Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 1509–1512

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Effects of 8-Chlorodeoxyadenosine on DNA Synthesis by the Klenow Fragment of DNA Polymerase I

Lisa S. Chen, Michael H. Bahr and Terry L. Sheppard*

Department of Chemistry and The Robert H. Lurie Comprehensive Cancer Center, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208-3113, USA

Received 18 January 2003; revised 21 February 2003; accepted 27 February 2003

Abstract—8-Chloro-2′-deoxyadenosine (8-Cl-dAdo) was incorporated into synthetic DNA oligonucleotides to determine its effects on DNA synthesis by the 3′–5′ exonuclease-free Klenow fragment of *Escherichia coli* DNA Polymerase I (KF^-). Single nucleotide insertion experiments were used to determine the coding potential of 8-Cl-dAdo in a DNA template. KF^- inserted TTP opposite 8-Cl-dAdo in the template, but with decreased efficiency relative to natural deoxyadenosine. Running-start primer extensions with KF^- resulted in polymerase pausing at 8-Cl-dAdo template sites during DNA synthesis. The 2′-deoxyribonucleoside triphosphate analogue, 8-Cl-dATP, was incorporated opposite thymidine (T) approximately two-fold less efficiently than dATP.

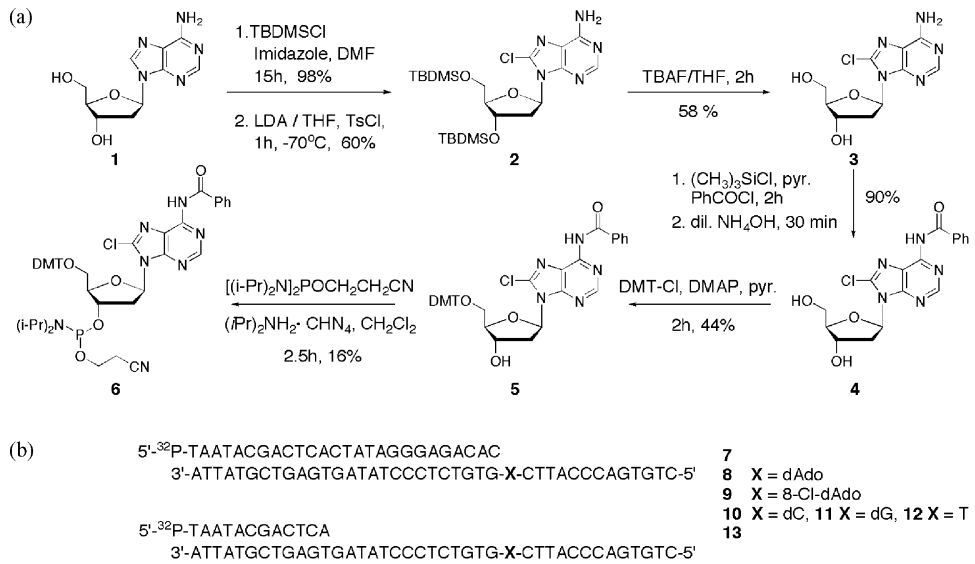
Nucleoside analogues have been used extensively as medicinal agents, particularly in anti-viral and cancer treatments.[1,2] Several 8-modified purine analogues have demonstrated antineoplastic activity and show significant promise as chemotherapeutic agents.[3,4] 8-Chloroadenosine (8-Cl-Ado) is currently under investigation as a potential treatment for multiple myeloma and leukemia. 8-Cl-Ado induces apoptosis in multiple myeloma cell lines in culture,[4] but the mechanism of action remains unclear. Apoptosis may result from 8-Cl-Ado-mediated inhibition of transcription or RNA processing, or by incorporation of the analogue into RNA, which may alter cellular RNA structure or function. In support of the latter hypothesis, our laboratory has shown that direct incorporation of 8-Cl-Ado into RNA oligonucleotides leads to RNA duplex destabilization.[5] However, apoptosis induction may involve 8-chloro-2′-deoxyadenosine (8-Cl-dAdo) and its metabolites through an indirect mechanism. After cellular uptake, 8-Cl-Ado is converted rapidly to 5′-phosphorylated 8-Cl-Ado derivatives.[3] These 8-Cl-Ado nucleotides then may enter the deoxyribonucleotide pool as 8-Cl-dATP by the combined action of ribonucleotide reductase and a kinase. As a potential substrate for cellular DNA polymerases, 8-Cl-dATP may be incorporated into newly synthesized DNA.

