Interaction of flavonoid topoisomerase I and II inhibitors with DNA oligomers†

Wojciech Bocian,*[b] Robert Kawęcki,[a] Elżbieta Bednarek,[b] Jerzy Sitkowski,[ab] Agnieszka Ulkowska[b] and Lech Kozerski*[ab]

Received (in Montpellier, France) 5th December 2005, Accepted 9th January 2006
First published as an Advance Article on the web 1st February 2006
DOI: 10.1039/b517245b

The binding affinities of flavonoids, genistein and quercetin, to DNA oligomers have been established by means of studying by NMR the diffusion coefficients of these compounds with and without the presence of DNA. Genistein was found to bind very weakly, $K_a = 1.54 \times 10^2$ M^{-1}, as compared to quercetin, $K_a = 5.75 \times 10^3$ M^{-1} and luteolin, $K_a = 2.17 \times 10^4$ M^{-1} (as reported in the literature). In the case of genistein a hydrogen bond between the $NH^{F,B}$ cytidine protons of the edge base pair and a genistein hydroxyl is proposed, based on the shape of the DOSY spectrum. MP2 and DFT calculations of genistein show a 9 kcal mol^{-1} excess energy of the planar conformation as compared with a twisted one. For this reason intercalation of the planar genistein into the base pairs is rather unlikely.

Introduction

A wide interest is observed in new classes of compounds in the pursuit to find a lead drug and better formulate an anticancer drug pharmacophore.[1–3] Topoisomerase I and II inhibitors attract much attention in biomedical research since they constitute important chemotherapeutic routes in combating cancer.[4] Along with other classes of chemical compounds,[5] naturally occurring flavone derivatives and their isosteres are intensely studied in this direction.[2,3] Diverse, wide biological activity is expressed by genistein, **1**,[6–10] luteolin, **2**,[11] and quercetin, **3**.[12] Genistein, in particular, is recognized as an inhibitor of tyrosine-specific protein kinase,[13] a topoisomerase II cleaving agent[14,15] and an immunosupressant *in vivo*.[16]

Luteolin was recently shown[11] to intercalate into DNA at high concentration (> 250 μM), resembling the camptothecin family mode of binding the DNA.[17–19] This finding supports the earlier suggestion[20] that flavonoids may fall into two categories as regards the DNA binding, *i.e.* intercalators and minor groove binders. Earlier reports seem to indicate that quercetin has low affinity to DNA,[21] as compared with typical intercalators (μM range of K_D value), and does not possess any preferred sites of binding in DNA.[22] To the contrary, recent studies claim that quercetin binds to the 24 kDa fragment of gyrase B of *Escherichia coli* with a K_D value of 15 μM[23] or even binds covalently to DNA.[24]

Although a vast amount of biochemical literature exists regarding the biochemical studies on a cell level, scarce information is available concerning the mechanism of action of this class of compounds on an atomic coordinate level. With respect to that, intramolecular properties of genistein complexes with amines[25–27] and other flavonoids were recently studied in order to establish the electronic distribution in the genistein moiety and evaluate the hydrogen bonding properties of the hydroxyl groups.

In view of the faint and contradictory evidence in the literature the present work was undertaken to gain more information on an atomic level regarding the interaction with DNA which is an important factor governing the flavonoids' activity in poisoning the enzymic action of topoisomerases.

The main goal of this work was the evaluation of binding affinities of the studied flavonoids to the DNA oligomers and defining the other sources of spectral information which may aid the understanding of the mode of binding.

Experimental

Materials and procedures

Genistein, **1**, was synthesized according to published procedure and used as purified in the synthesis. Luteolin, **2**, was purchased from Lancaster and was recrystallized from a methanol–chloroform mixture. Quercetin, **3**, was crystallized from an acetone–chloroform mixture.

DNA purification. The oligonucleotides $d(GCGTACGC)_2$ and $d(GCGATCGC)_2$ were purchased from Microsynth (Switzerland) and purified by ion-exchange chromatography on a HiTrap™-Q column (Pharmacia Biotech) using gradient elution with ammonium bicarbonate solution (0.1 M–0.9 M) and desalted on Sephadex G-10. Paramagnetic impurities were removed by treatment with Chelex 100 resin purchased from Bio-Rat Laboratories.

