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Genistein Binding Mode to Doubly Nicked Dumbbell DNA. Dynamic and Diffusion Ordered NMR Study

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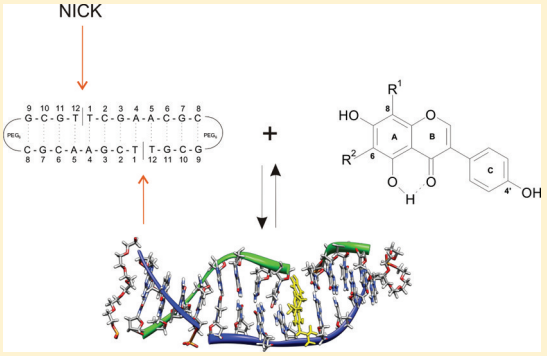
Supporting Information

ABSTRACT: New genistein derivatives were synthesized, which are fairly well soluble in water, with respect to parent genistein, and thus facilitate study of the interaction with dumbbell DNA dodecamer, mimicking the biological target for topoisomerase II inhibitors. A pulsed field gradient spin echo NMR experiment was used to check the binding and to estimate the association constants and its pH dependence of genistein with dumbbell DNA. Experimental restraints based on nuclear Overhauser spectroscopy spectra were used to calculate the NMR structure in solution in case of 6,8-disubstituted genistein with dimethylaminomethyl groups and were used in molecular modeling calculations. The structure is dynamic, and 10 molecular dynamics runs yield a family of conformations that essentially differ in a depth of inclusion of genistein into a nick. The paper experimentally shows evidence for binding, intercalation in the nick is proposed as a mode of genistein binding, and a model of the event is provided.



INTRODUCTION

A continuous interest is observed in new classes of compounds to find a lead and better formulate an anticancer drug pharmacophore.[1−5] Topoisomerases I and II poisons attract much attention in biomedical research in this direction since they constitute an important chemotherapeutic route in combating cancer.[6−9]

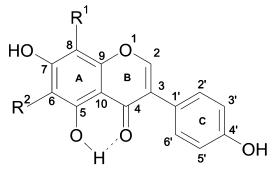
The various problems of disentangling DNA strands or duplexes in a cell are all rooted in the double-helical structure of DNA. Three distinct subfamilies of enzymes, known as the DNA topoisomerases, have evolved to solve these problems.[9]

A diverse, wide biological activity is expressed by genistein[10−13] and, in particular, anticancer activity[14] and topoisomerase II cleaving agent.[15,16] It also acts as an inhibitor of tyrosine-specific protein kinase.[17] Our interests in this area are related to the problem of targeting the secondary complex of DNA with topo II by the genistein,[18] since the mechanism of its action on an atomic level in this complex is not known.[19]

In this contribution, results are presented that concern the interaction of genistein and its derivatives (Scheme 1) with a dumbbell DNA. This study may aid in the greater understanding of genistein: double-nicked DNA motif binding. Its binary complex with topo II is targeted by genistein.

The choice of nucleotides around the nick in a dumbbell (Scheme 2) was based on the result of the biological assay, which disclosed that genistein, on DNA binding, requires thymidines at both −1 and +1 positions.[18]

Scheme 1. Structures of Parent Genistein and Its Derivatives Studied in This Contribution



compound	R^1	R^2
Genistein	H	H
Bismorpholino genistein	$-CH_2-N$ O	$-CH_2-N$ O
Bisdimethylamino genistein	$-CH_2-NMe_2$	$-CH_2-NMe_2$

In this contribution, there were no attempts undertaken to explain the full biological mechanism of topo II inhibition by genistein, because topo II is not involved. Therefore, it was only intended to address the two main issues of the genistein biological role in inhibiting topo II; that is, the evidence of

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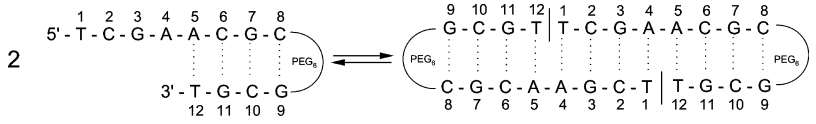
Scheme 2. Hairpin/Dumbbell Equilibrium



binding of genistein in a nick of a dumbbell DNA was presented, and intercalation of the nick was discussed as a mode of binding, proposed on the basis of calculated and nuclear Overhauser spectroscopy (NOESY) derived geometry of dumbbell/genistein complexes.

RESULTS AND DISCUSSION

Thymidine–Genistein Interaction Mode. Figure 1 shows theoretical modes of binding of genistein to thymidine

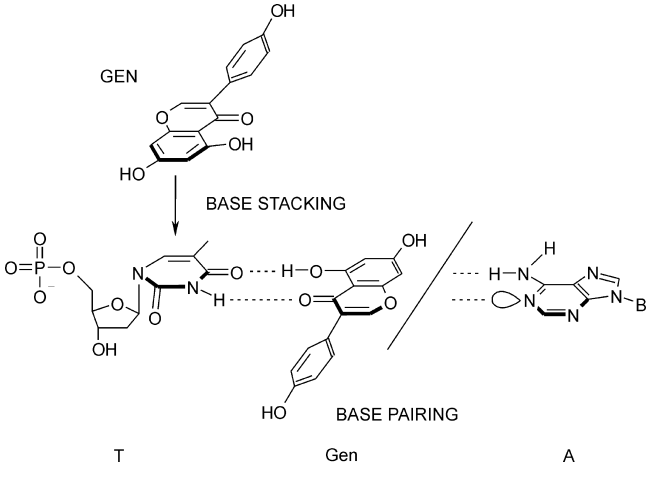


Figure 1. Possible site-specific binding modes of genistein and thymidine nucleotide.

