C8-Guanine Modifications: Effect on Z-DNA Formation and its Role in Cancer

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REVIEW

C8-Guanine Modifications: Effect on Z-DNA Formation and its Role in Cancer

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Base modifications are known to affect structure and function of DNA. C8-gaunine adducts from various carcinogenic compounds have been shown to be potent Z-DNA inducers. Hence, it has been hypothesized that Z-DNA plays a role in cancer and other genetic diseases. In this comprehensive review, Z-DNA and the effect of prevalent C8-guanine adducts on B-Z transition is addressed. The discoveries of Z-DNA binding proteins including ADAR1, E3L, DLM1, and PKZ have suggested the relevant of Z-DNA in living system. In addition, increasing evidence on the Z-DNA connection to gene transcription and inhibition reveals potential biological functions of the left-handed DNA. Finally, C8-guanine adducts that promote Z-DNA formation can be used as a tool to answer Z-DNA function and role in carcinogenesis.

1. Introduction

In 1979, Rich and his colleagues solved the structure of a self-complementary DNA hexamer $d(CG)_3$.[1] To their surprise, they discovered that under the conditions they had used to prepare the crystal, the molecule adopted a left-handed double helix with two antiparallel chains held together by Watson-Crick base pairs. The potential for the formation of this novel structure had been suggested by other spectroscopic measurements under conditions of high salt concentration.[2,3] This was the first left-handed double helix to be found and, to date, is the only type of DNA that adopts this unique left-handed helical structure.

The discovery of this novel DNA structure resulted in a decade of research regarding both *in vitro* and *in vivo* aspects of Z-DNA. The basic structure and factors responsible for stabilizing it were elucidated primarily from circular dichroism (CD), NMR and X-ray crystallographic studies. At the most fundamental level it was found that Z-DNA requires an alternating pyrimidine-purine sequence. The most favourable, and consequently the most commonly studied, are comprised of cytosine-guanine (C-G) sequences or 5-methylcytosine-guanine (C^{Me}-G).

Once the basic structure of Z-DNA was determined it became of interest to determine conditions that stabilize the Z-DNA conformation relative to other forms such as B-DNA. Assuming the basic structural requirements are met (alternating pyrimidine-purine sequence), the most import factor is the concentration and nature of the salt present. Most duplex structures are stabilized by the presence of salt. For example, B-DNA typically forms at relatively low salt concentrations and is stabilized in proportion to its concentration. Likewise, salt stabilizes Z-DNA formation but typically requires molar concentrations and is in equilibrium with the B-DNA form.

Modifications of the DNA bases have also found to play a significant role in the stability of Z-DNA. The most important of these modifications is either the replacement of cytosine by 5-methylcytosine or the substitution of guanine at the C8-position. Either of these modifications stabilizes the Z-form in Z-forming sequences. Further the effect of these modifications is cumulative such that as additional cytosines or guanines are replaced by a 5-methylcytosines or C8-substituted guanines, respectively, the equilibrium is further shifted toward the Z form.

The discovery of a biological function(s) for Z-DNA has been much more difficult. Part of the problem is that Z-DNA was discovered in the absence of a biological context. It is far more typical to study biological function, first, and then examine the underlying structures of the molecules involved. In some ways, this situation is similar to triplex DNA. This form of DNA was discovered in 1957 by Felsenfeld, et al.,[4] but elucidation of its biological function only slowly evolved over time. Nevertheless, there were hints of the involvement of Z-DNA in biological processes. For example, cells contain relatively high levels of spermine and spermidine and both of these polyamines are known to stabilize Z-DNA. DNA methylation of cytosine, involved in gene silencing, stabilizes DNA in the Z conformation. Negative supercoiling was discovered to stabilize Z-DNA. Negative supercoiling requires energy as it unwinds the B-DNA during transcription. The formation of Z-DNA reduces the number of supercoils and therefore the energy required is reduced and a portion of this energy is used to stabilize the resulting Z-DNA seqment.[5,6]

In 1992, 13 years after the original discovery of Z-DNA, more significant data for a biological function were reported. In particular, it was found that Z-DNA formation was associated with

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the expression of the oncogene *c*-myc and that Z-DNA forming sequences were found near promoter regions in U937 cells.[7] Similar observations where subsequently made. For example, Liu, et al.,[8] found that the activation of the CSF1 gene involved the formation of Z-DNA and this has been further verified by Mulholland[9] who concluded that Z-DNA formation near a promoter stimulates transcription.

In the mid 1990's, the enzyme Adenosine Deaminase Acting on RNA (ADAR1) was found to tightly bind to Z-DNA and in 1999 the structure of ADAR1 bound to a Z-DNA was solved. ADAR1 was the first of several proteins that have been found to bind to Z-DNA and are collectively known as Z-DNA Binding Proteins (ZBPs). The ability of ADAR1 to bind to Z-DNA was discovered by using an immobilized DNA that was stabilized in the Z conformation by partial bromination of guanine residues.[10] Obtaining a crystal structure on the ADAR1-Z-DNA complex was key as it helped to defined the elements involved in Z-DNA recognition by a protein.[11]

Since the time that ADAR1 was discovered, several other ZBPs have been discovered. Several were discovered by using the binding sequence of ADAR1 as a template to scan DNA sequences. For example, DLM1, also referred to as ZBP1 or DAI, which is up regulated in tissue in contact with tumors was discovered using this approach.[12] A third ZBP is E3L, found in poxviruses. E3L is required for poxvirus pathogenicity as if E3L is mutated or eliminated, it is no longer pathogenic.[13] Finally, a ZBP has been discovered in fish termed PKZ.[14,15] The protein was found to inhibit replication of the grass carp reovirus and to play a role in IFN-mediated antiviral response.[16]

Recent work regarding Z-DNA has led to the conclusion that Z-DNA plays a role in cancer and viral infections. As noted, ZBP have been found *in vivo* and their biological function has been demonstrated. Z-DNA has been tied to gene expression and may result in gene dysregulation, correlated with carcinogenesis. Since C8-adducts affect the B- to Z-DNA conformational interconversion and tend to drive the equilibrium toward the Z-form, it has been hypothesized that C8-guanine formation may play a role in carcinogenesis and, if true, would represent a new carcinogenesis mechanism. Significantly, Z-DNA has been identified as a potential therapeutic target. Finally, C8-guanine adducts are a useful tool for the structural and biological study of Z-DNA.