The NMR sample was prepared as described below.

Sample preparation. To overcome the problem of low solubility of flavonoids in water the DNA was dissolved in 500 μL of phosphate buffer, 50 mM in $K_3PO_4 \times H_2O$ and

[a] *Institute of Organic Chemistry, Polish Academy of Sciences, Kasprzaka 44, 01-224 Warsaw, Poland. E-mail: lkoz@icho.edu.pl; Fax: +48(22) 632 66 81; Tel: +48(22) 343 2018*
[b] *National Institute of Public Health, Chełmska 30/34, 00-725 Warsaw, Poland. E-mail: wbocian@il.waw.pl*

† Electronic supplementary information (ESI) available: Fig. 1S–3S and Table 1S. See DOI: 10.1039/b517245b

50 mM in NaCl, the pH was adjusted (*ca.* 8) and up to 150 μL of DMSO-d_6 (99.95% D content) was added to avoid precipitation of the added stock solution of flavonoid in neat DMSO-d_6. Aliquots of 10 μL of stock flavonoid solution were added to a 0.6–1.4 mM DNA solution to achieve the desired DNA–flavonoid ratio (1 : 1 or 1 : 2) using 50 μL stock solution. The resultant solution had at most 28% of DMSO. The dilution of the DNA solution in phosphate buffer by DMSO was performed and the resultant spectra analyzed to check the duplex formation was unaffected by organic solvent (Fig. 3S, supporting information†). A DNMR (dynamic nuclear magnetic resonance) study was also performed to check the stability of the duplex (Fig. 2S, Table 1S, supporting information†).

The samples of **2** are stable, even at pH 8.25, for only few hours, so that only titration could be performed and a diffusion coefficient experiment, lasting overnight, could not be acquired. Apparently **2** is too unstable to make any conclusions about its interaction with DNA from these types of study and therefore it is omitted from the discussion. On the other hand, a sample of **3** had enough solubility at pH 6.0, so that the increased stability of the sample at this pH allowed the running of the diffusion coefficient experiment. Although the concentration of DNA in the solution of **3** is half of that in **1**, nevertheless the ratio of ligand : DNA remains 2 : 1, which enables comparison of the binding affinities.

NMR spectra

The NMR spectra were run on a Varian 500 MHz INOVA spectrometer operating at 499 807 MHz for ^{1}H observation. The spectrometer was equipped with a Nalorac Z-spec IDG inverse detection probe. Calibration of ^{1}H chemical shifts was based on internal standard 3-(trimethylsilyl)propionic acid (TSPA).

NOESY spectra[28]. NOESY spectra were recorded in phase-sensitive mode,[29,30] with a 10 000 Hz spectral window in both dimensions, 4096 data points in f_2, 64 scans for each of 512 × 2 increments in f_1, a 250 ms mixing time and a 2 s delay before each scan. A z gradient (1 Gauss cm^{-1}, 5 ms) was used at the beginning of t_1 evolution. Spectra were apodized in both dimensions using a squared cosine bell. Linear prediction was applied in the f_1 dimension to extend the data twice, with zero filling to yield 4096 data points in the f_1 dimension. A binomial sequence was used to suppress the solvent resonance. To minimize zero quantum coherence peaks a small random variation in the mixing time between transients and between t_1 increments was used.[31]

TOCSY spectra[32]. TOCSY spectra with water suppression by gradient echo and DIPSI spinlock were acquired with a 10 000 Hz spectral window in both dimensions, 2048 data points, 16 transients for each of 512 × 2 increments in f_1 with a relaxation delay of 1.5 s. The mixing times for TOCSY spectra were 20 and 80 ms with a spin-lock field of 8000 Hz.