Structural modifications of deoxynucleoside triphosphates (dNTPs) may affect the fidelity of nucleotide insertion during enzymatic DNA synthesis. Purine nucleoside analogues modified at the C-8 position frequently adopt *syn* glycosidic torsion angles instead of the preferred *anti* conformation found in unmodified nucleosides.[6–8] For example, substitution of adenine H-8 with larger groups, in analogues such as 8-methoxy-2′-deoxyadenosine[9] or 8-bromoadenosine,[10] biases nucleoside torsional preferences toward the non-standard *syn* glycosidic bond conformation. NMR studies on 8-Cl-Ado nucleotides suggest that chlorine substitution at the C-8 position of adenine favors the *syn* base conformer.[11] By analogy to DNA damage lesions such as 8-oxo-dG,[12] the *syn* preference for 8-chloroadenine (8-Cl-A) bases may lead to base mispairing during enzymatic DNA synthesis or in transcription. To test this hypothesis experimentally, we synthesized DNA oligonucleotides containing 8-Cl-dAdo and used them to assess the effects of 8-Cl-dAdo derivatives on DNA synthesis. In this work, we address the coding potential of 8-Cl-dAdo in DNA templates and the insertion fidelity of 8-Cl-dATP using exonuclease free Klenow fragment (KF^-) from *Escherichia coli* DNA polymerase I. 8-Cl-dAdo derivatives pair with thymidine, but exhibit decreased nucleotide incorporation efficiencies and induce KF^- polymerase pausing during enzymatic DNA synthesis.

To incorporate 8-Cl-dAdo into synthetic DNA oligonucleotides, an 8-Cl-dAdo phosphoramidite derivative

*Corresponding author. Tel.: +1-847-467-7636; fax: +1-847-491-7713; e-mail: sheppard@chem.northwestern.edu

0960-894X/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0960-894X(03)00204-X



Scheme 1. (a) Chemical synthesis of 8-Cl-dAdo phosphoramidite **6**, and (b) sequences of standard and 8-Cl-dAdo modified DNA oligonucleotides.

was synthesized from deoxyadenosine (dAdo) in a six-step synthesis outlined in Scheme 1A. Deoxyadenosine (**1**) was disilylated with TBDMSCl (98%)[13] and subjected to chlorination using tosyl chloride and LDA to yield derivative **2** (60%).[14] The silyl ethers were cleaved with tetra-*n*-butylammonium fluoride (TBAF) in THF to give 8-Cl-dAdo (**3**, 58%). Transient protection of the nucleoside hydroxyls with TMSCl, followed by benzoylation[15] and aqueous work up afforded compound **4** (90%). Benzoylated nucleoside **4** was converted to the 4,4′-dimethoxytrityl (DMT) derivative **5** (44%) using DMT-Cl in the presence of DMAP in pyridine. 8-Cl-dAdo phosphoramidite **6** was synthesized from compound **5** using standard phosphoramidite chemistry.[16,17] Phosphoramidite analogue **6** was incorporated into DNA oligonucleotides using standard solid-phase synthesis methods. Oligonucleotide **9**, which contained a single 8-Cl-dAdo substitution (X = 8-Cl-dAdo), was synthesized and deprotected using standard methods (Scheme 1B).[18] Following oligonucleotide characterization by RP-HPLC and MALDI-TOF mass spectrometry,[19] **9** was utilized as a template to explore the effects of 8-Cl-dAdo substitution on DNA synthesis.[20]