2. Factors that influence Z-DNA formation

Z-DNA has been found to be less stable as compared to B-DNA and a considerable amount of research has been devoted to determining the factors that promote Z-DNA formation. The overarching factor that has been identified that is responsible for destabilizing the Z-DNA, relative to B-DNA, is the unfavourable electrostatics of Z-DNA due to the negatively charged phosphate groups being nearer to one another in Z-DNA than in B-DNA. Consequently, the external factors that influence the B-Z transition all provide positively charged functionality or functional groups that minimize the unfavourable phosphate-phosphate interaction by screening the charge. To this end, inorganic cations and polyamines are the most studied and are relevant to physiological conditions.

2.1 Inorganic salts

The effect of salts on the transition of B-DNA to Z-DNA was first demonstrated in 1972 by Pohl and Jovin[2]. They found that high sodium ion concentrations (~4 M) were required for poly(dG-dC)·poly(dG-dC)[17,2] and short CG repeat sequences[18] to completely convert B-DNA to a new form of DNA, subsequently shown to be Z-DNA (Table 1). Much lower concentrations of divalent cations such as Mg^{2+}[17,19,20], Ba^{2+}[17], Ca^{2+}[17,20], Ni^{2+}[21], Zn^{2+}[22], or Cu^{2+}[22] have since been shown to stabilize Z-DNA CG or modified CG repeat sequences including CG hexamers[19,20], poly(dG-dC)·poly(dG-dC)[17,19], and 5-methylcytosine containing oligonucleotides[17,22] at much lower concentrations.[23] The reason for the stronger effect by divalent cations is that they are bound by two phosphates, are more tightly bound, and, therefore, are more effective at screening the unfavourable inter-phosphate charge interactions relative to monocations.

The effect of trivalent Co^{3+} and Ru^{3+} in form of cobalt hexamine ($[Co(NH_3)_6]^{3+}$)[17,19,20,24] and ruthenium hexamine ($[Ru(NH_3)_6]^{3+}$)[24] has also been studied. Their effect is even more potent than that of the dications with respect to stabilizing Z-DNA. In particular, the concentration required to attain the midpoint of the B-Z transition for poly(dG-dC)·poly(dG-dC) was reported to be 50 μM[24] for $[Ru(NH_3)_6]^{3+}$ and 20 μM[17] for $[Co(NH_3)_6]^{3+}$. For monocations the midpoint is typically around 2 M and for dications, 0.7 M. Overall, a reduction in the unfavourable electrostatic interactions in Z-DNA by cations is the most comprehensive explanation for Z-DNA stabilization by inorganic cations. Nevertheless, non-electrostatic effects (e.g., Van der Waals interactions, internal strain, solvation, entropy) may also contribute to the effect of transition metals on B-Z transition.[23]

2.2 Polyamines and amine containing Z-DNA stabilizers

Although many metal ions such as Na^+ and Mg^{2+} can help to stabilize the Z-DNA conformation, the high salt concentrations that are required to promote the B to Z-DNA conversion of native DNA are unlikely to occur in living organisms (see Table 1). Therefore, investigators have examined other potential and biologically relevant Z-DNA promoters. Several endogenous polyamines including spermine and spermidine have been found to promote Z-DNA formation.[25-29] Like metal ions that have positive charges to screen the phosphate group electrostatic interactions present in Z-DNA, polyamines can also exert this effect since they exist in an ionized state (protonated amino groups) at physiological pH. Also, key to their stabilizing effect is the spacing of the amino groups in the polyamines. Midpoint concentrations of spermidine and

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spermine were 280 and 5 µM, respectively, with unmodified poly(dG-dC)·poly(dG-dC). Increasing or decreasing the methylene spacer between the amino groups increased the required polyamine concentrations, as shown in Figure 1. Therefore, upon their binding to Z-DNA, electrostatic repulsion between multiple phosphate groups in the narrow backbone of Z-DNA are simultaneously screened and the Z-DNA structure is thereby stabilized.

Micromolar concentrations of spermine and spermidine have also been reported to induce-DNA formation in other purine-pyrimidine alternating sequences including poly(dA-dC)·poly(dG-dT)[26] and poly(dG-dC^{Me})·poly(dG-dC^{Me}) (dC^{Me}; 5-methyl-2'-deoxycytosine).[17] The concentration of spermine and spermidine in living cells are reported to be around 1 mM[30] which is higher than the required transition concentration used in these examples. Consequently, Z-DNA may be present in cells.

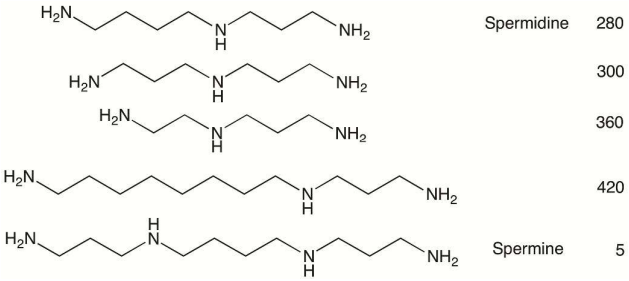


Figure 1. Spermidine, spermine, and related polyamines that have been found to stabilize the Z-DNA. The reported concentrations are those required for poly(dG-dC)·poly(dG-dC) to be present in a B-/Z-DNA ratio of 1:1 (midpoint concentration).

In addition to polyamines, oligopeptides containing basic side chain(s) have also been found to facilitate Z-DNA formation in poly(dG-dC)·poly(dG-dC) in aqueous methanol mixtures.[31] Finally, stacking and screening interactions of a cationic porphyrin into poly(dA-dT)·poly(dA-dT) stabilizes this sequence in the left handed Z conformation.[32-34] The effect of this cationic porphyrin, however, is sequence selective and it does not promote the B-Z transition in poly(dG-dC)·poly(dG-dC).