in the T-A base pair. This interaction has to be considered in view of the biological assays, which show that genistein requires thymidines at both −1 and +1 positions in a nick of the topo II binary complex. It can be hypothesized that two kinds of binding modes can be considered with the thymidine in the T-A base pair in the nick. First, genistein can intercalate, and second, it can replace the adenine in a base pair by forming two hydrogen bonds with thymidine. The latter binding mode, although geometrically feasible, requires breaking of the intramolecular hydrogen bond in genistein and breaking of the duplex forming T-A base pairing. On the other hand, intercalation of genistein into a nick results in stretching of the DNA backbone of the unbroken strand by creating the situation as if another base pair was introduced into the DNA sequence in a nick site. This situation has a precedence in molecular complexes of topotecan (TPT) intercalating the DNA nick, observed in solution in a study of TPT interaction with nicked decamer[20] and in a crystal structure of nicked DNA–TPT–topoI ternary complex.[21]

In the present case, the stacking interaction of electron-rich rings of genistein with the pyrimidine ring of thymidine can be considered as a factor favoring the intercalation or minor groove binding modes. Furthermore, evidence is presented that confirms the presence of the interaction, by means of acquiring pulsed field gradient spin echo (PFGSE) experiments, which monitor the translational diffusion coefficients of species in solution. A mode of interaction is probed by means of NOESY cross-peaks as a source of experimental constraints embedded in simulated annealing protocol.

Effect of Genistein on Hairpin/Dumbbell Equilibrium: Biological Relevance of the Results. Previously, the conditions for observing hairpin or dumbbell forms of DNA in solution were devised, Scheme 2.[22] Lowering the temperature and increasing the ionic strength and solute concentration (ca. 2 mM) increase the amount of dumbbell that can be observed as a sole form at 2 °C in 200/25 mM NaCl/K_3PO_4 buffer in a water/methanol solution (85/15 vol %).

At a low concentration of hairpin in solution, ca. 0.5 mM, and low buffer concentration, 25/25 mM K_3PO_4/NaCl, this form prevails in equilibrium and is dimerizing at lower temperatures, but the dumbbell population reaches only 25% (Figure 2, lower set of spectra). The assignment of the

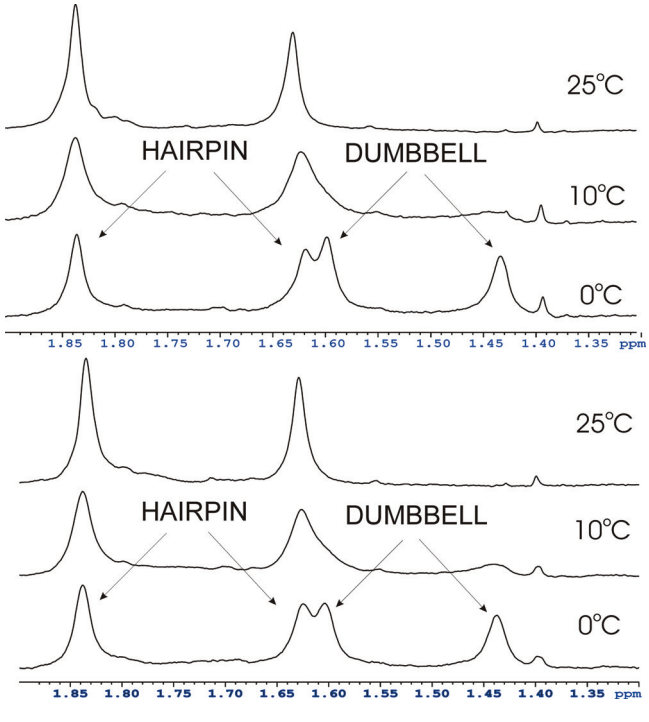


Figure 2. Effect of genistein (upper set) on hairpin (reference, lower set) dimerization to dumbbell observed on thymidine methyl resonances. The hairpin solution (lower set of spectra) was saturated with genistein (ca. 0.5 equiv with respect to hairpin DNA) and spectra recorded in the same conditions (see Figure 7S in the Supporting Information for more information on integrals and linewidths).

thymidine resonances to both forms was based on a customarily accepted fact that duplex formation leads to low frequency shifts due to stacking interactions of aromatic bases in DNA.

The hairpin solution was saturated with genistein to check its effect on the position of the hairpin/dumbbell equilibrium. The lower set of spectra in Figure 2 (reference experiment) shows the effect of lowering the temperature on a hairpin self-association to dumbbell DNA in ca. 0.4 mM solution. The spectrum at 25 °C represents the hairpin chemical shift of