Pulsed gradient spin echo (PGSE). PGSE experiments for compounds **1** and **3** were done according to the following conditions: 24 spectra were acquired using the BPPSTE[33,34] (stimulated echo sequence incorporating bipolar gradients) sequence modified with binomial water suppression. The gradient strengths were incremented as a square dependence in the range from 1 to 60 G cm^{-1}. The diffusion time (Δ) and the duration of the magnetic field gradients (δ) were 170 ms and 2 ms, respectively. Other parameters include the following: a sweep width of 6000 Hz, 32 K data points, 1024 transients and an acquisition time of 1.6 s and relaxation delay of 3 s. The data were processed using Varian DOSY[35] and DECRA[36,37] packages.

Dissociation constant. The dissociation constant, K_D, is extracted from these experiments as follows: given the initial concentrations of DNA and genistein c_{DNA} and c_{GEN}, the diffusion constant $D_{DNA/GEN}$, m^2 s^{-1}, for the complex DNA/GEN, the observed average diffusion constant D_{OBS} and diffusion constants, D_{DNA} and D_{GEN}, for free DNA and genistein, respectively, the population of the complex may be expressed as:

$$P_{DNA/GEN} = D_{GEN} - D_{OBS}/D_{GEN} - D_{DNA/GEN.}$$

The K_D value is given as

$$K_D = [DNA][GEN]/[DNA/GEN]$$

and

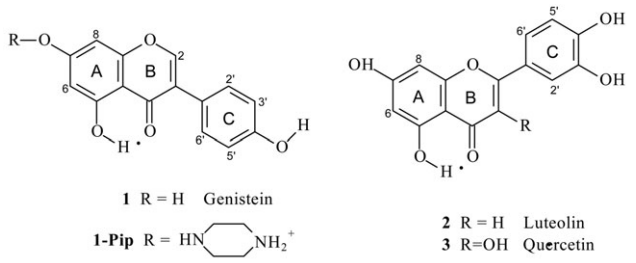
$$K_D = (c_{DNA} - [DNA/GEN])(c_{GEN} - [DNA/GEN])/[DNA/GEN]$$

where $[DNA/GEN] = P_{DNA/GEN}c_{GEN}$. This formal treatment of the data includes a simplification expressed as $D_{DNA/GEN} \cong D_{DNA}$ which is valid when there is a large difference between the diffusion constants of interacting species as is the case here.

Diffusion experiments. PGSE diffusion experiments for a piperazine complex with genistein (**1-Pip**) were run in D_2O solution. The reference sample for **1-Pip** was composed of 400 μl D_2O, 20 μl stock solution of **1-Pip** in DMSO-d_6 and 80 μl of neat DMSO-d_6 to dissolve all **1-Pip**. The sample of **1-Pip** with DNA, d(GCGTACGC)$_2$, was run in 400 μl D_2O, 60 μl DMSO plus 38 mM NaCl, using 1.4 mM DNA and 1.15 mM **1-Pip** from 40 μl of a stock solution in DMSO-d_6. 1D NMR diffusion experiments were run on a Bruker DRX 500 MHz spectrometer. The FIDs were acquired over 12 500 Hz with a 90° pulse width, using 0.6 s acquisition, and a 1 s delay after each of 64 scans. A sine-shaped gradient pulse was incremented from 0 to 45 G cm^{-1}.

Calculations

Calculations were carried out using the Gaussian 98 program package.[38] Geometry optimizations were performed using Hartree Fock (HF) and density functional (DFT) methods with the B3LYP exchange functional and the split-valence basis sets: 6-31G, 6-31G(d) and 6-31G(d,p). The full geometry optimizations of genistein were done for two structures representing two distinct minimum energy points during rotation along the C3–C1′ bond[25] and additionally two optimizations were performed with the C2–C3–C1′–C6′ angle constrained to 0 and 180°, representing fully flat structures. The single-point energy calculations were performed additionally for each



Scheme 1

structure at the MP2 level with the 6-31G(d,p) and 6-311G(d,p) basis sets. The zero-point energy (ZPE) corrections were calculated at the B3LYP/6-31G(d) level and with the scaling factor 0.9804, to account for the effects of molecular vibrations at 0 K.

Results and discussion

Scheme 1 shows the compounds discussed in this work. The interaction of these molecules was studied with the duplexes $d(GCGTACGC)_2$ and $d(GCGATCGC)_2$.