Table 1. The transition concentration of cationic Z-DNA inducers[a]

Z-DNA inducers	Conc. (mM)	DNA sequences	References
Na^+	2500	poly(dG-dC)·poly(dG-dC)	Pohl et al.[2]
	700	poly(dG-dC^{Me})·poly(dG-dC^{Me})	Behe et al.[17]
	2544	$d(CGCGCGCGCG)_2$	
	1690[b]	$d(CGCGCGCG)_2$	Sugiyama et al.[35]
	2600[b]	$d(CGCGCG)_2$	Chen et al.[36]
Mg^{2+}	700	poly(dG-dC)·poly(dG-dC)	Pohl et al.[2]
	0.6	poly(dG-dC^{Me})·poly(dG-dC^{Me})	Behe et al.[17]
$[Co(NH_3)_6]^{3+}$	0.02	poly(dG-dC)·poly(dG-dC)	Behe et al.[17]
	0.005	poly(dG-dC^{Me})·poly(dG-dC^{Me})	Behe et al.[17]
$[Ru(NH_3)_6]^{3+}$	0.05	poly(dG-dC)·poly(dG-dC)	Thomas et al.[24]
	0.004	poly(dG-dC^{Me})·poly(dG-dC^{Me})	Thomas et al.[24]
Spermine	0.002	poly(dG-dC^{Me})·poly(dG-dC^{Me})	Behe et al.[17]
Spermidine	0.05	poly(dG-dC^{Me})·poly(dG-dC^{Me})	Behe et al.[17]

[a]All data were obtained at ambient temperatures except for $d(CGCGCGCG)_2$ and $d(CGCGCG)_2$ where the data were collected at 283K.

2.3 Supercoiling

During replication[37] or transcription[38,39] the helical structure of DNA undergoes superhelical formation. The double stranded DNA region of the coding regions need to be unwound and open to two single stranded DNAs. The unwinding process puts torsional strain on the right handed DNA conformation, causing formation of supercoiled DNA.[40] Several studies on supercoiled DNA plasmids containing CG repeated sequences have shown that Z-DNA formation is promoted within supercoiled regions of DNA[6,41,42] under physiological conditions. The inducing effect of DNA superhelicity on Z-DNA formation is because switching from the right handed B-DNA to the left handed Z-DNA relieves the torsional strain developed at the replication fork and thereby relaxes the high energy supercoiled DNA. Due to the natural occurrence of negative supercoiled DNA during transcription, the potential biological relevance of Z-DNA has been studied in regard to the Z-DNAs role in or during gene expression.

3.Effect of C8 guanine modifications on Z-DNA formation

The effect of C8-guanine substituents on stabilizing the Z-DNA conformation have been extensively studied. The underlying reason for the Z-DNA stabilizing effect of C8-guanine adducts is due to the fact that C8-guanine modifications prefer to adopt the *syn* conformation (as adopted in Z-DNA) about the glycosidic bond due to an unfavourable interaction between substituents at the C8-guanine position and the H-2" proton of the same nucleoside. The interaction is dependent on the sterics of the C8-guanine substitute and thus selected modifications of the C8 position of guanine base can result in a shift to Z-DNA. Here we discuss the C8 guanine modifications that are known to facilitate Z-DNA formation.

3.1 Bromine adduct

Bromination of poly(dG-dC) was demonstrated to occur by Lafer, et al.[43] through the reaction of the polynucleotide and saturated

bromine water at room temperature. Bromination occurs at the C8 position of guanine and up to 40% of guanines can be brominated. The resulting brominated DNA adopts the Z-DNA conformation under physiological conditions[43,44] unlike the non-brominated poly(dG-dC) that requires ~4 M NaCl to cause the complete conversion to Z-DNA. A computational study utilizing molecular mechanics and thermodynamic perturbation methods revealed that bromination at C8 position of guanine stabilizes the Z-DNA conformation relative to its non-brominated counterpart.[45]

Part of the reason for the conformational shift from B- to Z-DNA for the 8-bromo modified guanine is steric. Substituents on the 8-position of guanine in B-DNA have an unfavourable steric interaction with H-2′′ of the attached sugar group. Further, C8-guanine substituents sterically interact with the phosphate backbone of DNA. Both of these interactions are relieved in the Z-DNA conformation due to a change in the glycosidic bond torsion of guanines in Z-DNA changing from *anti* in B-DNA to *syn* in Z-DNA. In addition, the C8 position of guanine in Z-DNA is located outside of the backbone (Figure 2) so there are no steric interactions between the C8-position and the phosphates or H-2″ proton.

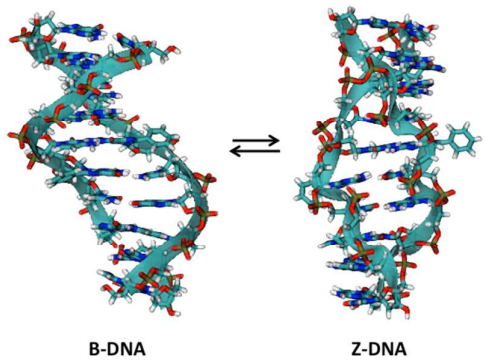


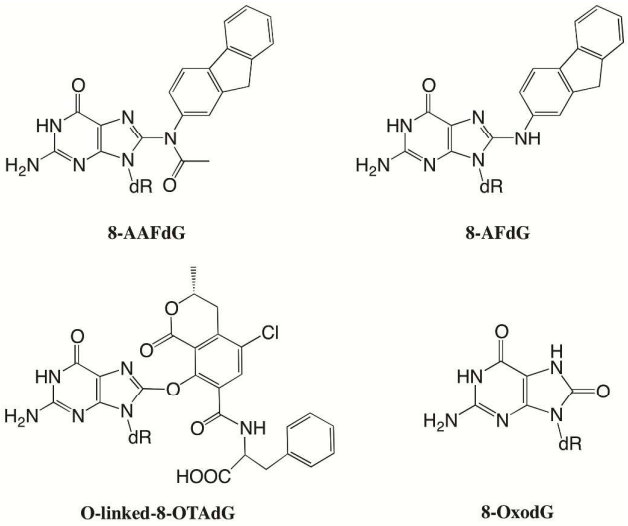
Figure 2. B-Z transition aided by C8-arylguanine adducts.

We have studied the effect of a single bromination in a CG decamer (d(CGCGCG^{8Br}CGCG)$_2$, G^{8Br} = 8-bromo-2′deoxyguanosine). The single brominated oligonucleotide was prepared with automated DNA synthesis utilizing phosphoramidite chemistry. The presence of the bromine adduct in the middle of oligonucleotide reduces the salt concentration (to approximately 400 mM NaCl) necessary to convert CG decamer to its Z-DNA conformation, a ten-fold reduction in the required salt concentration relative to the unmodified decamer.