Single nucleotide insertion experiments with KF$^-$ DNA polymerase were conducted to determine the nucleotide insertion preference opposite 8-Cl-dAdo compared with the natural dAdo nucleotide. DNA primer–template complexes (**7·8** or **7·9**, Scheme 1B) were incubated with KF$^-$ in the presence of a single deoxynucleoside triphosphate (dNTP) under standard conditions optimized for single insertion.[21] Aliquots were removed from the extension reactions at specific times, and the products were analyzed by denaturing polyacrylamide gel electrophoresis (dPAGE).[22] Results for experiments that tested the pairing preferences of dA and 8-Cl-dA bases in DNA templates are shown in Figure 1. The control DNA template **8** (X = dAdo, Fig. 1, top panel) was shown to insert exclusively TTP, the normal pairing partner of dAdo. The three natural triphosphates (dATP, dCTP and dGTP) were not substrates for single nucleotide extension opposite dAdo. Interestingly, single nucleotide insertion experiments with modified template **9** (X = 8-Cl-dAdo, Fig. 1, bottom panel) yielded similar results. TTP was the nucleotide most efficiently inserted opposite 8-Cl-dAdo, which suggests that the coding potential of the adenine base was not altered significantly by chlorine substitution at C-8. Although both dAdo and 8-Cl-dAdo coded for TTP insertion, analysis of the extended products indicated that KF$^-$ nucleotide insertion was more efficient when the template nucleotide was dAdo compared with 8-Cl-dAdo: template **8** (X = dAdo) showed 40% primer extension with TTP after 30 min, whereas modified template **9** (X = 8-Cl-dAdo) yielded only 26% extension product opposite 8-Cl-dAdo over the same time course. These results indicate that 8-Cl-dAdo shows the same nucleotide insertion preferences as dAdo, but exhibits lower incorporation efficiencies.

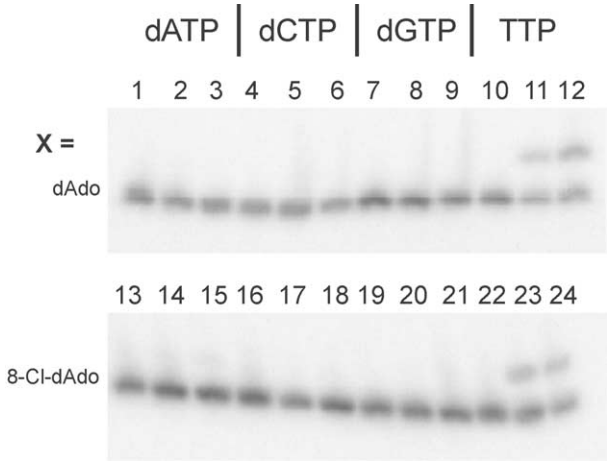


Figure 1. Gel electrophoretic analysis of single nucleotide insertion by KF$^-$. Time points at 0, 15, and 30 min for reaction of 5′–^{32}P radiolabeled DNA primer **7** with control template **8** (top) or 8-Cl-dAdo template **9** (bottom) are shown for each dNTP.

Single nucleotide insertion experiments with template **9** showed that 8-Cl-dAdo is recognized by KF$^-$ as deoxyadenosine. However, as demonstrated by Kool and co-workers,[21] the coding potential for an unnatural base analogue in a DNA template may not be the same as the pairing preference for the modified base as a dNTP analogue. Thus, while 8-Cl-dAdo codes for TTP, misincorporation of 8-Cl-dATP opposite bases other than thymidine may provide an alternate mechanism for mutagenesis. To determine the insertion fidelity of 8-Cl-dATP, single nucleotide insertion experiments were conducted using KF$^-$ and DNA templates containing natural bases. Either dATP or 8-Cl-dATP (TriLink Biotechnologies, Inc.) was incubated with a primer/template complex comprised of primer **7** and a template containing one of the natural bases (Fig. 1B, X = dAdo (**8**), dC (**10**), dG (**11**) or T (**12**)) in the presence of KF$^-$. Aliquots from the reactions were removed at specific times, and the products were analyzed by dPAGE.[22] Results with triphosphate derivatives were consistent with those observed with oligonucleotide templates **8** and **9**. 8-Cl-dATP was inserted only when T was the coding base (template **12,** X = T); no extension was observed when the template bases were dAdo, dC, or dG (data not shown). As illustrated in Figure 2 for the T-containing template (**12**), 8-Cl-dATP showed decreased insertion efficiency relative to dATP. Enzymatic incorporation of 8-Cl-dATP yielded 22% of extended product after 2 h of incubation (Fig. 2, lanes 5–8), compared with 41% extension for the natural triphosphate, dATP (lanes 1–4).