Estimation of DNA binding affinity

The pulsed gradient spin echo (PGSE) experiment is especially sensitive for measuring the weak binding of a molecule of low molecular weight, such as the studied flavonoids ($M_w \approx 400$ Da) to a molecule of much higher molecular weight (DNA, $M_w \approx 2.8$ kDa). Applications of the method to solute binding have been reviewed.[39] Treatment of chemical exchange phenomena has also been published.[40]

In order to evaluate the binding affinities of the studied flavonoids to DNA we have performed a series of PGSE experiments. Titration experiments of DNA with flavonoids were performed to observe the mutually induced shifts and NOESY spectra were run to observe the possible dipolar contacts.

Fig. 1 shows a PGSE plot of **1-Pip** and **1-Pip** in the presence of $d(GCGTACGC)_2$. Data for linear correlations presented in

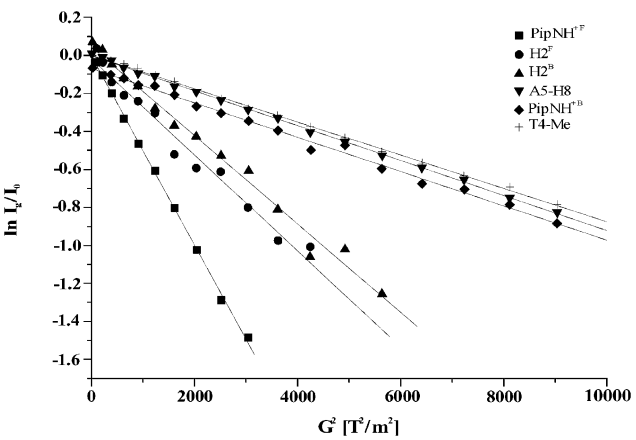


Fig. 1 A PGSE plot of **1-Pip** and **1-Pip** in the presence of $d(GCGTACGC)_2$.

Table 1 Gradient strength dependence of solute proton intensities in absence (^{F}) and presence (^{B}) of $[d(GCGTACGC)_2]^a$

Signal observed	Slope[b]	Correlation coefficient R
$PipNH^{+F}$	$-5.0 \times 10^{-4} \pm 6.2 \times 10^{-6}$	0.9997
$H2^F$	$-2.5 \times 10^{-4} \pm 1.0 \times 10^{-5}$	0.9877
$H2^B$	$-2.3 \times 10^{-4} \pm 7.5 \times 10^{-6}$	0.9938
A5-H8 (DNA)	$-0.9 \times 10^{-4} \pm 7.5 \times 10^{-7}$	0.9995
$PipNH^{+B}$	$-0.9 \times 10^{-4} \pm 1.5 \times 10^{-6}$	0.9972
$T4\text{-}CH_3$ (DNA)	$-0.9 \times 10^{-4} \pm 8.5 \times 10^{-7}$	0.9992

[a] Signals marked B in superscript refer to the solution with DNA present, signals marked F in superscript refer to free genistein anion and piperazine cation $PipNH^+$. [b] Slope and correlation coefficient R from least squares fit of the lines shown in Fig. 1.

Fig. 1 are collected in Table 1. Following published procedures[41,42] we have calculated the binding constant for **1-Pip** to DNA oligomer as 1.01×10^2 M^{-1} (Table 2).

We have also used the automated program DECRA, implemented in the Varian software package, to process the PGSE spectra of **1** and **3**. The data are shown in Table 2 and the example spectrum is shown in the supplementary material (Fig. 1S).†

The instability of flavonoids **2** and **3**, even at high pH, does not allow their comparison with genistein, **1**, at exactly the same conditions. For luteolin **2**, the PGSE experiment could not even be performed in a reproducible manner. We therefore, relate our results for **1** and **3**, to the literature data which used other techniques and DNA sources. In the case of **3** this is hardly even possible as the contradictory conclusions from biological results predict either no specific interaction or covalent bonding (*vide* introduction). Nevertheless, excluding the ambiguous literature information on **3**, our results are consistent and predict much weaker binding of genistein as compared to luteolin or quercetin, whose binding affinities are at least an order of magnitude larger.