3.2 C8-N/O-guanine adducts formed from carcinogenic compounds

Acetylaminofluorene (AAF)[46-49] and aminofluorene (AF)[46,50] adducts are among the first C8 guanine modifications extensively studied that are related to carcinogenesis. These adducts have been found to influence Z-DNA formation. Both of these C8-guanine adducts contain a C-N covalent bond to the C8 position of guanine (C8-AAF and C8-AF, Figure 3) and are major products that form from exposure to the carcinogenic compounds N-acetoxy-N-2-acetylaminofluorene and N-hydroxy-N-2-acetylaminofluorene, respectively.[46] AAF modified poly(dG-dC) was shown to be more prone to form Z-DNA than with AF modification. It has been argued that for both the AAF and the AF adducts in poly(dG-dC), the B-DNA form is destabilized for steric reasons[50] and this may make adoption of the Z-DNA conformation more favourable. In line with the argument of steric destabilization of the B-DNA form containing either of these adducts is the greater preference of the Z-DNA form for the larger adduct AAF, which bears two substituents on the nitrogen attached to C8, versus AF with only one substituent. However, this does not explain how adduct formation overrides the unfavourable electrostatics of Z-DNA. Steric destabilization of the B-DNA form by adduct formation does not have to adopt a double-stranded structure and could simply form a single-stranded DNA.

Figure 3. Various C8-N-/O-linked deoxyguanosine adducts from carcinogens that may promote B-Z transition



Much like AAF and AF, ochratoxin A (OTA), carcinogenic mycotoxin produced by *Aspergillus*, can react with guanine to form C8 adducts[51,52] and may promote Z-DNA formation.[53] Exposure to OTA lead to formation of both C-linked-8-OTBdG (see next section) and O-linked-8-OTAdG.[52] Similar results have been found for other phenolic compounds including the probable carcinogen pentachlorophenol[54] and the related di- and tri-chlorophenols.[55] However, in these latter cases the effect of the O-linked adducts on the B/Z-DNA conformational preferences has not been investigated.

Oxidative stress due to mutagenic agents[56,57] or radiation[57,58] can cause oxidation of the guanine base to form the 8-oxoguanine

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adduct (a.k.a. 8-hydroxyguanine). A study of 8-oxoguanosine affect on Z-DNA formation has been conducted in a CG hexamer. It was found that this adduct supports Z-DNA formation to some extent.[59] A related adduct is the C8-aminoguanine adduct. This adduct was found to form from 2-nitropropane.[60] However, to date, there is no data on this adduct with respect to its effect on the B-/Z-DNA equilibrium. Both the C8-oxo and C8-amino groups are small and their effect on the B-/Z-DNA equilibrium is expected to be small.

3.3 C8-C-guanine adducts formed from carcinogens

Alkyl adducts. Alkylation of nucleotide bases is one of possible mechanism of action for carcinogens to cause DNA damage and mutagenesis. In many cases, alkylation is an electrophilic process and results in N- or O-alkylation. In contrast, N-methyl hydrazine and related methyl hydrazines have been shown to lead to the formation of a methyl adduct on the C8 position of guanine through a methyl radical intermediate.[61] The adduct, C8-methylguanine, has been found to be a strong Z-DNA stabilizer[35,36,62] such that it markedly reduces the salt concentration required for CG hexamer to exclusively adopt the Z-DNA conformation. It has been reported that at 30 mM salt this hexamer exists entirely in the Z conformation and therefore will be in the Z-DNA conformation under physiological conditions. Thermodynamic parameters calculated from CD data of d(CGCG^{8Me}CG)$_2$ (G^{8Me} = 8-methyl-2'-deoxyguanosine) showed that the C8-methyl guanine adduct reduces the free energy required to shift the modified nucleoside monomer to *syn* conformation[35,] thereby sterically stabilizing the Z-DNA conformer. Finally, in addition to methyl adducts, the effect of similar groups such as alkylamino and alkynyl have been studied. These groups display similar effects and have been shown to be effective at promoting the B to Z conversion of the CG hexamer.[63]

Aryl adducts. Interest in C8-arylguanine adducts developed due to their toxicological relevance. There are a number of carcinogens that result in the formation of C8-arylguanines. As noted above, ochratoxin A (OTA), produces the C8-guanine adduct C-linked-8-OTBdG (Figure 4). This adduct has been studied by molecular dynamics and free energy calculations[53] though only in the B-DNA conformation. However, the same study reveal that *syn* and *anti* conformations of C-linked-8-OTBdG nucleoside are nearly energetically equivalent, while unmodified 2'-deoxyguanosine only adopts the *anti* conformation. These findings from the calculations, then, indicate the potential for Z-DNA formation in the presence of C-linked-8-OTBdG adduct on C8 position of guanine base due to the observed conformational preferences.

Polyaromatic hydrocarbons have been shown shown by many workers to be carcinogenic and result in a range of DNA damage. A unique polyaromatic hydrocarbon adduct was discovered by Cavalieri, et al.[64] These workers found that under electrochemical oxidation or treatment with HRP, C8 adducts (C8-BaPd, Figure 4) are formed. However, the effects of this adduct on the B/Z DNA equilibrium have not been computationally or experimentally examined.