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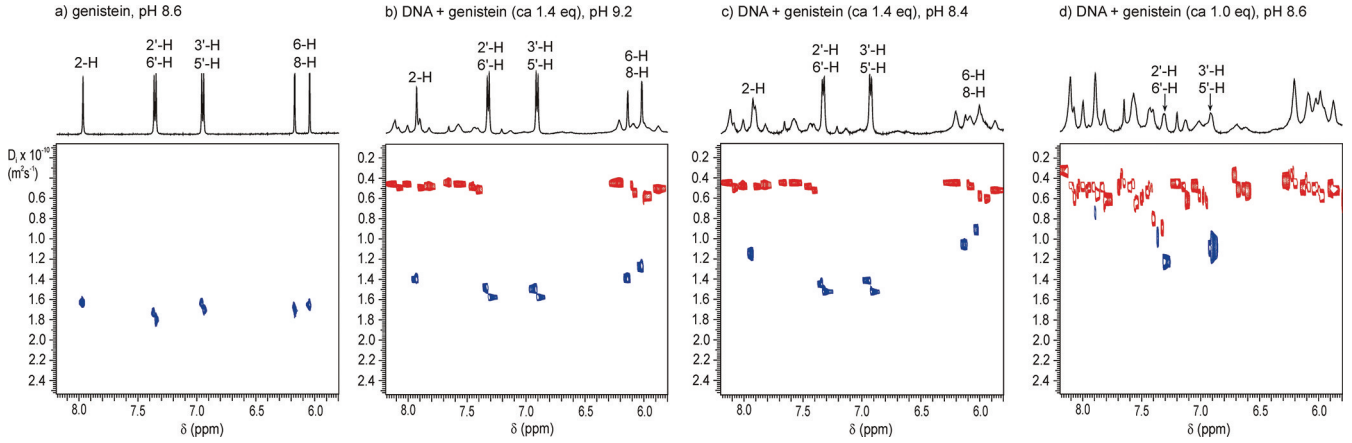

Figure 3. Genistein (in blue) diffusion coefficient dependence on the solution pH and molar excess relative to dumbbell DNA (in red), established at 0 °C in buffered water/methanol (82/18 vol %) solution. Genistein signals are marked in horizontal traces of each experiment.

Table 1. PFGSE Data for the Binding of Genistein and Bisdimethylamino Genistein Derivative to Nicked Dumbbell DNA Duplex[a]

				$m^2\ s^{-1}$				
no.	sample	pH	concn (mM)	D_i (Gen) (free) × 10^{-10}	D_i (Gen) (complexed) × 10^{-10}	D_i (DNA) × 10^{-10}	K_a (mM^{-1}) $P_{complex}$[b]	signal observed
1	genistein	8.6	C_{Gen}, 1.0	1.75 ± 0.05				3′-H
2	DNA/genistein	9.2	C_{DNA}, 1.0			0.45 ± 0.05	0.31	T-1 CH_3
			C_{Gen}, 1.4		1.53 ± 0.05		0.17	3′-H
3	DNA/genistein	8.4	C_{DNA}, 1.0			0.46 ± 0.05	0.57	T-1 CH_3
			C_{Gen}, 1.4		1.45 ± 0.05		0.23	3′-H
4	DNA/genistein	8.6	C_{DNA}, 1.0			0.45 ± 0.05	0.77	T-1 CH_3
			C_{Gen}, 1.0		1.25 ± 0.05		0.38	3′-H
5	DNA	8.6	C_{DNA}, 1.0			0.45 ± 0.05		T-1 CH_3
6	bisdimethylamino genistein	6.3	*c*	1.51 ± 0.05[c]			0.15[d]	3′-H
7	DNA/bisdimethyl amino genistein	6.0	C_{DNA}, 1.9		0.98 ± 0.05	0.45 ± 0.05	0.71[e]	T-1 CH_3
			C_{Gen}, 1.0				0.50	3′-H

[a]The samples were studied in H_2O/methanol-d_3 (82/18 vol %), in a buffer solution, 200/25 mmol NaCl/K_3PO_4 at given pH and 2 °C. The hairpin concentration of DNA is cited, as measured by UV on a solution from NMR sample. [b]Population of a complex, a mole fraction. K_a values were established using diffusion constants of free genistein and in a complex with DNA. For the sake of simplicity, it was assumed that the DNA diffusion constant changes negligibly on genistein complexation, which is what is really observed in experiment. [c]Diffusion coefficient at infinite dilution (see Figure 3S in the Supporting Information). [d]Self-association binding constant of bisdimethylamino genistein from dilution experiment using PFGSE technique (see Figure 3S in the Supporting Information). [e]Association constant calculated considering the self-association of bisdimethylamino genistein.

thymidine methyl resonances in a hairpin, at 1.63 and 1.84 ppm. At 0 °C, a second pair of thymidine methyl groups appears at 1.44 and 1.60 ppm, assigned to the dumbbell structure. Both forms are in the slow exchange regime with equilibrium populations of 75:25% in favor of the hairpin.

The upper set of spectra in Figure 2 shows the effect of genistein on dimerization of hairpin DNA. A trace at 0 °C, when compared with the respective spectrum in a reference set, shows ca. 10% increase of thymidine resonances integrals at 1.44 and 1.60 ppm assigned to the dumbbell structure in slow exchange with a hairpin. Because the spectra are compared at the same temperatures (0 °C), this change of population can only be assigned to influence genistein on the dumbbell population in equilibrium. The half-widths of the signals are the same because kinetics of exchange are not changed at the same temperature, but only the thermodynamic stability of both forms is modulated, what finds reflection in intensity changes. The same results were obtained with parent genistein and its bismorpholino derivative (Scheme 1). Therefore, the rise of the population of a dumbbell form in equilibrium on addition of the flavonoid was observed. This result can be interpreted as a thermodynamic stabilizing effect of genistein on dumbbell formation. The observation has a significant biological relevance because it suggests that the dumbbell bound to genistein is thermodynamically more stable than the neat dumbbell. A possible rationale of the mechanism of the effect shown in Figure 2 may point to the presence of the interaction inside the nick with flanking thymidines. It also suggests that genistein affinity to the two thymidines flanking a nick is a determining factor of its binding in the dumbbell DNA; therefore, this is a site-specific binding. This interaction would give additional stabilization of the dumbbell by strengthening the dimer interface. This hypothesis was confirmed by acquiring the NOESY spectrum of the dumbbell/bisdimethylamino genistein complex at 0 °C, which shows the intermolecular interactions in a nick (vide infra). This feature distinguishes genistein as a topo II inhibitor from a number of other chemical species that could bind DNA.