Single nucleotide insertion data suggest that 8-Cl-dAdo derivatives exhibit decreased incorporation efficiencies, both as a template nucleotide and as a nucleoside triphosphate. The impaired insertion efficiency opposite 8-Cl-dAdo nucleotides may stall DNA polymerase and impede further enzymatic DNA synthesis. To assess the ability of KF$^-$ to efficiently synthesize DNA past 8-Cl-dAdo sites, a series of 'running-start' primer extension studies were performed. These experiments utilized a 12-nucleotide primer (**13**, Scheme 1B), with either template **8** or **9**, and all four natural dNTPs. Template **8** or **9** places the dAdo/8-Cl-dAdo site 14 nucleotides downstream from the primer terminus, which requires that the polymerase advance on the template prior to insertion opposite dAdo/8-Cl-dAdo. The results for running-start extension with KF$^-$ in the presence of the four natural dNTPs are given in Figure 3.[23] Primer extension on the control template **8** (X = dAdo) proceeded to

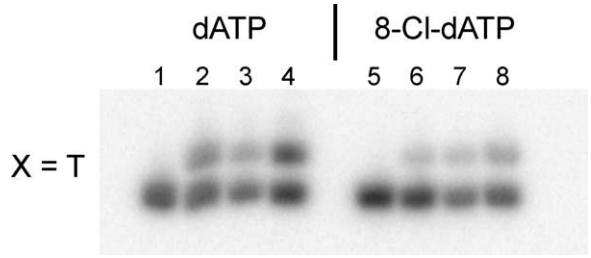


Figure 2. Gel electrophoretic analysis of nucleotide insertion by KF$^-$ opposite thymidine. Products of extension after 0, 30, 60 and 120 min of incubation of 5′–^{32}P radiolabeled DNA primer **7** and template **12** with dATP (lanes 1–4) or 8-Cl-dATP (lanes 5–8) are shown.

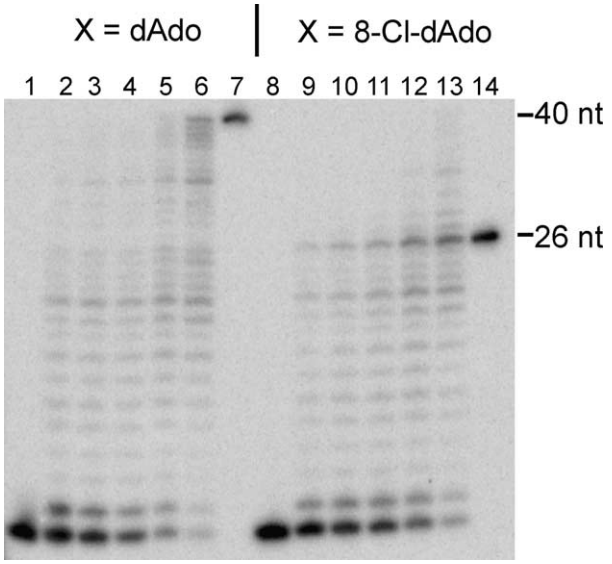


Figure 3. Gel electrophoretic analysis of running-start primer extension by KF$^-$. Primer extension time points at 0, 5, 10, 15, 30 and 60 min of incubation of 5′–^{32}P radiolabeled DNA primer **13** with control template **8** (lanes 1–6) or 8-Cl-dAdo template **9** (lanes 8–13) are provided. Radiolabeled DNA standards were included in lane 7 (40 nt) and lane 14 (26 nt).

completion over one hour (Fig. 3, lanes 1–6) leading to full-length product, as shown by a 40-nucleotide DNA marker (lane 7). Incubation of KF$^-$ with modified template **9** (X = 8-Cl-Ado) gave rise to a major termination product over the same time course (Fig. 3, lanes 8–13). The termination product co-migrates with the 26 nucleotide DNA, **7**, (Scheme 1B; Fig. 3, lane 14), indicating that termination occurs at the 8-Cl-dAdo site in the template. This truncated product suggests that KF pauses directly opposite the 8-Cl-dAdo site, resulting in termination of DNA synthesis.