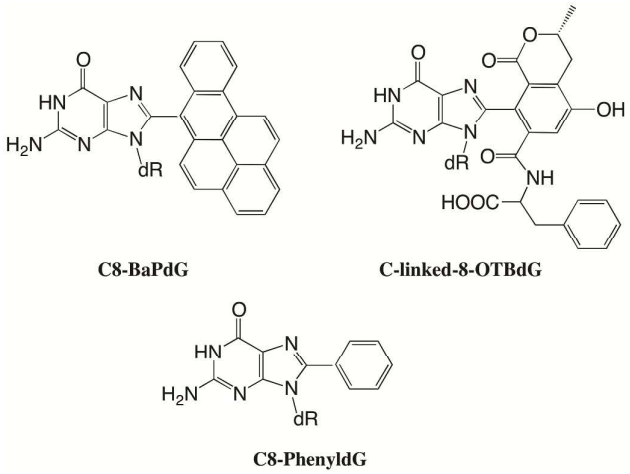
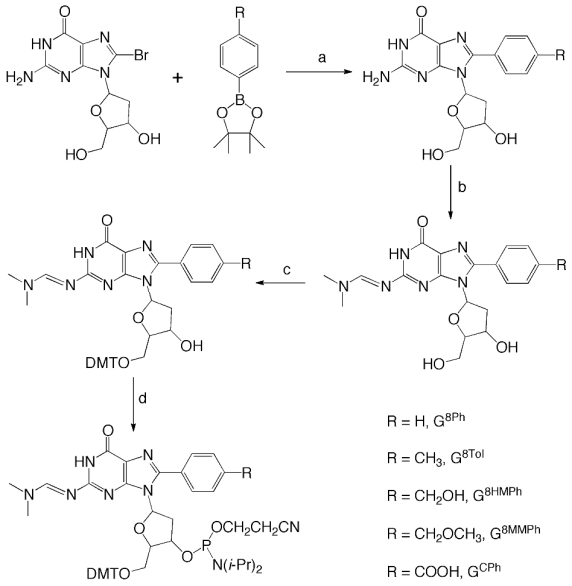


Figure 4. Various C8-C-linked deoxyguanosine adducts from carcinogens that may promote B-Z transition

C8-Arylguanine adducts have been shown to form from carcinogenic arylhydrazines.[65,66] Metabolism of arylhydrazines first leads to the formation of an arenediazonium ion and then an aryl radical.[67] Either of these reactive intermediates has been found to lead to the formation C8-purine adducts (both C8-guanine and C8-adenine). The relative amounts of the two adducts depends on the aryl group though slightly greater amounts of the C8-aryladenine adduct in DNA typically observed.[66] The mechanism underlying the carcinogenicity of arylhydrazines is still unknown even though it has received extensive study. C8-aryl adducts have been examined for their potential to cause mutations by misreading or to induce frameshifts.[68] These types of mechanisms do not appear to be playing a significant role in arylhydrazine carcinogenesis. An alternative mechanism is based on Z-DNA formation as it has been shown that C8-arylguanine adducts promote the conversion of B-DNA to Z-DNA. For example, a series of C8-arylguanine nucleosides[69] and CG decamers containing C8-arylguanine adducts were synthesized[70] by means of phosphoramidite chemistry and automated DNA synthesis (Scheme I). The unmodified and modified CG decamers were characterized to elucidate the effect of aryl adducts on B to Z-DNA transition.[70-72]

The C8-phenylguanine adduct was the first of the series to be shown to be effective at promoting Z-DNA formation.[70] Subsequent studies examined a series of C8-arylguanine adducts, based on the phenyl adduct, in which the para position of the aryl ring, bore a range of substituents including *p*-CH_3, *p*-CH_2OH, *p*-CH_2OCH_3, and *p*-COOH.[72] These adducts all form from carcinogenic arylhydrazines. All of these adducts were found to have significant impact, though differential, on stability of both the B-DNA and Z-DNA forms and the

stabilization effect was correlated with arylhydrazine carcinogenicity. Among C8-arylguanine adducts studied, the *p*-carboxyphenyl adduct was the most potent at promoting Z-DNA formation. In fact, under conditions that were similar to physiological[72] (Na^+, 121 mM, T = 310 K) resulted in a 1:1 B-DNA:Z-DNA mixture. Further, at a temperature of 283 K, almost no salt was required (Table 2). The notable effect of *p*-carboxyphenyl adduct may be due to carboxylate residue, which is negatively charged and in close proximity to the negatively charged phosphate backbone in the B-DNA conformation. The combination of both the steric destabilization of the C8-guanine substitution and the unfavourable electrostatics results in the Z-DNA form becoming the preferred conformation.



Scheme 1 Synthesis of C8-aryldG phosphoramidites a.) $Pd(OAc)_2$, TPPTS, Na_2CO_3, methanol, b.) N,N-dimethylformadinedimethyl acetal, methanol, c.) 4,4'-dimethoxytrityl chloride (DMT-Cl), TEA, pyridine, and d.) $(i\text{-}Pr_2N)P(Cl)OCH_2CH_2CN$, TEA, dichloromethane.

The C8-arylguanine adducts are also capable of promoting Z-DNA formation in the hairpin turn DNA models.[73] The hairpin forming duplex d(5′-CGCGCG*CGCGTTTTCGCGCGCGCG-3′) (G* = C8-arylguanine), forms 1:1 B-DNA:Z-DNA mixtures with salt concentrations in the range of 600-800 mM depending on the specific aryl group. The addition of physiological concentrations of Mg^{2+} and spermine lower the required salt concentrations such that a 1:1 ratio would form under physiological conditions.

Table 2. Transition concentration for B-Z transition of the unmodified and modified CG oligomers

DNA sequences[a]	Transition Conc. (mM NaCl) at 283K	Transition Conc. (mM NaCl) at 310K
$d(CGCGCG)_2$[b]	2600	n/a
$d(CGCG^{8Me}CG)_2$[b]	30	n/a
$d(CGCGCGCG)_2$[b]	1690	n/a
$d(CGCG^{8Me}CGCG)_2$[b]	34	n/a
$d(CGCGCGCGCG)_2$	n/a	3180
$d(CGCGCG^{8Br}CGCG)_2$	159	422
$d(CGCGCG^{8Ph}CGCG)_2$	114	585
$d(CGCGCG^{8Tol}CGCG)_2$	88	926
$d(CGCGCG^{8HMPh}CGCG)_2$	113	710
$d(CGCGCG^{8MMPh}CGCG)_2$	106	675
$d(CGCGCG^{8CPh}CGCG)_2$	13	121

[a] G^8 refers to C8-guanine adduct where Me = methyl, Br = bromo, Ph = Phenyl, Tol = *p*-tolyl, HMPh = *p*-hydroxymethylphenyl, MMPh= *p*-methoxymethylphenyl, and CPh = *p*-carboxyphenyl.