It was attempted to acquire more detailed information on parent genistein binding affinity to a nicked dumbbell. Parent

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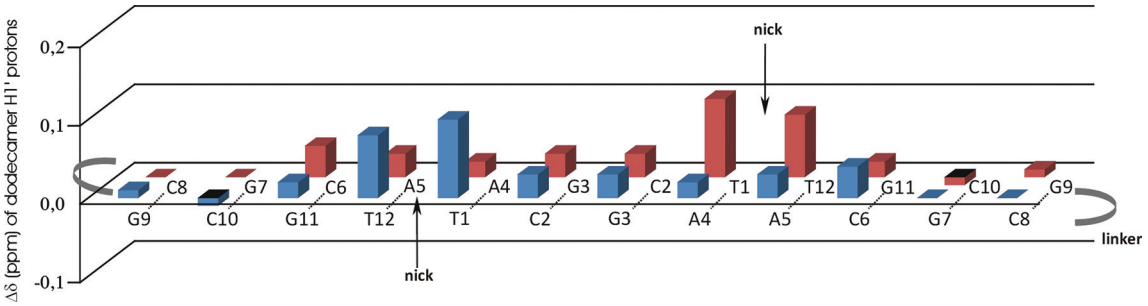

Figure 4. Chemical shift changes $\Delta\delta = \delta$(dodecamer) − δ(dodecamer/bisdimethylamino genistein) for H1′ protons in dumbbell dodecamer in the presence of bisdimethylamino genistein.

genistein is very poorly soluble in water at biological pH. The pH of the dumbbell solution at increased ionic strength, 200 mM NaCl, was therefore raised to 9.2 to increase the amount of better solubility in water−sodium phenolate anion of genistein. According to Zielonka et al.,[23] genistein is characterized by three pK_a values at pH 7.2, 10.0, and 13.1. Therefore, at pH 9.2, one of the OH groups should be ionized. Methanol-d_3 was added (18 vol %) to enhance the genistein solubility. Also, the hairpin concentration, 1 mM, in the hairpin was kept low to allow the highest genistein/dumbbell ratio but high enough to observe only the dumbbell structure at 2 °C.

To quantitatively establish the genistein binding to dumbbell DNA, the PFGSE method was used (Figure 3). The results are given in Table 1. The data give clear evidence of binding, which is further confirmed by the spectral processes shown in Figure 3. At high excess of genistein to dumbbell DNA (items 2 and 3 in Table 1), the D_i coefficient of the 3′-H signal is very close to the one in pure genistein (item 1). This is because there is a large amount of uncomplexed genistein, averaged with the DNA/genistein complex. Lowering the pH from 9.2 to 8.4 results, with the same DNA/genistein ratio, in diminishing the D_i coefficient and broadening of the 3′-H signal (item 3, Table 1 and Figure 3c). By lowering the pH, we diminish the amount of sodium phenolate, which apparently is less effectively bound to dumbbell DNA, and simultaneously, the phenol OH groups are restored, which increases the amount of a complex. As the negative charge is reduced, one can reason that it could be due to reduction of electrostatic repulsion because DNA is negatively charged. Alternatively, this also could be treated as a hint that OH groups in genistein are involved in DNA binding. Interestingly, the latter explanation would have confirmed earlier results concluding that the 7-OH group of parent genistein may be involved in interaction with NH of the edge guanosine in the d(GCGATCGC)$_2$ duplex.[24] To further confirm the binding, the excess of genistein was substantially lowered, which increases the amount of a binary complex. As expected, this lowers the value of D_i to 1.25×10^{-10} m^2/s (item 4, Table 1) and broadens the 3-H′ signal (Figure 3d). Table 1 also shows that genistein is very weakly bound to DNA (K_a value). This may explain the fact that there is small amount of a complex (ca. 17%) in fast exchange with uncomplexed DNA; therefore, negligible shifts are induced on DNA protons upon binding.

The data in Table 1 also show that genistein and bisdimethylamino genistein behave in a different way in the water solution. The diffusion coefficient, D_i, for genistein, item 1 in Table 1, is higher than for bisdimethylamino genistein at infinite dilution, even after accounting for the smaller molecular weight of genistein (item 6, Table 1). This suggests that genistein weakly self-associates in the water solution. For this reason, self-association of genistein was not considered in calculations of the binding constant of the genistein/DNA complex, but the self-association constant of bisdimethylamino genistein was taken into account for calculation of the binding constant of the bisdimethylamino genistein/DNA complex.

Nevertheless, the results presented above are in agreement with the biological assays, suggesting the inhibition of topo II action by genistein as the evidence is presented that parent genistein and its derivatives are bound to dumbbell DNA. This property probably distinguishes the genistein moiety from a number of small molecules one can envisage as possible DNA binders. What was essentially arranged experimentally was mixing three molecules (two hairpins and one genistein), which in a concerted manner have created the binary complex, mimicking a component of biological ternary complex composed of topoII/dumbbell/inhibitor. Binding of genistein in a nick prevents religation of both strands of DNA and thus inhibits the topo II action. Although the binding affinity of parent genistein is established as small, one can expect that this affinity could be strengthened in the presence of topo II, which may supply an additional stabilization factor by binding OH groups of genistein.

