR) and dilazep (Figure 4), two inhibitors of hENT1, to block the uptake of FuPmR (53). In addition, live cell imaging of FuPmR was performed against a panel of human cancer cell lines possessing variable hENT1 activity (53). Time courses in the fluorescence changes associated with the uptake of FuPmR were biphasic. The fast first phase, representing initial uptake, was complete with 20 seconds while the second phase was considerably slower (53). Equally important, these authors were able to use time-lapse confocal microscopy to show the intracellular accumulation of FuPmR in organelles such as the nucleus and mitochondria which rely heavily on nucleosides for optimal activity (53). In general, the use of autofluorescent nucleosides using time-lapse confocal microscopy provides a novel strategy to study transport activity.

Another advantage of fluorescence detection is the ability to define the localization and distribution of nucleoside transporters. For instance, a report by Robins *et al.* describes the

synthesis and applications of several fluorescent 5'-thioadenosine analogs as probes that can localize and quantify the distribution of hENT1 on cancer cells (54). The rationale behind this approach was to attach fluorescent moieties to the tight binding analog, NBMPR, to produce a selective and sensitive probe to determine hENT1 content in different cell lines. Indeed, conjugating the fluorescent probe fluorescein-5-yl isothiocyanate (FITC) to NBMPR analogs produced a series of spectroscopically active compounds that bind hENT1 with nanomolar affinity (54). Furthermore, these authors applied these probes with flow cytometry techniques to quantify the amount of hENT1 present on leukemia cells (54). With these tools, the authors could predict the efficacy of the anti-cancer agent, gemcitabine, which is a known substrate for hENT1 (54). Collectively, these results demonstrate how fluorogenic probes against specific nucleoside transporters can be used as prognostic indicators to predict the effectiveness of nucleoside analogs used as anti-cancer agents.

Our efforts in this area have focused on developing metal-containing nucleoside analogs that function as fluorogenic surrogates for natural nucleosides. One recently developed analog, designated Ir(III)-PPY nucleoside, contains iridium embedded within a bis-cyclometallated scaffold attached to a deoxyriboside (Figure 4) (55,56). Cyclometallated complexes of iridium(III) were chosen as these organometallic complexes are highly stable and can withstand the aqueous environment of a living cell (57). In addition, these complexes are high-yielding phosphorescent compounds which can be rationally designed to emit light at various wavelengths (58). In particular, spectroscopic measurements of these metal-containing nucleosides can be performed with excitation wavelengths greater than 380 nm which are far beyond the wavelengths for most biological fluorophores. Although the large size of Ir(III)-PPY nucleoside suggests that it would function as a poor substrate for any nucleoside transporter,

modeling studies show that the three dimensional structure of Ir(III)-PPY nucleoside is surprisingly compact and spherical (56). In fact, the volume of Ir(III)-PPY nucleoside is 596.4 Å³ and only ~2.5 fold larger than deoxyadenosine (228.5 Å³) (56). To verify that Ir(III)-PPY nucleoside functions as a transportable substrate, we treated cervical cancer cells (KB3-1) with variable concentrations of the metal-containing nucleoside and measured fluorescence associated its uptake at variable times ranging from 4 to 24 hours (56). Our results show that the intracellular accumulation of Ir(III)-PPY nucleoside is both time- and dose-dependent. Efficient uptake of low concentrations (10 µM) is observed at short time intervals (less than 4 hours). To determine which nucleoside transporter is involved in the uptake of Ir(III)-PPY nucleoside, we first measured uptake in the presence of pharmacological inhibitors of nucleoside transporters. Our results showed that inhibitors against hENT1, namely dipyrimadole and NBMPR, blocked the cellular uptake of Ir(III)-PPY nucleoside. We further verified the role of hENT1 using siRNA to knockdown its expression levels. Western blot analyses demonstrated that cells treated with siRNA against hENT1 have significantly lower levels of the nucleoside transporter compared to cells treated with scrambled siRNA. Fluorescent microscopy studies confirmed that Ir(III)-PPY nucleoside uptake is significantly reduced in siRNA treated cells compared to cells treated with DMSO. Collectively, data obtained using genetic approaches and pharmacological inhibitors validate that hENT1 catalyzes the cellular uptake of Ir(III)-PPY nucleoside.