[b] Data of the unmodified and modified CG hexamers and CG octamers are referenced from Sugiyama et al.[35] and Chen et al.[36]

4. Z-DNA binding proteins (ZBP)

The biological relevance or function(s) of Z-DNA in living systems has been at the center of Z-DNA research since its discovery in 1979[1]. Antibodies specific to Z-DNA produced in sera of animals immunized with brominated poly(dG-dC).poly(dG-dC)[43,74] or poly(dG-dC).poly(dG-dC) modified with chlorodiethylenetriamino platinum(II) chloride[75] were reported. The Z-DNA antibodies were found to be specific to the left-handed Z-DNA and did not recognize B-DNA.[43,74] Interestingly, Z-DNA antibodies were found in sera of patients with systemic lupus erythematous (SLE)[76], which in turn suggests the involvement of Z-DNA as immunogen in this autoimmune disease. However, the finding of Z-DNA binding proteins (ZBP) that bind specifically to Z-DNA is amongst the strongest evidence that Z-DNA plays a physiological role.

To date, several ZBP have been discovered and their biological roles related to Z-DNA such as gene regulation and progression of disease have been studied and will be discussed below. It is important to note that these ZBP have gene expression regulatory roles that are dependent upon the presence of the Z-DNA conformation. The formation of C8-guanines can augment the formation of the Z-DNA conformation and therefore there may be an interplay between an adduct, ZBP, and gene expression. In turn, these processes may have relevance to cancer.

4.1 Adenosine deaminase acting on RNA (ADAR)

Adenosine deaminase acting on RNA (ADAR)[77] protein family is a deaminase enzyme that is responsible for converting adenosine to inosine[78] at specific sites in double strand RNA (dsRNA) and was first

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discovered in 1987.[79] The editing process can cause dsRNA unwinding because of the presence of the less stable I:U base pair instead of the normal A:U base pair.[80] In addition, change of A:U base pair to an I:C base pair is also observed[81] due to inosine being recognized as guanosine in the translation process and results in the production of different proteins from the same pre-mRNA.

Active ADAR was isolated from chicken lung cells and the enzyme was found to have *in vitro* Z-DNA-binding properties.[82] The Z-DNA binding domain (Zα) of ADAR1 has been identified.[11] Binding studies between Zα and poly(dC-dG) or a plasmid containing the CG repeat unit have shown that the optimal binding ratio between ADAR1 and Z-DNA was at one Zα domain per five base pairs. NMR and CD studies on $d(CGCGCG)_2$ have revealed that one Zα domain of human ADAR1 binds to one strand and another Zα domain binds to the opposite strand[83] (i.e., a 2:1 ratio, Figure 5). The results suggest that two Zα domain bind to Z-DNA duplex and involve interactions between the protein and five to six base pairs.

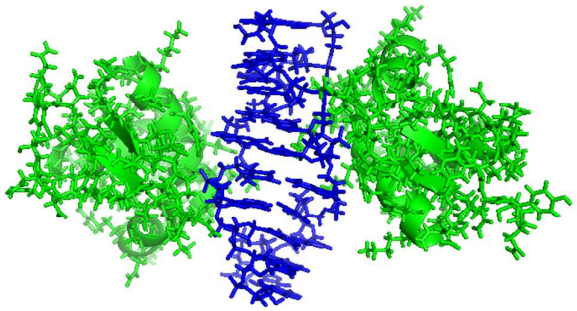

Figure 5. Structure of Z DNA (blue) bound to two Zα binding domains (green). Stabilization occurs by interactions between amino acid residues in the ZBP and 5-6 DNA bases (based on 1J75.PDB).

Sequence preference for Zα of ADAR-1 binding has been observed and ADAR1 prefers to bind to the CG repeat over other pyrimidine-purine alternated sequences.[84] In addition to the Zα domain, a second domain, termed Zβ, has also been identified and a crystal structure has been reported.[85] Notably, mutation of the Z-DNA binding domain diminishes deaminase activity[86] and therefore implies that ADAR1 binding to Z-DNA may be involved with ADAR1's enzymatic activity. Interferon (IFN) induces expression of ADAR1 which further suggests that ADAR1 has a role in the immune mechanism to fend off viral infection[87,88] by impeded function of viral RNA.

4.2 E3L

Vaccinia virus produces the E3L protein, a ZBP, as a countermeasure to excretion by host cells of IFN, produced as a defence against viral infection.[89] The inhibitory effect of E3L on IFN activity has been hypothesized to involve interfering with ADAR1 activity. Due to the high degree of homology in the Z-DNA binding domain between ADAR1 and E3L[87], the viral protein can bind to ADAR1 substrates and thereby inhibit deaminase activity associated with ADAR1. In E3L the Zα domain is located near the N-terminus and has been shown to bind to Z-DNA both *in vitro*[90] and *in vivo*.[91] Further, the Z-DNA binding domain has been shown to be essential for virus pathogenicity and mutation. Removal of the Z-DNA binding domain diminishes or eliminates virus virulence. In contrast, replacing Zα of E3L with Zα of other proteins such as ADAR1 maintains virus virulency.[13] Two monomeric Zα domains of E3L from Yatapoxvirus have been shown to bind to the Z-DNA duplex of $d(CGCGCG)_2$ very much like Zα of human ADAR1.[92] Finally, this study also revealed that E3L not only binds to Z-DNA but also aids in the transition of B-DNA to Z-DNA.

4.3 DLM1/ZBP1/DAI

The DLM1 gene was first isolated from cancer induced mice.[93] The novel gene produces the cancer associated protein DLM1 that was also identified as DNA-dependent activator of IFN-regulatory factors (DAI[94]) and Z-DNA binding protein 1 (ZBP1). The Z-DNA binding properties of DLM1 were discovered from a search for proteins that have structural homology to ADAR1.[12] The crystal structure of DLM1 bound to Z-DNA confirmed the presence of a Zα domain that was located near N-terminus. To date the Zα domain is conserved for all Z-DNA binding proteins. Binding of two monomeric Zα domains to $d(TCGCGCG)_2$ has been reported.[12] The biological function of DLM1 has been proposed and the ZBP is believed to be involved in the DNA mediation of the innate immune response.[94,95] A second Z-DNA binding domain in DLM1, Zβ, has also been discovered[96,97] and has been shown to bind to both Z-DNA and B-DNA. Binding of Zβ domain to B-DNA appears to promote the B-Z-DNA transition in $d(CGCGCG)_2$.[98] The finding of Zβ binding mode hints that ZBP may also stabilize Z-DNA.