Mode of Modified Genistein Moiety Binding the Hairpin/Dumbbell DNA. There were synthesized two analogues of parent genistein bearing two methyl morpholine or methyl dimethylamino groups in positions 6 and 8 (Scheme 1, bismorpholino genistein and bisdimethylamino genistein, respectively), which are much more soluble at physiological pH due to presence of two nitrogen atoms undergoing protonation. This allows us to study these derivatives at pH 6 with appreciable excess with respect to dumbbell DNA. Also, three unionized phenolic groups are available for binding.

Therefore, the interaction of DNA hairpin motif with bismorpholino genistein was studied at 25 °C since at this temperature there is only one DNA form that allows the interpretation of the chemical shift changes, and NMR experiments based on scalar couplings can be run. It was anticipated that thymidines T-1 and T-12 shall be the primary targets for genistein binding, according to biological assay disclosures.[18] Figure 1S in the Supporting Information shows the fingerprint of the NOESY spectrum of hairpin motif with ca. 1.5 equiv of bismorpholino genistein. There were negligible shifts observed on DNA signals and no intermolecular cross-peaks between 2-H and 2′-H or 3′-H of bismorpholino genistein and DNA protons at room temperature.

The side projection of the NOESY spectrum (bottom trace in Figure 2S in the Supporting Information) shows a broad signal at 7.92 ppm assigned to the 2-H proton of bismorpholino

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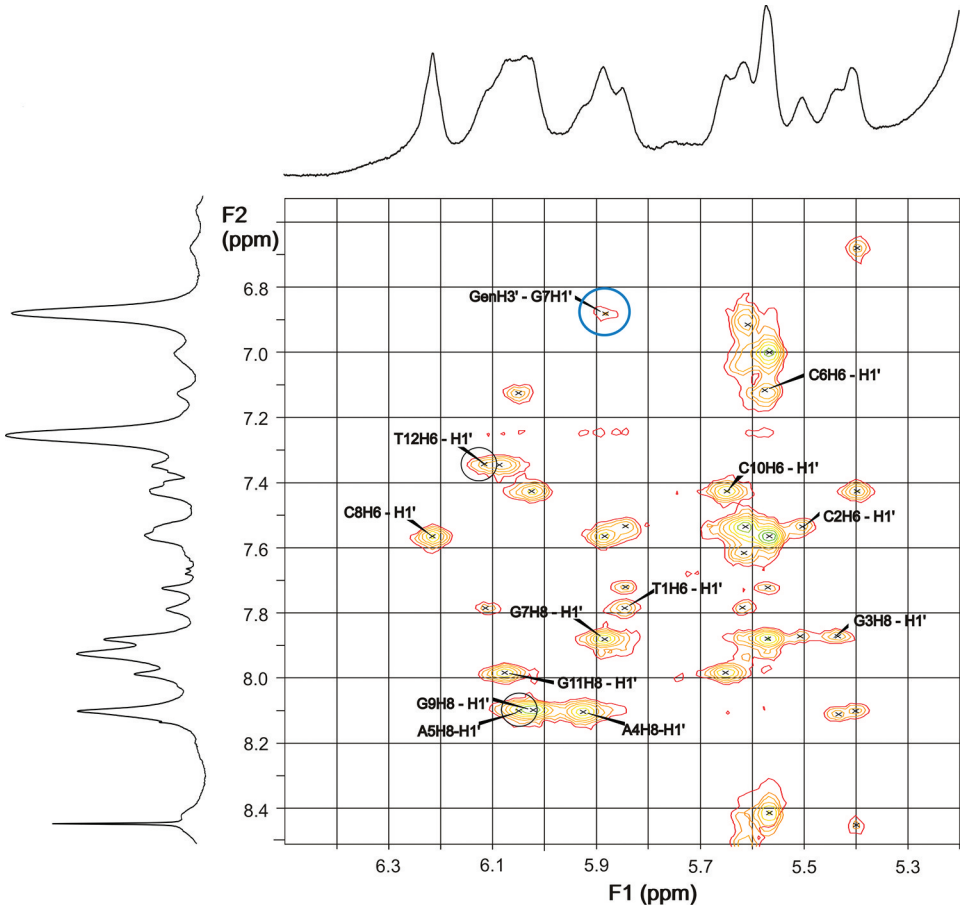

Figure 5. Fingerprint region of NOESY spectrum of dumbbell DNA at 2 °C, 1.9 mM in hairpin, pH 6, in 25/200 mM K_3PO_4/NaCl buffer (H_2O/D_2O, 9:1) in the presence of 2 equiv of bisdimethylamino genistein. The intermolecular cross-peak, Gen3′-H/G7H1′, is indicated by a blue circle. The Gen2-H/A5H2 cross-peak is shown in Figure 3S in the Supporting Information.

genistein, which is observed as a sharp singlet at 8.04 ppm in neat bismorpholino genistein in the same solvent and pH. Likewise, protons 2′-H and 3′-H move to lower frequencies by 0.12 and 0.09 ppm, respectively. Low frequency shift of signals indicates the intercalation of bismorpholino genistein into a DNA base pair and a broadening can be due to the intermediate exchange rate with DNA. This broadening, however, also excludes the possibility of observing the cross-peaks due to intermolecular interaction.