The Role of Nucleoside Metabolism in DNA Replication. In the following sections, we describe the utility of nucleotide analogs to study DNA synthesis. DNA replication is the biological process by which DNA is duplicated to produce two copies of an organism's genetic information. This complex process is catalyzed by a confederation of enzymes and proteins that collectively define a replication fork (59-61). However, the most important enzyme involved in this process

is the DNA polymerase which adds mononucleotides into a growing primer using nucleic acid templates to guide each incorporation event (Figure 5) (62). At the molecular level, the process of faithfully replicating DNA is based upon the mutual recognition of adenine (A) by thymine (T) and of guanine (G) by cytosine (C). These interactions involve complementarity in hydrogen-bonding interactions between each nucleobase. Using these interactions, most replicative DNA polymerases display incredible fidelity, making only 1 mistake every million opportunities (63). It is even more impressive that polymerases can maintain this accuracy when replicating DNA at remarkably high speeds of >500 base pairs/second (64).

Mammalian cells possess at 15 different DNA polymerases that can be classified based on their biological function (65). For example, pol α , pol δ , pol ϵ , telomerase, and pol γ are involved in chromosomal or mitochondrial DNA synthesis. These polymerases are typically considered "high-fidelity" enzymes as they efficiently and faithfully replicate undamaged DNA. A second group consists of pol β , pol λ , pol μ , and terminal deoxynucleotidyl transferase (TdT) and are involved in various DNA repair pathways including base excision repair and double-strand DNA break repair. Finally, the most diverse group of DNA polymerases includes pol η , pol ι , pol θ , pol ζ , pol κ , and the Rev1 protein which are coined "specialized" due to their ability to replicate a wide variety of structurally distinct DNA lesions. Originally, these polymerases were classified as "error-prone" since each showed a remarkable lack of fidelity when replicating undamaged DNA (66-68). For example, pol η displays a high error frequency of 1 mistake every 100 to 1000 opportunities when replicating undamaged DNA (69). However, pol η is highly proficient at replicating DNA lesions such as thymine dimers, abasic sites, O⁶-methyl-guanine, 8-oxoguanine, and crosslinked DNA lesions such as cisplatinated DNA under *in vitro* and *in vivo* conditions (70-73).

Using Radioactive Nucleosides to Measure DNA Synthesis. Cell-based studies to monitor DNA synthesis have generally employed radioactive nucleosides such as ^3H -thymidine. Experiments are performed as similarly described using radiolabeled nucleosides to monitor the activity of nucleoside transporters (vide supra). As before, cells are treated with variable concentrations of a nucleoside cocktail containing both unlabeled and tritiated thymidine. At variable times, cells are harvested and liquid scintillation counting techniques are used to measure the amount of radioactive nucleotide incorporated into DNA. Alternatively, DNA can be purified from cells and imaged using autoradiography techniques to monitor DNA synthesis. While extensively used to measure DNA synthesis, this technique suffers from many of the complications described earlier (discontinuous assay, time consuming, safety requirements). As a result, other techniques including the use of nucleosides containing either halogens or alkyne groups have essentially replaced radioactive nucleosides to measure DNA synthesis. These two technologies are further described below.

Antibody detection of halogenated nucleoside analogs. Thymidine analogs including halogenated pyrimidines such as 5-bromo-2-deoxyuridine (BrdU) and 5-chloro-2-deoxyuridine (CldU) replaced the use of radiolabeled nucleosides as chemical agents to study the extent and timing of DNA synthesis during replication, repair, and recombination. BrdU has been the most extensively used nucleoside analog applied experimentally to label DNA at the cellular and organismal level (reviewed in 74 and 75). The experimental paradigm for using BrdU and other halogenated nucleosides is depicted in Figure 6A. Experiments are typically performed in a similar fashion as those described above using radioactive nucleosides. Briefly, cells are treated with a fixed concentration of BrdU for brief periods of time (<4 hours) which allows for efficient uptake of the analog. After enzymatic conversion to the corresponding nucleoside triphosphate,

the analog is stably incorporated into DNA. The labeling of cells is followed by antibody staining directed against the modified nucleobase.

