Functionalized DNA Hot Paper

DOI: 10.1002/anie.201404742

# 7-Aryl-7-deazaadenine 2′-Deoxyribonucleoside Triphosphates (dNTPs): Better Substrates for DNA Polymerases than dATP in Competitive Incorporations**

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***Abstract:*** *A series of 7-substituted 7-deazaadenine and 5-substituted cytosine 2′-deoxyribonucleoside triphosphates (dNTPs) were tested for their competitive incorporations (in the presence of dATP and dCTP) into DNA by several DNA polymerases by using analysis based on cleavage by restriction endonucleases. 7-Aryl-7-deazaadenine dNTPs were more efficient substrates than dATP because of their higher affinity for the active site of the enzyme, as proved by kinetic measurements and calculations.*

Base-functionalized DNA has a wide range of applications and can be efficiently prepared by the incorporation of base-modified 2′-deoxyribonucleoside triphosphates (dNTPs) with polymerases, either by primer extension (PEX) or the polymerase chain reaction (PCR).[1,2] These procedures use a modified dNTP in the absence of its natural counterpart to ensure that the polymerase incorporates the modified nucleotide. Little attention has been paid to incorporations of a modified nucleotide in competition with the natural one, presumably because it was supposed that the non-natural dNTPs must be much worse substrates for polymerases and result in a low frequency of incorporation. However, any prospective in vivo applications of modified dNTPs inherently require competitive incorporation in the presence of an excess of all four natural dNTPs. Therefore, we report here on the competitive incorporations of 5-modified dCTP and 7-modified 7-deaza-dATP derivatives (Figure 1).

Competitive in vivo incorporation of modified dNTPs is performed in DNA metabolic labeling, which traditionally uses 5-bromo-dUTP,[3] and has recently been complemented by ethynyl- (EdU,[4] EdC,[5] or 7-ethynyl-7-deaza-dATP[6]) or azido-substituted dNTPs,[7] but no quantitative or kinetic study was reported. Detailed mechanistic and structural studies on the incorporation of modified dNTPs were performed by Marx and co-workers,[8,9] including a competitive study of single nucleotide incorporations, which were monitored by the different mobility of natural and modified extended primer in gel electrophoresis (PAGE). Most modified dNTPs were worse substrates for polymerases than the natural ones,[8] with the exception of 7-[(hydroxydecanoyl)aminopentynyl]-7-deaza-dATP, which was a slightly better substrate for KlenTaq polymerase than dATP, but the authors did not comment on it further.[9] However, this PAGE-based method can only be applied for the analysis of single nucleotide incorporation with bulky modifications, where the differences in mobility are distinctive. Therefore, a new method needed to be developed for the analysis of competitive primer extension and multiple incorporations (including PCR).

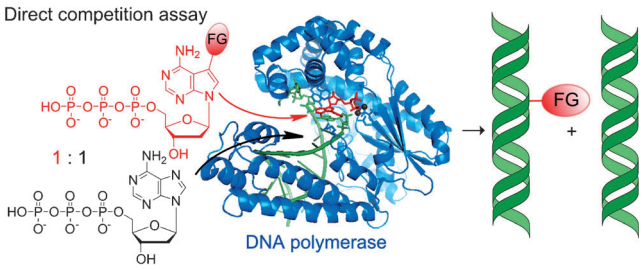


***Figure 1.*** Competitive incorporation of **dA$^{X}$TP** versus dATP.

Recently, we studied the influence of major-groove modifications on the cleavage of DNA by restriction endonucleases (REs) and found that some REs do not tolerate any modifications, whereas other REs are blocked only by bulky groups.[10] Therefore, we envisaged the use of REs[11] for analyzing the products of competitive incorporations since, for any modification, we know specific REs which do not cleave the modified recognition sequence. Thus, PEX can be performed with a mixture of modified and natural dNTPs, followed by cleavage using the appropriate RE and PAGE analysis (Figure 2). This enables the proportion of modified to unmodified sequence to be quantified by the intensity of the two spots corresponding to the products.