The most soluble in water of the three studied compounds, bisdimethylamino genistein, allows gathering more detailed information on a mode of genistein moiety binding to dumbbell DNA, even if the only source of information is the NOESY, (for chemical shifts see to Table 1S in the Supporting Information), because broadening of the signals at 2 °C makes useless total correlation spectroscopy (TOCSY) and heteronuclear single quantum correlation (HSQC) and HMBC experiments.

Nevertheless, the NOESY spectrum allowed assignment of chemical shifts in DNA induced by bisdimethylamino genistein and comparison of them with chemical shifts in solution of neat dumbbell measured at the same temperature (Figure 4).

As seen in Figure 4, these shifts concentrate symmetrically around the nick. The ligand is in fast exchange with DNA, and its C2 symmetry is not lifted upon bisdimethylamino genistein binding in a nick. This graph allows the statement that the ligand is bound specifically in a nick, flanking the two thymidines.

More importantly, there were several intermolecular cross-peaks (for example, GenH2/A5H2 and GenH3′/G7H1′; see Figure 5 and Figure 4S and Table 2S in the Supporting Information) found in a NOESY spectrum of a dumbbell DNA at 2 °C. These cross-peaks give essential information in defining the experimental restraints used in molecular modeling to construct the DNA/ligand complex. The interaction of bisdimethylamino genistein with DNA induces shifts on some of the DNA protons; that is, on H1′ (see Figure 4) and H6/H8 (see Figure 4S in the Supporting Information) as well as on bisdimethylamino genistein (see Table 1S in the Supporting Information). Both shifts are to lower frequencies what is customarily interpreted as arising from stacking interactions of aromatic rings in intercalation process in DNA.

All three kinds of information gathered, that is, establishing binding constants of dumbbell and ligand, specific mutual shifts induced on both, and intermolecular cross-peaks between them, are consistent with bisdimethylamino genistein intercalating between T-A base pairs in a nick of a dumbbell, as shown in Figure 6. Using the mentioned cross-peaks, it was possible to dock the bisdimethylamino genistein to dumbbell DNA and compute the formal structure.

The closer inspection of the geometry of the complex bisdimethylamino genistein/dumbbell DNA in Figure 6 allows the above-discussed statement that genistein is intercalating in the nick, flanking both T-A base pairs. The intercalation is perpendicular to the base pair axis so that bulky dimethylamino groups are in a major groove and ring C is in a minor groove.

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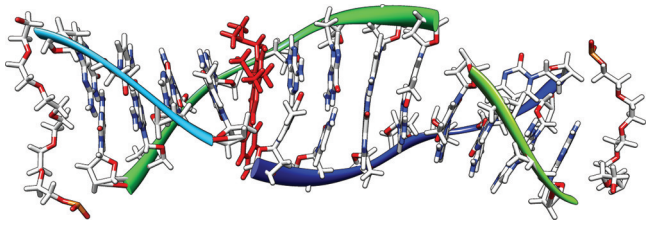

Figure 6. Dumbbell DNA dodecamer in the presence of bisdimethylamino genistein (red) intercalating between the TA base pairs in the nick. The PEG6 linker is open in this presentation.

The suggested earlier phenomenon of noncoplanarity of ring C in genistein moiety[19] apparently does not preclude the intercalation. The molecular modeling gave a cluster of conformations similar to that shown in Figure 6 (Figure 6S in the Supporting Information) in which bisdimethylamino genistein is to a different degree embedded in a nick. This is a consequence of the low affinity of genistein to DNA (see Table 1), which is a cause of the dynamic nature of a complex. Also important is the fact that the substituents in position 6 and 8 are sticking outside the nick and do not interfere with the DNA. This fact can be considered as a hint that the geometry of bisdimethylamino genistein/dumbbell can also be representative for the mode of binding of parent genistein. The poor quality of the spectra at 2 °C preclude acquiring more information, which could enable detailed discussion of the changes induced on the DNA environment. The stoichiometry of a molecular complex can only be addressed qualitatively. Because of the C2 symmetry of a dumbbell, one can treat the two parts as independent, assuming that monointercalation does not influence thermodynamics of second intercalation. Therefore, pure statistical approach, in the case of 50% of a complex, would predict 33% of monointercalation, 17% of double intercalation, and 50% of free DNA in fast exchange.

SUMMARY AND CONCLUSIONS

In this contribution, spectral results are presented that concern the interaction of parent genistein and its derivatives (bismorpholino genistein and bisdimethylamino genistein) with the model mimicking their natural biological target, that is, dumbbell DNA dodecamer with two nicks in both strands of the duplex. It was earlier shown that this model in applied here conditions (water/methanol 82/18 vol % at 2 °C, pH 6.0, and 25/200 mmol K_3PO_4/NaCl buffer concentration) exists solely in a form of a dumbbell. The poor solubility of parent genistein in water and its low affinity to DNA was overcome by introducing, through the methylene linker, two dimethyl amino groups or morpholines in positions 6 and 8. These derivatives are fairly well soluble in water and thus allow studying the interaction with DNA using appreciable excess of the ligand.

The association constants of genistein with dumbbell DNA and its pH dependence were established in the PFGSE NMR experiment. It was shown that phenolate anion has the lower affinity to DNA than phenol. Chemical shifts induced on DNA protons by bisdimethylamino genistein were found indicative of the mode of binding. For this derivative, experimental constraints based on cross-peaks volumes from NOESY spectra were used to calculate the NMR structure in solution. It was also possible to observe several intermolecular cross-peaks, which confirm the geometry of bisdimethylamino genistein/dumbbell complex. It was found that bisdimethylamino genistein is intercalating the nick in a dumbbell, flanking the two T-A base pairs. It is discussed that despite the different geometry of the ligand, with comparison to parent genistein, its binding mode can be similar to that found in this study for bisdimethylamino genistein.