Since this technique does not rely on radioisotopes, it is far more convenient than using ^3H -thymidine. Antibody detection of DNA provides additional benefits since DNA synthesis can be quantified using spectroscopic methods in 96-well plate formats or via flow cytometry techniques, both of which are conducive for high throughput analyses. Equally important, the use of fluorescently labeled antibodies provides a convenient and sensitive way to visualize DNA synthesis using confocal fluorescence microscopy. Furthermore, applying kinetic approaches such as pulse-chase methods can increase the dynamic range of these agents by allowing researchers to differentiate between DNA synthesis occurring during S-phase (replication) versus other stages of the cell cycle (DNA repair and/or recombination). Collectively, the combination of versatility and convenience made BrdU staining the gold standard for measuring cellular DNA synthesis. Unfortunately, the BrdU assay suffers from several deficiencies that limit its overall sensitivity and widespread use. One particular deficiency is the low efficiency for directly binding anti-BrdU antibodies to native DNA containing BrdU. The low efficiency requires that harsh chemicals or additional enzymes be used to disrupt the structure of DNA in order to generate better interactions between the two molecules. The obvious drawback is that this requirement increases the number of experimental manipulations and hinders the ability to observe native DNA structures such as nucleosomes.

Measuring DNA synthesis using “click” chemistry. In 2008, Salic and Mitchison described an improved method for detecting DNA synthesis (76). This technique is based on applying “click” chemistry, the Cu(I)-catalyzed variant of the Huisgen [3+2] cycloaddition between a terminal alkyne and an azide (77-79). The key innovation of this method is the use of 5-ethynyl-2'-

deoxyuridine (EdU), a thymidine analog in which the methyl group is replaced with an alkyne (Figure 6B). The corresponding nucleoside triphosphate, EdUTP, is incorporated into DNA opposite A like dTTP or BrdUTP. However, the advantage is that the alkyne moiety of EdU reacts both efficiently and selectively with azide-containing fluorophores in the presence of Cu(I) catalyst. This reaction leads to an increase in the sensitivity and speed for performing assays to monitor DNA synthesis. Both features are achieved since most fluorescent azides are much smaller than monoclonal antibodies, and the reduced size provides a greater degree of probe penetration into DNA which correlates with increased sensitivity and improved resolution. In addition, the “click” chemistry method does not require sample fixation or DNA denaturation as does the BrdU assay. This is a significant advancement as the ability to retain the integrity of DNA allows researchers to visualize detailed features of nucleic acid ranging from its helical properties to nucleosome structures to identifying banding patterns in chromosomes. Furthermore, the milder labeling conditions used in “click” chemistry allows for the concomitant utilization of other biochemical probes to directly compare EdU incorporation with cellular biomarkers such as proteins associated with DNA replication and DNA repair including PCNA and γ H2AX, respectively (80,81). Due its superiority compared to halogenated thymidine analogs, EdU is now considered the premier method for labeling nucleic acid and has found far-reaching applications toward quantifying DNA synthesis at the cellular and organismal level. Provided below are a few recent highlights demonstrating the utility of EdU in these areas.

Many basic science and clinical laboratories evaluate the proliferative response of cells to various chemotherapeutic agents. In the past, the gold standard for measuring the potential cytostatic effects of an agent was routinely performed by measuring ^3H -thymidine incorporation into DNA. This is now being replaced by EdU as it functions as an acceptable nucleoside

surrogate for high throughput testing. For example, Poujol *et al.* compared the performance of EdU incorporation for measuring lymphocyte proliferation versus ^3H -thymidine (82). These authors verified that EdU incorporation assays are highly reproducible and show excellent correlations with results obtained using ^3H -thymidine incorporation assays (82). In addition, their work validated that the EdU assay is both stringent and versatile enough to maintain several important clinical laboratory constraints such as delayed stimulation, low number of cells, and delayed analysis after staining (82).

The labeling efficiencies of EdU versus halogenated pyrimidines have also been extensively studied at the cellular level using fission yeast as the model organism (83). In this study, Anda *et al.* demonstrated that both nucleoside analogs adversely affect cell cycle progression (83). However, these effects can be minimized by modifying the concentrations of each analog. In addition, applying pulse-chase techniques allowed for the sequential labelling of two consecutive S-phases of the cell cycle (83). Results from this study demonstrated that using these analogs to “double label” nucleic acid during replicative DNA synthesis provides a much more sensitive method to measure cell-cycle progression compared to conventional flow cytometry approaches employing propidium iodide as a marker for DNA content (83).