Thus the data in Table 2 show that the affinity of genistein to DNA is much weaker than those of the other flavonoids. The genistein moiety in a labile piperazine complex, **1-Pip**, which is better soluble in water than **1**, binds with the same strength as genistein itself, the piperazine cation being strongly bound in a DNA groove, as judged by data in Table 1 and Fig. 1. A possible explanation for the weak genistein binding is the overall non-planarity of the genistein moiety as compared to other flavonoids. This is judged from the X-ray structure comparison outlined below.

The X-ray structures of genistein[43] and its complexes with bases, morpholine[25] and piperazine, **1-Pip**,[27] show that ring C is not coplanar with rings A and B and is twisted from their plane by *ca.* 57°. In addition, the strong intramolecular hydrogen bond of the C-5 hydroxyl to the C-4 carbonyl group adds a six-membered ring moiety to the structure. While the latter property would suggest the molecule was prone to intercalation the overall non-planarity creates steric hindrance to intercalation and hence one may anticipate that the molecule will behave rather as minor groove binder. The X-ray structure of quercetin[44] shows the planar arrangements of all

Table 2 The PGSE data for the binding of **1** to d(GCGATCGC)$_2$ and **1-Pip**, **2** and **3**, to the d(GCGTACGC)$_2$ octamer duplexes[a]

Ligand	Concentration [mM]	D_i(free ligand) $\times 10^{-10}$[m^2 s^{-1}]	D_i(ligand)[b] $\times 10^{-10}$[m^2 s^{-1}]	D_i(DNA)[c] $\times 10^{-10}$[m^2 s^{-1}]	K_a [M^{-1}]	Signal observed
1-Pip	C_{DNA}, 1.4, C_{LIG}, 1.15	—	—	—	1.01×10^2	H-2
1	C_{DNA}, 1.63, C_{LIG}, 3.26	3.54 ± 0.05	3.13 ± 0.10	1.1	1.54×10^2	H-3′
2	—	—	—	—	(2.17×10^4)[d]	—
3	C_{DNA}, 0.675, C_{LIG}, 1.35	2.63 ± 0.05	1.90 ± 0.10	0.85	5.75×10^3 (6.7×10^4)[e]	H-6′

[a] D_i denotes diffusion coefficient, K_a is a binding constant. In the case of **1** and **3** only these ligand signals were used for the K_a calculation, which have no overlap with the other signals in the spectrum and therefore could secure calculation of the true binding constant in the PGSE experiment (see also Fig. 2). [b] Diffusion coefficient for ligand in the presence of DNA. [c] Diffusion coefficient for DNA in the presence of ligand. [d] Binding constant established from fluorescence quenching, ref. 9. [e] Binding constant to 24 kDa fragment of DNA gyrase of *Escherichia coli* established by fluorescence quenching, ref. 22.

aromatic rings and therefore, based on the above argument, one could predict the intercalation in this case as more likely to occur.