4.4 PKZ

Protein kinase containing Z-DNA binding domains (PKZ) is a member of double-stranded RNA dependent protein kinase (PKR) that can be activated by host IFN or binding to foreign RNA or DNA. Hence, PKRs has been studied for their antiviral function.[16] Phosphorylation of proteins, such as the α subunit of eukaryotic translation initiation factor 2 (eIF2α), has been proposed to be a defensive mechanism of PKR to halt viral protein activity.[99,100] Discoveries of PKZ gene have been mainly in fish. The expressed PKZ has been shown to be homologous with the Z-DNA binding domains, Zα and Zβ are present and are located near the N-terminus, as is typical of ZBP.[101,102] The Z-DNA binding kinases expressed have been found in zebrafish[103] and goldfish[104,14] and have been shown to bind to CG repeated sequences. The Z-DNA binding domain has been shown to be mandatory for PKZ defence against viral infection.[16]

4.5 ZBP Binding Mechanism and Structure

Owing to common findings from ZBP binding studies, the mechanistic pathway of ZBP binding to DNA has been proposed (Figure 6).[83] The ZBP can bind to either/both B-DNA and Z-DNA, which, in former case, will promote the B-Z transition. The Zβ domain would most likely be the ZBP binding domain to bind to B-DNA as Zα appears to only bind to Z-DNA. Two Z-DNA binding domains are present in ZBDs, the Zα and Zβ domains, and both have been shown to bind to Z-DNA. Therefore, it is possible that either a single ZBP binds utilizing both binding domains or two separate ZBP may bind to the left-handed DNA.

Since DNA is typically in the B conformation, then, the process shown in Figure 6 likely starts with a ZBD Zβ domain binding to the B form and forming B-ZBD. This binding may induce conformational interconversion of the B DNA in B-ZBD and then form Z-ZBD. From this point, the Z-ZBD may bind to either Zα or Zβ with the result being Z-$(ZBD)_2$. This pathway has been explored by NMR and rate constants for the B/B-ZBD/Z-ZBD/Z-$(ZBD)_2$ with ADAR1 and the ZBD have been determined.[83] Alternatively, since Z-DNA may be present *in vivo,* for example, during transcription or due to the formation of C8 guanine adducts, binding may occur directly with either the Zα or Zβ binding domain such that the Z/Z-ZBD/Z-$(ZBD)_2$ path is followed.

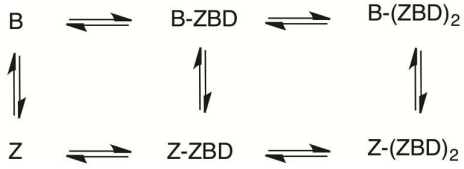

Figure 6. Binding mechanism of ZBP and Z-DNA. B is B-DNA, Z is Z-DNA, and ZBD is Z-DNA binding domain.[83]

Regardless of the specific pathway followed, the formation of C8-guanine adducts will alter the relative rate constants between the various species and therefore the corresponding equilbria. Since C8-guanine adducts favour the Z-DNA form, the amount of Z-$(ZBD)_2$ formed may increase if the only factor involved is the B/Z-DNA ratio. However, there may be steric factors that disfavour the formation of Z-$(ZBD)_2$ and thus the Z-DNA would not be bound to a ZBD. Either of these affects will likely alter gene expression and thus there is a potential link between C8-guanine adduct formation, interaction with ZBD, and cancer.

5.Biological Role of Z-DNA

Determining the role of Z-DNA in living systems has long been a major focus of Z-DNA research. Studies of the potential biological role(s) of Z-DNA have surged in the past two decades. Although the significance of the left-handed Z-DNA remains equivocal and will require additional research, several findings support a biological function for Z-DNA. The reports of CG islands and other Z-DNA prone pyrimidine-purine alternated sequences located at or near several promotor regions[7,105], the presence of Z-DNA *in vivo*[106], the *in vivo* occurrence of Z-DNA antibodies, and the discoveries of ZBP are among the key findings that point toward the possibility of Z-DNA function in gene regulation.

The early evidence that indicated involvement of Z-DNA during transcription was with regard to the c-*myc* gene.[7] The up regulation of c-*myc* has been associated with Z-DNA formation located at Alu*I* restriction fragments near a promotor region. The negative supercoiling generated during transcription has been shown to stabilize the left-handed Z-DNA as Z-DNA formation relieves the negative helical torsional strain.[42,107] The negative helical torsional strained supercoil can also be relieve by topoisomerase I and this was found to also diminished Z-DNA formation and down regulated c-myc expression.[108]

The activation of human colony stimulating 1 (CSF1) gene has also been related to formation of Z-DNA in the CSF1 promotor region promoted by BAF complex.[8] In this case the pyrimidine-purine repeat TG is found in the promotor region of CSF1 gene and is mandatory for expression. Replacing TG with CG repeats results in similar activity as compared to the wild type CSF1. *In vivo* Z-DNA formation in the CSF1 promotor has been reported and the BAF complex was identified as an enhancer of Z-DNA formation. Thus, the apparent requirement for Z-DNA formation during gene transcription underscores the relevance of Z-DNA in gene regulation.

In addition to the gene activation role of Z-DNA, recent studies on the family of a disintegrin and metalloprotease (ADAM) proteins has suggested Z-DNA as a repressor factor.[109] ADAM12 has been characterized and shown to have proteolytic activity.[110] A basal level of ADAM12 has been measured in non-proliferating tissues and found to be elevated in highly proliferating tissues, like placenta tissue during pregnancy.[109] ADAM12 has been implicated in the pathogenesis of several diseases including cancer.[111,112] Elevated levels of ADAM12 in urine of breast cancer patients has been reported and detection of ADAM12 has been studied for the potential to use it in cancer prognosis.[111]

A highly conserved negative regulatory element (NRE) in the 5'-untranslated region of the human ADAM12 gene has been shown to contain an alternating pyrimidine-purine track. Sequence homology of the Z-DNA prone track has been found in the human, mouse, rat, and bovine ADAM12 gene. Binding of ZBP to the NRE has been identified as a key step in ADAM12 transcription inhibition. Further, the regulation of ZBP concentration has been shown to be directly related to ADAM12 activity. In placenta tissue, where ADAM12 expression was high, negligible levels of ZBP were found and the NRE was expected to be inert. On the other hand, elevated concentrations of ZBP were found in the tissues with low ADAM12 activity and this indicates that the NRE has been activated by ZBP binding, hence, ADAM12 repression resulted. Identification and characterization of the implied ZBP will be a necessary and a

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next step to understand the role of Z-DNA in human ADAM12 gene regulation. Finally, the epigenetic regulator MeCP2, a methylcytosine binding protein, has been observed to play a part in ADAM12 inhibition in breast cancer cells.[113] Binding of MeCP2 to the NRE causes the NF1 transcription factor to bind to an upstream site near the NRE and triggers suppression of ADAM12. Notably, the inhibition complex was formed at significantly lower levels in breast cancer cells as compared to normal cells.