These studies demonstrate that 8-Cl-dAdo preferentially pairs with thymidine bases both as a template base and as an incoming nucleoside triphosphate derivative. Thus, chlorine substitution at C-8 does not significantly alter the coding ability of deoxyadenosine. The results obtained with 8-Cl-dAdo show similarities to other modified adenosine derivatives. The halogenated nucleoside analogue, 2-chloro-2′-deoxyadenosine (2-Cl-dAdo or cladribine), currently is used for hairy cell leukemia treatment.[24] In contrast with 8-Cl-dAdo, 2-Cl-dAdo is chlorinated at C-2, on the non-Watson–Crick pairing face of adenine. In vitro experiments have shown that 2-Cl-dATP is inserted with fidelity opposite thymidine by bacterial polymerases.[25] Other studies have shown that DNA templates containing 2-Cl-dAdo are transcribed with high fidelity.[26] Thus, chlorine-substitution at the adenine C-2 position does not appear to affect the coding potential of dAdo. In addition, other studies have demonstrated that 8-oxo-deoxyadenosine (8-oxo-dAdo) does not mispair during enzymatic DNA synthesis.[27,28] The similarity in the pairing preferences of 8-Cl-dAdo and 8-oxo-dAdo offer a striking contrast to the oxidative DNA damage lesion, 8-oxo-dG, which shows both standard dC incorporation and mutagenic dAdo insertion.[12]

This work also has illustrated that KF^- mediated DNA synthesis undergoes premature chain termination at sites of 8-Cl-dAdo in the template strand. Thus, although 8-Cl-dAdo pairs normally with thymidine, chain termination at 8-Cl-dAdo sites may have biochemical consequences. For example, decreased DNA replication efficiency may affect cell division and has been shown to contribute to the cytotoxic effects of nucleoside antimetabolites.[29] Additional studies with 8-Cl-Ado and 8-Cl-dAdo will be conducted to assess the effects of 8-modified derivatives on RNA biochemistry and will explore the potential of 8-modified nucleotides for the treatment of human disease.

Acknowledgements

We thank our collaborators Dr. Steven Rosen, Dr. Nancy Krett, and Dr. Varsha Gandhi for insightful discussions. We acknowledge the Keck Biophysics Facility and the Analytical Services Laboratory of Northwestern University, and the University of Illinois, Urbana-Champaign Mass Spectrometry Laboratory. This work was supported by the National Institutes of Health (RO1 CA85915-02) and the Leukemia and Lymphoma Society (6505-00).