Further expanding upon this technique, Hoshi and Ushiki used EdU incorporation to analyze replicating banding patterns in human chromosomes during metaphase (84). In this study, human lymphocytes were first synchronized using excess thymidine and then treated with EdU during the late S-phase (84). “Click” chemistry was then applied to covalently attach fluorogenic azides to the incorporated EdU, and this provided a way to visualize chromosomes during metaphase (84). Subsequent microscopy imaging showed that chromosomes contained EdU and displayed a banding pattern similar to G-banding observed in human chromosomes

(84). This result validates the ability of EdU to provide high resolution structures of DNA. A different study by Haines *et al.* demonstrated that EdU could quantify mitochondrial biogenesis by measuring rates of mitochondrial DNA replication (85). This technique couples EdU incorporation with a tyramide signal amplification protocol to produce a convenient yet sensitive assay that can visualize mitochondrial DNA replication (85). Since mitochondria play important roles in regulating cellular energy and apoptosis (86-88), the application of EdU to define mitochondrial biogenesis provides a novel technique to study its function. This is especially important in defining how defects mitochondrial function can cause pathological conditions such as aging, cancer, and neurodegenerative disease.

EdU has also been used as a biomarker for cellular proliferation at the organismal level. One interesting study reported by Asano *et al.* used BrdU and EdU as dual labels to measure the migration rates of cells in the small intestines of rats (89). This represents an important achievement as epithelial cells that comprise the small intestine are a self-renewing system. In this case, intestinal cells cycle through proliferation, differentiation, and death in a sequential process during their migration from the intestinal crypt to the villus. In the study by Asano *et al.*, rats were first injected with fixed concentrations of BrdU and EdU. At variable time intervals, intestinal tissue sections were removed and examined using immunohistochemistry techniques to measure BrdU-positive cells while Edu-positive cells were identified using "click" chemistry to attach fluorogenic probes (89). These studies showed that BrdU- positive cells completely overlap with EdU-positive cells in rats that received both nucleosides simultaneously (89). More importantly, cell migration rates could be measured by dividing the distance travelled in BrdU- and EdU-positive cells by the time interval between nucleoside injections. Their results show

that epithelia cells in the jejunum migrate nearly twice as fast compared to epithelia cells in the ileum (compare 9 versus 5 $\mu\text{m/hr}$, respectively) (89).

These organismal studies have paved the way for more complicated endeavors such as monitoring stem cell migration. This is an important biological area as stem cell replacement promises to be a new therapeutic strategy to treat degenerative diseases such as neurological and cardiovascular disorders (90, 91). However, one limitation in stem cell therapy is the ability to properly target these cells to their desired location. Toward this goal, EdU has been used as a novel method to label stem cells and subsequently track their location and capabilities to proliferate and differentiate. To date, there are mixed reports on utilizing EdU as a tracking agent in stem cell replacement therapies. For example, Lin *et al.* used EdU as a probe to label and track adipose tissue-derived stem cells (ADSC) (92). Labeling was achieved via intraperitoneal injection of EdU followed by “click” chemistry and examination using histological and fluorescence microscopy to identify cells positives for EdU incorporation. While this technique showed that EdU is incorporated into ~50% of ADSC, the percentage of cells remaining EdU-positive decreased as function of time. This reduction could result from the elimination of stem cells by apoptosis or by “dilution” of the Edu-positive cell population caused by cellular proliferation. Although the answer to this question is currently not known, it was shown that EdU-positive cells were found in both epithelia and connective tissues several hours post injection. However, long-term tracking (over 2 to 6 weeks post-injection) showed that only connective tissue contained EdU-positive cells. Collectively, this study suggests that EdU can function as a novel probe to monitor stem cell migration and proliferation.