Besides the involvement of Z-DNA in genetic regulation and cancer, it also has been found to play a role in the pathogenesis of several other diseases including viral infection[91] and autoimmune diseases.[76,114] The etiology of Z-DNA related pathogenesis has been hypothesized to be due to the finding of the involvement of ZBP in disease states. The virulence of vaccinia or poxvirus infection has been associated with the E3L protein, a ZBD that has conserved Zα domains. Inhibition of host defence through INF secretion was postulated to rely on the ability of E3L to inhibit the function of ADAR1.

The design and discovery of novel therapies that can inhibit E3L have been proposed.[91,115] In addition, Z-DNA has been known to be highly immunogenic. Antibodies toward Z-DNA can be induced upon Z-DNA exposure[43] and have been found in patients with autoimmune disease such as rheumatoid arthritis and SLE.[76,114] Recent studies with DLM1 or DAI have shown that the ZBP promotes lupus nephritis by activation of the immune system. Inhibition of ZBP has been reported to alleviate the SLE condition.[116] Thus, nearly four decades since the discovery of Z-DNA structure, research on Z-DNA has led to the current finding that Z-DNA plays a putative role in living systems and now may represent a therapeutic target.

6. C8-guanine adducts, Z-DNA, and cancer

The effect of different C8-guanine adducts on the B to Z transition have been established and their presence in pyrimidine-purine alternating sequences promotes Z-DNA formation to various degrees.[35,43,71,72,73] Some of the adducts, e.g. C8-methyl guanine[35] and C8-carboxyphenyl guanine[73], are very potent Z-DNA promoters that can cause Z-DNA formation under physiological concentrations of salt. Most of the C8-adducts that facilitate Z-DNA formation can be generated by exposure of DNA to carcinogens such as polyaromatic hydrocarbons, aryl amines, phenolic compounds and alkyl- or arylhydrazines. Therefore, inappropriate Z-DNA formation and carcinogenesis are postulated to be correlated.

The amount of data that supports the role of Z-DNA in gene regulation continues to grow as is the discovery, of *in vivo* ZBP that regulate transcription or gene inhibition. Nevertheless, whether Z-DNA formation is a key event in carcinogenesis is still unclear and requires further investigation. The C8-guanine adduct formed from carcinogens could be utilized in a model systems to answer pivotal questions regarding Z-DNA role in carcinogenesis. With high levels of exposure to carcinogens, C8-guanine adducts form and may overwhelm cellular defence and repair mechanisms. In turn, the C8-guanine modification may influence genetic expression and may be analogous to what is observed in the case of cytosine methylation, which is an epigenetic modulator and also a Z-DNA promoter.

The idea of gene silencing by C8-guanine adduct is possible and may be involved carcinogenesis if silencing occurs on a tumor suppressor gene. Moreover, C8-guanine adducts may also induce carcinogenesis by promoting transcription of certain oncogenes as Z-DNA has been shown to promote transcription. A clear, solid relationship between the presence of C8-guanine adduct, Z-DNA formation, and findings of cancer related genes regulated by the previously stated conditions would be strong evidence for carcinogenesis orchestrated by Z-DNA. Ultimately, a gene constructed with C8-guanine adduct and real time detection of Z-DNA *in vivo* could provide tools to observed Z-DNA in action.

Another notable area is the growing number of ZBP that have been identified along with an identified or proposed role in living cells. The interaction between ZBP and C8-guanine containing Z-DNA will be an interesting point to investigate since ZBP have been demonstrated to be an integral part of several biological functions such as gene regulation and viral infection. Additionally, a clear understanding of ZBP function would help to clarify the significance of Z-DNA in vivo. The merits of understanding the effects of C8-adduct on Z-DNA formation and carcinogenesis is that it will help elucidate the role of Z-DNA in cancer and possibly other genetic diseases.

7. Conclusions

The role and function of Z-DNA in biological systems has been at the center of Z-DNA research for decades. The discovery of the ZBP family that contain homologous Z-DNA binding domains has fueled ongoing searches for new ZBP and Z-DNA functions. The finding of Z-DNA involvement in gene transcription and inhibition has strengthened the theory that it plays an important role in gene regulation. Although a significant amount of work and further investigation is required, research on Z-DNA and ZBP as drug targets has been initiated. C8-guanine adducts, potent Z-DNA inducers, can be used as a model to decipher and gain understanding regard to Z-DNA function in living systems and will be useful for drug discovery and design based on the left-handed Z-DNA.[91,115]

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Notes and references

Abbreviations

ADAR1 = Adenosine Deaminase Acting on RNA
CD = circular dichroism
C^{Me} = 5-methyl-2′-deoxycytosine
dA = 2′-deoxyadenosine
DAI = DNA-dependent activator of IFN-regulatory factors
dC = 2′-deoxycytidine
dG = 2′-deoxyguanosine
DLM1
dT = 2′-deoxythymidine
E3L
G^{8Br} = 8-bromo-2′-deoxyguanosine
G^{8CPh} = 8-*p*-carboxyphenyl-2′-deoxyguanosine
G^{8HMPh} = 8-*p*-hydroxymethylphenyl-2′-deoxyguanosine
G^{8MMPh} = 8-*p*-methoxymethylphenyl-2′-deoxyguanosine
G^{8Me} = 8-methyl-2′-deoxyguanosine
G^{8Ph} = 8-phenyl-2′-deoxyguanosine
IFN = Interferon
PKZ = Protein kinase containing Z-DNA
TEA = Triethylamine
TPPTS = 3,3′,3″-Phosphanetriyltris(benzenesulfonic acid) trisodium salt
ZBD = Z-DNA binding domain
ZBP-1 = Z-DNA binding protein 1
ZBP = Z-DNA binding protein

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