References and Notes

1. Mitsuya, H.; Yarchoan, R.; Broder, S. *Science* **1990**, *249*, 1533.
2. Plunkett, W.; Gandhi, V. *Hematol. Cell Ther.* **1996**, *38*, S67.
3. Gandhi, V.; Ayres, M.; Halgren, R. G.; Krett, N. L.; Newman, R. A.; Rosen, S. T. *Cancer Res.* **2001**, *61*, 5474.
4. Halgren, R. G.; Traynor, A. E.; Pillay, S.; Zell, J. L.; Heller, K. F.; Krett, N. L.; Rosen, S. T. *Blood* **1998**, *92*, 2893.
5. Chen, L. S.; Sheppard, T. L. *Nucleosides, Nucleotides Nucleic Acids* **2002**, *21*, 599.
6. Tavale, S. S.; Sobell, H. M. *J. Mol. Biol.* **1970**, *48*, 109.
7. Uesugi, S.; Nagura, T.; Ohtsuka, E.; Ikehara, M. *Chem. Pharm. Bull.* **1976**, *24*, 1884.
8. Uesugi, S.; Shida, T.; Ikehara, M. *Biochemistry* **1982**, *21*, 3400.
9. Eason, R. G.; Burkhardt, D. M.; Phillips, S. J.; Smith, D. P.; David, S. S. *Nucleic Acids Res.* **1996**, *24*, 890.
10. Ikehara, M.; Uesugi, S.; Yoshida, K. *Biochemistry* **1972**, *11*, 830.
11. Chen, L. S.; Sheppard, T. L., unpublished observation.
12. Shibutani, S.; Takeshita, M.; Grollman, A. P. *Nature* **1991**, *349*, 431.
13. Ogilvie, K. K. *Can. J. Chem.* **1973**, *51*, 3799.
14. Hayakawa, H.; Tanaka, H.; Haraguchi, K.; Mayumi, M.; Nakajima, M.; Sakamaki, T.; Miyasaka, T. *Nucleosides Nucleotides* **1988**, *7*, 121.
15. Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316.
16. Characterization of phosphoramidite **6**. ^1H NMR (400 MHz, CD_3CN) δ: 9.28 (s, 1H, NH), 8.52 (s, 1H, H2), 7.98 (d, 1H, $J = 7.6$ Hz, Bz_a), 7.63 (dd, 2H, $J = 7.2$ Hz, $J = 7.2$ Hz, Bz_b), 7.53 (t, 2H, $J = 7.6$ Hz, Bz_c), 7.17 (m, 9H, DMT, −OPh), 6.74 (m, 4H, DMT), 6.42 (m, 1H, H1′), 5.19 (m, 1H, H2′), 4.19 (q, 1H, $J = 5.2$ Hz, 3′OH), 3.72 (d, 6H, $J = 4.0$ Hz, $-OCH_3$), 3.70-3.58 (m, 1H, H3′), 3.34 (m, 1H, H4′), 3.19 (m, 2H, H5′), 2.54 (m, 1H, $-OCH_2CH_2CN$), 2.32 (m, 1H, $-OCH_2CH_2CN$), 2.17 (s, 1H,-$NH(CH_3)_2$), 1.19 (t, 12H, $J = 6.4$ Hz, $-NH(CH_3)_2$). ^{31}P NMR (CD_3CN) δ: 148.6.
17. Caruthers, M. H.; Barone, A. D.; Beaucage, S. L.; Dodds, D. R.; Fisher, E. F.; McBride, L. J.; Matteucci, M.; Stabinsky, Z.; Tang, J. Y. *Methods Enzymol.* **1987**, *154*, 287.
18. Dey, S.; Sheppard, T. L. *Org. Lett.* **2001**, *3*, 3983. Unmodified oligonucleotides **7**, **8**, **10**–**13** were synthesized by Integrated DNA Technologies, purified using standard methods, and characterized by MALDI-TOF MS.
19. Characterization of oligonucleotide **9**. MALDI-TOF MS (m/z): $[M]^-$ calculated, 12261.4; found, 12261.5.
20. Stability of 8-Cl-dAdo DNA oligonucleotide. Chlorine substitution at C-8 enhances the rate of acid-catalyzed depurination of 8-Cl-dAdo (*J. Am. Chem. Soc.* **1978**, *100*, 7620.) However, after DNA synthesis and purification, no abasic site formation was observed either by MALDI-TOF MS or by radiolabeling and PAGE analysis of oligonucleotides containing 8-Cl-dAdo.
21. Morales, J. C.; Kool, E. T. *J. Am. Chem. Soc.* **2000**, *122*, 1001.
22. Single nucleotide insertion with KF^-. Radiolabeled (5′–^{32}P) primer **7** was annealed to the appropriate template in a solution containing 100 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 2 mM DTT and 0.1 mg/mL BSA. A solution containing a single dNTP in the same reaction buffer was added to the annealed primer, and primer extension was initiated by adding KF^-. Aliquots were removed from the reaction at specific times, and the products were analyzed by 20% dPAGE. Final extension conditions were 4 μM primer/template duplex, 50 nM KF^-, 10 μM dNTP, 150 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 1 mM DTT and 0.05 mg/mL BSA.
23. Running start primer extensions with KF^-. Radiolabeled (5′–^{32}P) primer **13** was annealed to the appropriate template in a solution containing 100 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 2 mM DTT and 0.1 mg/mL BSA. Primer extension was initiated by adding KF^- to the annealed duplex, followed by a dNTP solution in the same buffer. Aliquots were removed from the reaction at 30, 60, and 120 mins, and the products were analyzed by 20% dPAGE. Final extension conditions were 200 nM primer/template duplex, 1 nM KF^-, 20 μM each dNTP (dATP, dCTP, dGTP, TTP), 150 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 1 mM DTT and 0.05 mg/mL BSA.
24. Piro, L. D.; Carrera, C. J.; Carson, D. A.; Beutler, E. *N. Engl. J. Med.* **1998**, *322*, 1117.
25. Hentosh, P.; McCastlain, J. C.; Blakley, R. L. *Biochemistry* **1991**, *30*, 547.
26. Hentosh, P.; Tibudan, M. *Mol. Pharmacol.* **1995**, *48*, 897.
27. Duarte, V.; Muller, J. G.; Burrows, C. J. *Nucleic Acids Res.* **1999**, *27*, 496.
28. Shibutani, S.; Bodepudi, V.; Johnson, F.; Grollman, A. P. *Biochemistry* **1993**, *32*, 4615.
29. Huang, M. C.; Ashmum, R. A.; Avery, T. L.; Kuehl, M.; Blakley, R. L. *Cancer Res.* **1986**, *46*, 2362.