We selected a series of eight previously reported 7-substituted 7-deaza-dNTPs and six 5-substituted dCTPs bearing various bulky functional groups. They included ethynyl and phenyl derivatives,[10] redox labels (**dN$^{NO2}$TP** and **dN$^{NH2}$TP**),[12] fluorescent labels (**dA$^{BFU}$TP**, **dA$^{ABOX}$TP**),[13] reactive groups (**dC$^{FT}$TP**),[14] and very bulky groups (**dN$^{STr}$TP**).[15] They were incorporated through competitive PEX by different DNA polymerases (Pwo, KOD XL, Taq, Vent(*exo*-), Klenow, Bst, and human Pol α) to different

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[**] This work was supported by the ASCR (RVO: 61388963) and by the Czech Science Foundation (P206-12-G151).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201404742.

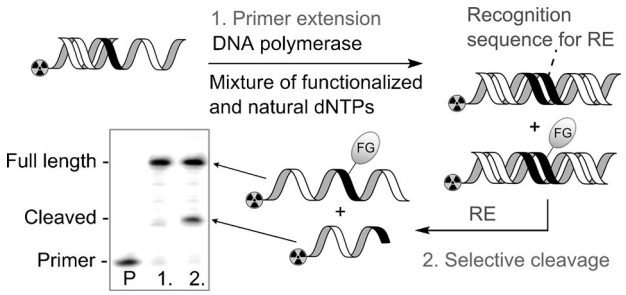
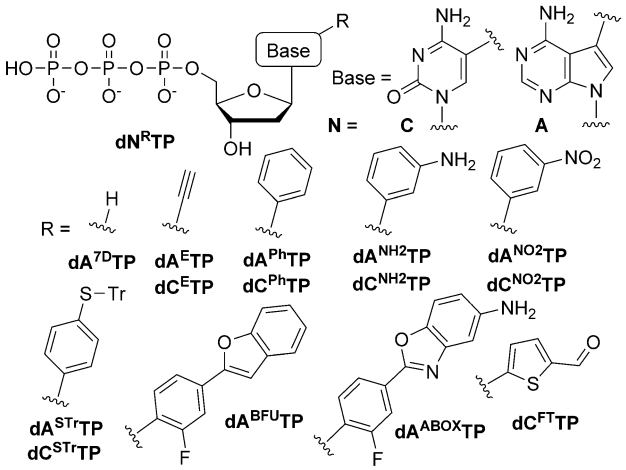



***Figure 2.*** Analysis of competitive PEX using REs and PAGE.




sequences in different ratios of modified **dN$^{X}$TP** to unmodified dNTP (1:1 and 10:1). The PAGE analysis of RE cleavage was first compared with MALDI analysis of the products of competitive PEX experiments using **dA$^{Ph}$TP** and **dC$^{Ph}$TP** and Pwo polymerase to validate the method. A clear correlation between the two methods is seen (see Figures S1 and S2 and Table S1 in the Supporting Information) and, therefore, the facile PAGE analysis of the RE cleavage (ClaI was used for **C$^{X}$**, BamHI for most **A$^{X}$**, XhoI for **A$^{E}$**, and ScaI for **A$^{7D}$**) was then used for all other experiments (for results, see Tables S4–S10 and Figures S3–S8 in the Supporting Information).

Generally, the 5-substituted **dC$^{X}$TP**s were worse substrates than dCTP for most polymerases,[8e] whereas some modified **dA$^{X}$TP**s were, surprisingly, better substrates. Table 1 shows the efficiency of the competitive incorporation of **dA$^{Ph}$TP** and dATP (1:1) with different polymerases. Except for Pwo, all other polymerases preferentially incorporated **dA$^{Ph}$TP** (up to 67% of **dA$^{Ph}$** for Klenow and Bst). On the other hand, **dC$^{Ph}$TP** was a worse substrate than dCTP for all enzymes, except for Bst (see Table S4 in the Supporting Information). Competitive incorporations of all modified **dN$^{X}$TP**s with Bst polymerase are presented in Table 2 to show that **dA$^{X}$TP**s bearing aromatic groups (except for the very bulky **A$^{STr}$**) or ethynyl were more efficient substrates than dATP for Bst polymerase. On the other hand, the unsubstituted 7-deaza-dATP (**dA$^{7D}$TP**) was a poorer substrate than dATP. The analysis of competitive PEX in different ratios of dNTPs with Bst polymerase showed that **dA$^{Ph}$TP** is efficiently incorporated (34%) even in the presence of a large excess of dATP (1:10; see Table S8 and Figure S8 in the Supporting Information). The efficiency of incorporation of **dA$^{Ph}$** and **dC$^{Ph}$** was sequence-dependent (see Tables S9 and S10 in the Supporting Information), but it always followed the same trend. The same approach was also used for analysis of competitive PCR with **dA$^{Ph}$TP** or **dC$^{Ph}$TP** using KOD XL polymerase and RsaI for cleavage, which led to the same conclusion (see Figure S9 and Table S11 in the Supporting Information).