Other groups have reported differing results that dampen enthusiasm for applying EdU as a tracking agent for stem cell therapy. For example, Andersen *et al.* found that stem cells

treated with EdU showed reduced viability (93). In addition, stem cells labeled with EdU showed low survival rates after transplantation into regenerating muscle. Consistent with these observations, other groups have demonstrated that EdU treatment reduces cell viability in time- and dose-dependent manners. A report by Kohlmeier *et al.* demonstrated that EdU incorporation does not adversely affect the rate of DNA synthesis catalyzed by the moving replication fork (95). However, cellular effects of EdU exposure range from delayed cell cycle progression to complete cell cycle arrest (95). The magnitude of these effects depends upon the type of cell line used. Mouse embryonic stem cells displayed the most severe phenotype as treatment with EdU caused cell-cycle arrest at G2/M followed by apoptosis (95). Furthermore, Zhao *et al.* showed that EdU incorporation causes several DNA damage responses including phosphorylation of repair proteins such as ATM histone H2AX, p53, and Chk2 (96). Consistent with these features, confocal microscopy analysis of cells pulsed with EdU for 1 hour and then grown for an additional 48 hours showed an increased number of foci containing γ H2AX and p53BP1 which are markers for DNA double-strand breaks (96).

In retrospect, the cytostatic and cytotoxic effects produced by EdU should not come as a major surprise as these effects were initially reported by DeClerq and colleagues decades earlier (97,98). In particular, this group synthesized several 5-substituted uracil derivatives such as 5-vinyl-2'-deoxyuridine and 5-ethynyl-2'-deoxyuridine and tested these agents as anti-cancer and antiviral agents (98). 5-ethynyl-2'-deoxyuridine displayed the highest antiviral potency amongst a large number of analogs tested (98). At the time, these authors proposed that the cytotoxic effects of 5-ethynyl-2'-deoxyuridine was caused by inhibiting thymidylate synthase, the enzyme that converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (98). Inhibiting this enzyme would deplete dNTP pools and thus

adversely affect DNA synthesis. While inhibiting thymidylate synthase could ultimately account for an induction of DNA damage, more research is needed to validate the exact molecular target(s) of EdU.

Based on these concerns, several attempts have been made to develop additional alkyne-containing nucleosides that can effectively measure DNA synthesis without producing toxic cellular effects. One promising agent is the deoxycytidine analog, 5-ethynyl-2'-deoxycytidine (EdC), which has been shown through *in vitro* and *in vivo* techniques to measure DNA synthesis with sensitivities comparable to EdU (99). Furthermore, EdC is much less cytotoxic than EdU (99), suggesting that it could be used for long-term detection of proliferating cells.

Using “Click” Chemistry to Measure Translesion DNA Synthesis. Cellular DNA is constantly exposed to a large variety of internal and external DNA damaging agents. The resulting DNA lesions can either be corrected by various repair pathways or misreplicated by DNA polymerases in a complex biological process known as translesion DNA synthesis (TLS). In humans, TLS can compromise the integrity of an individual's genome and have devastating consequences with respect to disease development. In addition, unregulated TLS can interfere with the cell's ability to properly respond to DNA damaging agents used in anti-cancer treatments. Despite the biological importance and obvious medical ramifications of TLS, relatively little is known regarding the molecular mechanism(s) by which various DNA polymerases misreplicate damaged DNA.

Much of this deficiency arises from the commonality in dNTP utilization during both normal and translesion DNA synthesis. For example, several groups have shown that dATP is preferentially incorporated opposite non-instructional DNA lesions such as abasic sites (100-