The evidence on an atomic level on the mode of genistein binding the doubly nicked DNA, mimicking its biological target, is presented. In this contribution, the information was gathered that can be used as a hint in addressing the two main issues of the genistein biological role in inhibiting topo II; that is, evidence of specific binding of genistein in a nick of a model dumbbell DNA is presented and intercalation, as the mode of binding the nick, is proposed. Specific affinity of genistein to a nick having thymidines at −1 and +1 positions is manifested by thermodynamic stabilization of a dumbbell form in the presence of flavonoid. The PFGSE experiment allows establishing the association constant of a complex. Although this is small, one can expect that this affinity could be strengthened in the presence of topo II, which may supply additional stabilization factor by binding OH groups of genistein. The intercalation is perpendicular to the base pair axis so that bulky dimethylamino groups are in a major groove and ring C is in a minor groove. This geometry makes genistein hydroxyl groups in ring A easily available to hydrogen bonding with topo II, which can stabilize the biologically relevant ternary complex. Further studies to synthesize new genistein derivatives that would confirm the mode of binding to dumbbell DNA that is suggested here are ongoing.

EXPERIMENTAL SECTION

The purity of the compounds studied was >95%. The elemental analysis and HPLC were used for purity assessment.

The hairpin dodecamer was purchasd from Symbios as 5′-TCGAACGC/iSp18/GCTG/3Phos/-3′ dodecamer with PEG_6 linker (iSp18). The oligonucleotide was purified by ion-exchange chromatography on a HiTrap-Q column (Pharmacia Biotech) using gradient elution with ammonium bicarbonate solution (0.1−0.8 M) and desalted on Sephadex G-10 filled column (purity >98%). Genistein was purchased from Sigma and used as received [purity >98% (HPLC)]. Genistein derivatives were synthesized according to given procedures.

Bisdimorpholino Genistein: 6,8-Bis-(morpholin-4-ylmethyl)-genistein. Genistein (100 mg, 0.370 mmol) and morpholin (64 mg, 0.740 mmol) were mixed, dissolved in methanol, and heated to 65 °C. Formaldehyde (138 mg, 1.850 mmol) was added to this solution. The obtained mixture was stirred for 6 h at 65 °C. Removal of the solvent and purification by column chromatography (silica gel, eluent: CH_2Cl_2/MeOH 15:1) gave compound **2** in 49% yield (85 mg). HR-MS (ESI): calculated for $C_{25}H_{29}N_2O_7$ $[M + H]^+$, 469.19593. Found, 469.1959. ^1H NMR ($CDCl_3$) δ: 2.64 (b, 8H, CH_2-12, CH_2-12′), 3.77 (b, 8H, CH_2-13, CH_2-13′), 3.85 (s, 4H, CH_2-11, CH_2-11′), 6.78−6.84 (m, 2H, CH-3′, CH-5′), 7.29−7.35 (m, 2H, C-2′, CH-6′), 7.68 (s, 1H, CH-2). ^{13}C NMR ($CDCl_3$) δ: 50.5 (CH_2-11), 52.2 (CH_2-11′), 52.8 (CH_2-12), 53.1 (CH_2-12′), 66.3 (CH_2-13), 66.4 (CH_2-13′), 99.8 (C-8), 103.7 (C-6), 104.5 (C-10), 115.6 (CH-3′, CH-5′), 121.7 (C-1′), 123.0 (C-3), 129.9 (CH-2′, CH-6′), 152.1 (CH-2), 155.4 (C-9), 157.0 (C-4′), 159.7 (C-5), 164.6 (C-7), 180.8 (C-4).

Bisdimethylamino Genistein: 6,8-Bis-(*N*,*N*-dimethylamino-methyl)-genistein. This compound is independently reported.[25] Genistein (58 mg, 0.21 mmol) and acetic acid (0.5 mL) were dissolved in methanol (22 mL). To this solution were added dimethylamine in methanol (40%, 0.2 mL, 1.40 mmol) and formaldehyde (37%, 151 mg, 1.87 mmol). The reaction mixture was stirred for 2 h at RT. The solvents were removed under vacuum. The residue was dissolved in methanol (5 mL) and evaporated. This step was repeated twice. The product was unstable and used after reaction. HR-MS (ESI): calculated for $C_{21}H_{25}N_2O_5$ $[M + H]^+$, 385.17736. Found, 385.1774. ^1H NMR (H_2O + 15% CD_3OH) δ: 2.82 (s, 12H, NMe_2-12, NMe_2-

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12′), 4.15 (s, 2H, CH_2-11′), 4.17 (s, 2H, CH_2-11), 6.83 (m, 2H, CH-3′, CH-5′), 7.21 (m, 2H, CH-2′, CH-6′), 7.78 (s, 1H, CH-2). ^{13}C NMR (H_2O + 15% CD_3OH) δ: 42.9 (NMe_2-12 or NMe_2-12′), 43.0 (NMe_2-12 or NMe_2-12′), 52.2 (CH_2-11, CH_2-11′), 97.8 (C-8), 103.7 (C-6), 116.1 (CH-3′, CH-5′), 122.9 (C-3), 123.4 (C-1′), 131.3 (CH-2′, CH-6′), 153.3 (CH-2), 157.0 (C-4′), 159.0 (C-9), 162.9 (C-5), 180.3 (C(O)-4). C-7 and C-10 were not assigned.