The surprisingly high efficiency of 7-aryl **dA$^{X}$TP**s in competitive incorporations prompted us to study the kinetics of single nucleotide incorporations.[16] Tables 1 and 2 (and Tables S12 and S13 in the Supporting Information) summarize the results. In most cases (except for KOD XL and Vent (*exo*-)), the $K_M$ values for **dN$^{X}$TP**s which were more efficiently incorporated were lower than those of the corresponding dNTPs, which indicated a higher affinity of those **dN$^{X}$TP**s to the active site of the polymerase (a similar correlation was reported for analysis of misincorporations[17]). Conversely, the $V_{max}$ values of **dN$^{X}$TP**s were mostly lower

***Table 1:*** **dA$^{Ph}$TP** incorporation with different DNA polymerases.

| Polymerase | Competition[a] | Discr.[b] | $V_{max}/K_m$ | $K_m$[c] | $V_{max}$[d] |
|---|---|---|---|---|---|
| Pwo | 43 | 0.4 | 0.03 | 24 | 0.81 |
| (dATP)[e] | – | – | 0.08 | 10 | 0.85 |
| KOD XL | 57 | 0.6 | 0.17 | 38 | 6.3 |
| (dATP)[e] | – | – | 0.26 | 18 | 4.6 |
| Taq | 63 | 1.1 | 0.042 | 8.9 | 0.371 |
| (dATP)[e] | – | – | 0.039 | 12 | 0.49 |
| Vent(*exo*-) | 65 | 0.4 | 0.03 | 24 | 0.73 |
| (dATP)[e] | – | – | 0.08 | 8.7 | 0.67 |
| Klenow | 67 | 2.4 | 0.23 | 2.1 | 0.49 |
| (dATP)[e] | – | – | 0.1 | 2.5 | 0.25 |
| Bst | 67 | 3 | 0.18 | 6.8 | 1.19 |
| (dATP)[e] | – | – | 0.06 | 28 | 1.8 |
| H. Pol. α | 46 | – | – | >100 | – |
| (dATP)[e] | – | – | 45 | 0.00095 | $2^{-5}$ |

[a] Percent of functionalized DNA. [b] Discrimination is defined as $(V_{max}/K_m)_{fnc}/(V_{max}/K_m)_{natural}$. [c] The units for $K_m$ are μM. [d] The units for $V_{max}$ are 10 pmol $s^{-1}$ $U^{-1}$. [e] dATP was incorporated by the same polymerase as in the row above.

***Table 2:*** Incorporation of functionalized **dN$^{X}$TP**s with Bst polymerase.

| dN | Competition[a] | Discrimination[b] | $V_{max}/K_m$ | $K_m$[c] | $V_{max}$[d] |
|---|---|---|---|---|---|
| **dA** | – | 1 | 0.06 | 28 | 1.8 |
| **dA$^{STr}$** | 16.9 | – | – | >1000 | – |
| **dA$^{7D}$** | 32 | 0.3 | 0.02 | 60 | 1 |
| **dA$^{ABOX}$** | 73 | 1.8 | 0.11 | 8 | 0.94 |
| **dA$^{E}$** | 67 | 2 | 0.12 | 13 | 1.51 |
| **dA$^{NH2}$** | 61 | 2.3 | 0.14 | 7 | 1.02 |
| **dA$^{Ph}$** | 67 | 3 | 0.18 | 6.8 | 1.19 |
| **dA$^{BFU}$** | 67 | 3.7 | 0.22 | 5.7 | 1.27 |
| **dA$^{NO2}$** | 70 | 3.8 | 0.23 | 6.5 | 1.57 |
| **dC** | – | 1 | 0.27 | 4.4 | 1.2 |
| **dC$^{NH2}$** | 32 | 0.2 | 0.06 | 21 | 1.34 |
| **dC$^{E}$** | 54 | 1.5 | 0.41 | 3.1 | 1.29 |
| **dC$^{Ph}$** | 58 | 2 | 0.54 | 2.4 | 1.32 |