102). However, dATP is also inserted opposite every templating T present in DNA. Furthermore, the higher frequency for inserting dATP opposite T (average of 1 in 4 bases) versus an abasic site (estimated to occur in 1 of 100,000 bases) makes it essentially impossible to distinguish translesion DNA synthesis from normal replication. Our efforts to deal with this problem have focused on engineering non-natural nucleotides that can be efficiently and selectively inserted opposite specific DNA lesions. The goal is to alleviate complications from common dNTP utilization during normal and translesion DNA synthesis to provide transformative chemical tools that can easily monitor the replication of damaged DNA at the cellular and organismal level. Much of our work has focused on the misreplication of non-instructional DNA lesions such as abasic sites. As indicated above, most high-fidelity DNA polymerases preferentially incorporate dATP opposite this lesion (103-105). Using this information, we synthesized a series of indolyl nucleotide analogs that mimic the core structure of dATP but that are devoid of classical hydrogen-bonding groups that are required for normal DNA synthesis. This negative selection mechanism allowed us to identify one particular analog, 5-nitroindolyl-2'-deoxyriboside triphosphate (5-NITP) (Figure 7) which is efficiently and selectively inserted opposite abasic sites by several DNA polymerases including pol δ , pol η , and TdT (106, unpublished results (JSC & AJB)). In fact, the efficiency for insertion opposite an abasic site is ~1,000-fold greater than measured for dATP, the preferred natural substrate. 5-NITP also retains high selectivity as it is incorporated opposite an abasic site ~10,000-fold more efficiently than any templating base.

We next converted this analog into a chemical probe to visualize and quantify the replication of damaged DNA under cellular conditions (Figure 7). This was achieved again by applying "click" chemistry to tag the non-natural nucleotide with fluorogenic probes after its incorporation opposite the DNA lesion. Specifically, an ethynyl moiety was placed at the 3-

position of 5-NITP to generate 3-Eth-5-NITP. Similar to EdU, this non-natural nucleotide can react with an azide-containing dye in the presence of Cu(I) catalyst to covalently attach a fluorogenic probe to the nucleotide inserted opposite the DNA lesion (106). Cell-based experiments demonstrated that the corresponding "clickable" nucleoside, 3-Eth-5-NIdR, functions as a probe that measures the cellular activity of TdT, a specialized DNA polymerase that randomly incorporates nucleotides at double-stranded DNA breaks (106). Human cancer cells that overexpress TdT were treated with 3-Eth-5-NIdR for 2 days. After this time, the cells were fixed, permeabilized, and washed to remove unbound nucleoside and/or nucleotide. The cells were treated with azide-containing fluorescent dyes and Cu(I) to initiate "clicking" of the azide fluorophore to any ethynyl-containing nucleotide incorporated into DNA. The amount of "clicked" DNA was quantified via dual parameter flow cytometry using propidium iodide staining to detect genomic DNA coupled with fluorescence detection of ethynyl-containing nucleotide incorporated into DNA. Cells treated with DMSO have low levels (<0.5%) of fluorescently labeled DNA while those treated 3-Eth-5-NIdR show higher levels of fluorescently labeled DNA (>5%). More importantly, the level of "clicked" DNA measured in leukemia cells correlated with the expression levels of TdT. Thus, this non-natural nucleoside highlights the ability to quantify the activity of specialized DNA polymerases involved in translesion DNA synthesis.

Future Directions. This article highlights some of the classic and contemporary approaches used to measure the uptake and metabolism of nucleosides. The application of radiolabeled and alkyne-containing nucleosides has provided new insight into measuring two important steps in nucleoside metabolism – the uptake of nucleosides and their incorporation into DNA. However, there are still many biological processes that could benefit from these probes, especially

nucleosides that possess biophotonic properties. In particular, it would be interesting to apply these analogs to better understand nucleoside metabolism such as the conversion of nucleosides to their mono, di, and triphosphate metabolites. Another area of interest is to apply these analogs to study the dynamic behavior of RNA metabolism. Finally, it should be noted that some of the novel nucleoside analogs described here could have a significant impact on clinical medicine in the future. In particular, cellular levels of nucleoside transporters are proposed to be predictive factors for patient responses to nucleoside analogs such as gemcitabine. However, there are several technological problems that currently exist using information regarding nucleoside transporter activity as a predictive index for therapeutic response. Much of this challenge arises from difficulties in easily identifying which transporters are responsible for the uptake of nucleoside analogs due to the existence of multiple family members coupled with their promiscuity in substrate utilization. This combination makes it difficult to selectively monitor the activity of one transporter versus the activity of others. As such, an important endeavor is to develop biophotonic nucleosides that are selectively utilized by specific nucleoside transporters. Another important goal is to develop additional biophotonic nucleotides that can differentiate translesion DNA synthesis from normal replication. Developing these tools is important since this aberrant activity is associated with the development of drug resistance to chemotherapeutic agents that damage DNA. The information obtained from these tools could be used to better understand how cells respond to DNA damage by invoking the activity of specialized DNA polymerase to “misreplicate” various DNA lesions. In addition, this technology could be applied in translational studies to accurately predict patient responses to DNA damaging agents used in chemotherapy.