Sample Preparation. Samples for NMR were prepared by dissolving the oligonucleotide in buffer containing 25 mM K_3PO_4 and a variable amount of sodium chloride (25−200 mM) to study the ionic strength on the hairpin/dumbbell equilibrium. The pH was adjusted to 6.0 or as otherwise indicated. Samples in H_2O contained 10% v/v D_2O. The concentration was measured by the UV absorption. TSP-d_4 was added to monitor the changes of chemical shifts at different temperatures. No EDTA was added to DNA samples, which were purified from paramagnetic impurities on a Chelex 100 column packing purchased from Bio-Rad Laboratories.

NMR Experiments. ^{1}H NMR spectra (500 and 600 MHz) in H_2O on Varian NMR spectrometers were run using a 12 or 16 kHz spectral window, DPFGSE water suppression pulse sequence, and presaturation for samples in D_2O. NOESY spectra[26] as (DPFGSE_NOESY) experiment with water suppression by gradient echo and ZQ artifact suppression during mixing) were recorded using the States-TPPI method[27,28] (mixing time 200 ms). TOCSY spectra[29,30] as (WGTOCSY) with selective H_2O one-lobe sinc pulse with flipback were acquired; the mixing times for TOCSY spectra were 90 ms with a DIPSI-2 spin-lock field of 7 kHz. ^{1}H/^{13}C-HSQC spectra[31] were acquired as the echo-antiecho phase sensitive ^{1}H−^{13}C HSQC (heteronuclear single quantum coherence, adiabatic version) with a relaxation delay of 1.2 s and ^1J(C,H) = 135 Hz.

PFGSE experiments were performed in H_2O according to the following conditions: 16 spectra were acquired using the BPPSTE[32,33] (stimulated echo sequence incorporating bipolar gradients). The gradient strengths were incremented as a square dependence in the range from 1 to 50 G/cm. The diffusion time (Δ) and the duration of magnetic field gradients (δ) were 250 and 2 ms, respectively. Other parameters include the following: a sweep width of 12000 Hz for H_2O, 32K data points, 128−1024 transients, depending on sample concentration, and relaxation delay of 2 s. The data were processed using Varian DOSY.[34]

Calculation Procedures. The 390 NOE cross-peaks volumes were assigned from the NOESY spectra at 200 ms mixing time, at 2 °C, using SPARKY[35] for assignment of cross-peaks and integration of their volumes. The NOE volumes were converted to upper and lower bound restraints using MARDIGRAS (version 5.21).[36,37] Intermolecular cross-peaks volumes can not be accurately transferred to distance due to poor S/N ratio, a small amount of complex, and exchange with free DNA. Therefore, it was assumed that the presence of an intermolecular cross-peak indicated that the interproton distance is less than 6 Å.

The MARDIGRAS algorithm converts the intensity matrix (nonobservable intensities are supplied from the model) to a relaxation rate matrix, which is improved by an iterative procedure. Distances are then calculated from final cross-relaxation rates. This yielded 610 restraints considering *C*2 symmetry of the molecule. MD refinement in AMBER 9[38] involved additionally 52 Watson−Crick restraints. Thirteen additional constraints from NOESY intermolecular cross-peaks in a complex DNA−Gen3 were incorporated. The generalized Born (GB) solvent model[39,40] approach was used in the refinement protocol using molecular dynamics simulated annealing.

Refinement Protocol. The energy minimization with GB model was first performed on 10 starting dumbbell molecules built using A and B DNA type canonical structures and intermediates with the genistein molecule placed close to the nick. This was followed by two cycles of 15 ps simulated annealing with GB performed by SANDER (from AMBER): 0−1000 steps heating the system from 10 to 1100 K, 1001−3000 steps leaving at 1100 K, 3000−15000 steps cooling to 0 K, 0−3000 steps tight coupling for heating and equilibration (TAUTP = 0.2), 3001−11000 steps of slow cooling (TAUTP = 4.0−2.0), 11000−13000 steps of faster cooling (TAUTP = 1.0), and 13000−15000 steps of fast cooling, like a minimization (TAUTP = 0.5−0.05); in steps 0−3000, the restraints were slowly increased from 10 to 100% of its final values. A cutoff value was set to 15 Å. From the resulting 10 structures, two were chosen that yielded best fitting of the 13 constraints from intermolecular cross-peaks between dumbbell and genistein. Both structures represent the situation where genistein is intercalating into the nick. Both of these structures, and eight additional, based on these two, in which genistein was modified to model another orientation of a ligand within the nick, were chosen for further calculations. The whole procedure of MARDIGRAS and simulated annealing calculations was repeated on these structures. Finally, 10 structures were subjected to 10 ns molecular dynamic run in explicit water, relevant trajectories were averaged, and energy was minimized. From these, the best four final structures were chosen that yielded a best fit to the 13 experimental restraints (Table 2S and Figure 6S in the Supporting Information).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NOESY spectra of a dumbbell in the presence of bisdimethylamino genistein, DNA and bisdimethylamino genistein chemical shifts at 2 °C from NOESY spectrum, intermolecular cross-peaks, computed structures of a dumbbell/bisdimethylamino genistein complex, and dilution curve of bisdimethylamino genistein in water solution. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

NOESY, nuclear Overhauser spectroscopy; PFGSE, pulsed field gradient spin echo; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation

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