[a]–[d] See footnote of Table 1.

than those of natural dNTPs, thus showing a slower incorporation. However, the PEX (and even more so PCR) is a much more complex process, where the efficiency depends not only on the rate of incorporation but also on the presence or absence of proofreading (no correlation was observed for this factor) and on further extension of the primer.

To explain the higher affinity of the 7-aryl **dA$^{X}$TP**s to the active site of polymerases, we performed molecular modeling studies. Both dATP and **dA$^{Ph}$TP** were docked to the complex of Bst polymerase with primer and template using the known crystal structure (pdb 4BDP).[18] The obtained tertiary complexes were studied using a semiempirical quantum mechanical scoring function.[19] The resulting optimized structures are shown in Figure 3. The phenyl ring of **dA$^{Ph}$TP** filled the space

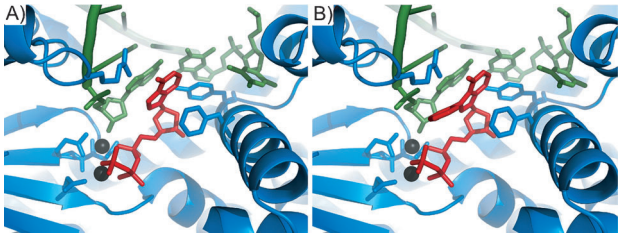



***Figure 3.*** Bst polymerase active site with modeled A) **dATP** and B) **dA$^{Ph}$TP**.

between the aliphatic chain of Arg629 and the phenyl group of Phe710. The **dA$^{Ph}$TP** had a larger affinity (more negative score) than dATP (−72.8 and −65.0 kcal mol$^{-1}$, respectively), which indicates a possible increase in π-π stacking as a result of the 7-phenyl group. A similar binding mode and increased stacking is expected for other 7-aryl-7-deaza-dATP analogues and it is in accord with the high affinity of these **dA$^{X}$TP**s to this polymerase.

In conclusion, we have developed a method[11] that is based on cleavage of the DNA by RE and PAGE analysis for the analysis of products generated from competitive incorporations of a modified **dN$^{X}$TP** in the presence of the corresponding natural dNTP. Our systematic study of competitive PEX using different **dA$^{X}$TP**s and **dC$^{X}$TP**s in different ratios with the natural dATP and dCTP revealed a surprising and counterintuitive result that most 7-aryl **dA$^{X}$TP**s are better substrates for most polymerases than dATP, whereas most **dC$^{X}$TP**s are worse substrates. The kinetic study and semiempirical calculations explain this by increased affinity of the 7-aryl **dA$^{X}$TP**s to the active site of the polymerase complex with the primer and template because of increased stacking. These findings are important for better understanding the mechanism of the incorporation of modified dNTPs by polymerase, as well as for the design of modified nucleosides and nucleotides for in vivo synthesis of base-modified DNA. Unfortunately, except for the known **dA$^{E}$** nucleoside,[6] the 7-aryl **dA$^{X}$** nucleosides cannot be directly used for metabolic labeling, presumably because of poor intracellular phosphorylation to dNTPs and/or limited cellular uptake. The solution can be either synthesis of masked **dN$^{X}$TP** prodrugs or the development of transport systems for **dN$^{X}$TP**s through cell membranes. These studies are currently under way.

Received: April 28, 2014
Published online: May 30, 2014

**Keywords:** DNA · enzymes · kinetics · nucleoside triphosphates · nucleotides

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