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Figure Legends

Figure 1. (A) Structures of natural nucleosides (adenosine, cytosine, guanosine, and uridine/thymidine). (B) Structures of representative nucleoside analogs that function as anti-viral agents. (C) Structures of representative nucleoside analogs that function as anti-cancer agents.

Figure 2. Summary of nucleoside transporters and their substrate utilization. The human ENT family of membrane proteins has four members designated hENT1-4. hENT1, hENT2, and hENT3 show similar kinetic behavior for using purine and pyrimidine nucleosides as substrates. hENT2 and hENT3 also transport nucleobases whereas hENT1 cannot. hENT4 selectively transports adenosine as well as organic cations. There are three hCNT isoforms that transport nucleosides in a Na^+ -dependent manner. hCNT family members bind nucleosides with ~10-fold higher affinity than hENT family members. hCNT1 prefers pyrimidine nucleosides while hCNT2 prefers purines. hCNT3 shows broad substrate specificity. With the exception of hENT4, all nucleoside transporters can tolerate diverse, large structural perturbations to the nucleobase portion of the nucleoside.

Figure 3. Summary of experimental methods used to define nucleoside uptake using radiolabeled nucleosides as substrates. Cells are treated with variable concentrations of a nucleoside cocktail containing both radiolabeled and unlabeled substrate. At variable times, cells are harvested and fixed to terminate nucleoside movement into or out of cell. Liquid scintillation counting techniques are used to measure the amount of radioactive nucleoside inside the cells. The total amount of uptake is calculated by dividing the measured CPMs in each sample by the specific activity of the nucleoside cocktail. The amount of nucleoside uptake is then plotted as a function

of time to define rates of transport at as a function of nucleoside substrate. Rates are plotted as a function of nucleoside concentration to define the dose-dependency on the rate of uptake. The kinetic parameters, $V_{\max}$, K_m , and $V_{\max}/K_m$, are empirically determined from the resulting Michaelis-Menten plot.

Figure 4. Comparison of the structures of deoxyadenosine with fluorescent analogs (dFuPmR and Ir(III)-PPY nucleoside) used as biophotonic probes to measure the activity of nucleoside transporters.

Figure 5. Summary of DNA polymerases function and their biological roles during replication, repair, recombination, and translesion DNA synthesis. See text for further details.

Figure 6. (A) Summary of experimental methods used to measure DNA synthesis via BrdU incorporation. Cells are treated with a fixed concentration of BrdU for brief periods of time. Following uptake of the analog, the halogenated nucleoside is enzymatic converted to the corresponding nucleoside triphosphate. BrdUTP is then stably incorporated into DNA by the action of one or several different DNA polymerases. Cells are then harvested and fixed for subsequent staining with antibodies directed against BrdU incorporated into DNA. (B) Summary of experimental methods used to measure DNA synthesis via EdU incorporation. Cells are treated with a fixed concentration of EdU for brief periods of time. Following uptake of the analog, the modified nucleoside is enzymatic converted to the corresponding nucleoside triphosphate. EdUTP is then stably incorporated into DNA and cells are then harvested, fixed, and permeabilized. Cells are then treated with azide-containing fluorophores in the presence of

Cu(I) catalyst which allows for the covalent attachment of the fluorescent dye to DNA at sites where EdU was inserted.

Figure 7. Comparison of the structures of dATP with 5-nitroindolyl-2'-deoxyriboside triphosphate (5-NITP) and 3-ethynyl-5-nitroindolyl-2'-deoxyriboside triphosphate (3-Eth-5-NITP) which are non-natural nucleotides selectively incorporated opposite non-instructional DNA lesions such as abasic sites.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

A

B

C

Figure 5.

Figure 6.

Figure 7.

Adenosine-2'-deoxyribose triphosphate (dATP)
5-nitroindolyl-2'-deoxyriboside triphosphate (5-NITP)
3-ethynyl-5-nitroindolyl-2'-deoxyriboside triphosphate (5-NITP)

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