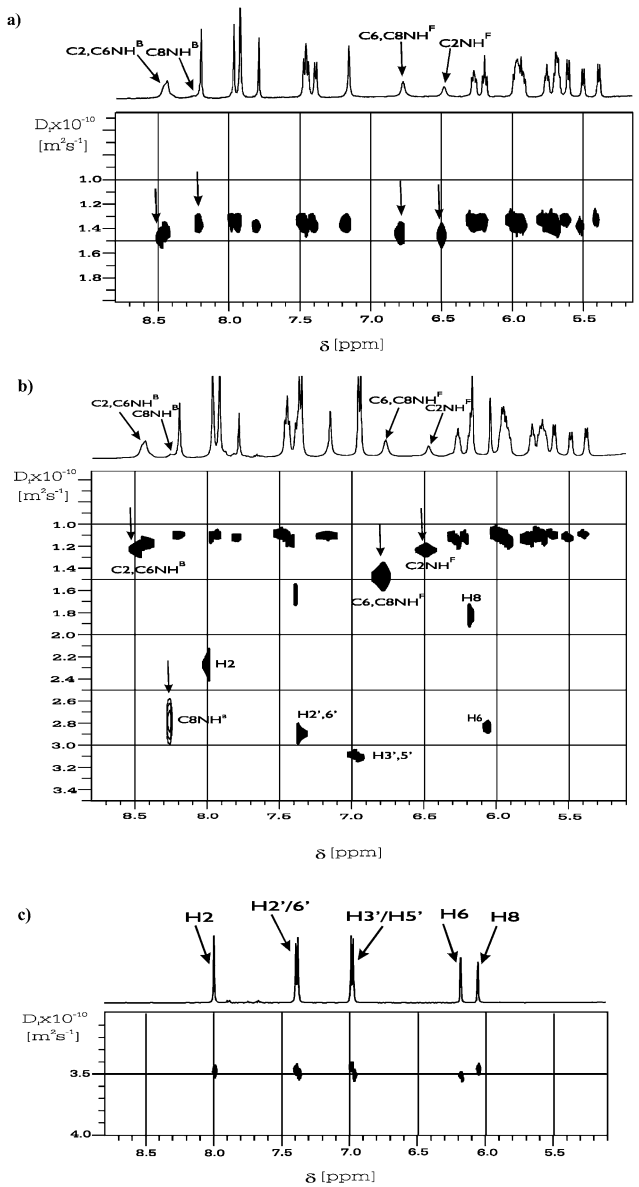
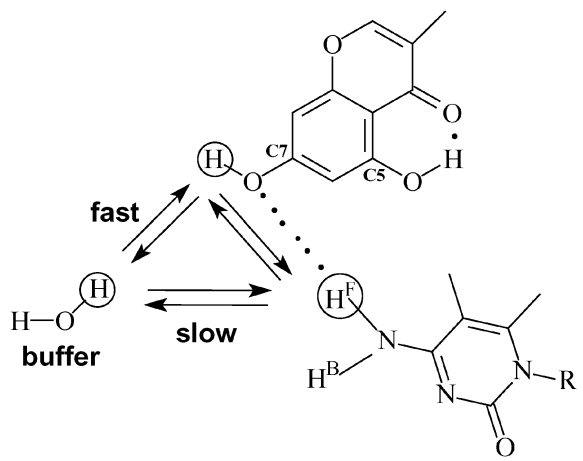


Fig. 2 DOSY spectra, from top to bottom, of free DNA oligomer (a), in the presence of genistein (b) and of the free genistein (c), showing diffusion constant changes on binding of the two species in solution.

Genistein–DNA interaction

The genistein is very weakly bound to a duplex DNA oligomer and not unexpectedly intermolecular crosspeaks were not found in the NOESY spectrum, thus excluding the possibility of more detailed structure elucidation. Also, due to the low equilibrium concentration of a complex in a fast exchange with unbound DNA, negligible changes of ribose and imino proton chemical shifts were observed. Nevertheless, the PGSE experiment performed on genistein–octamer DNA brings rarely observed information which hints at the possible binding mode. As we pointed out in our recent account,[27] the hydroxyl groups of ligands are of primary importance since they form hydrogen bonds with the biomolecule which stabilize the complex. Due to fast exchange with water in a buffer, especially at high pH, as in the present case for OH phenolic groups of genistein, they are not seen and their dipolar contacts are not easy to establish. However, in fortunate cases their interaction with DNA can be traced in a diffusion scale of the NMR experiment.

In Fig. 2 DOSY spectra of the DNA oligomer, d(GC^{2}GATC^{6}GC^8)$_2$, free and in the presence of genistein, are compared. It can be seen that in the spectrum of the free DNA duplex the cytidine NH^F and NH^B water exchangeable protons have larger, compared to the rest of the DNA signals, diffusion coefficients but the diffusion coefficients are uniform for the C-2, C-6 and C-8 bases. This is due to averaging of the diffusion coefficients of NH and bulk H_2O. The exchange is



Scheme 2

Table 3 Comparison of calculated energies for genistein twisted and coplanar structures

Method	C2–C3–C1′–C6′ angle[a]	Electronic energy (hartree)	Δ Energy (kcal mol^{-1})
HF/6-31G//HF/6-31G	46.44°	−947.77127	4.58
	0°	−947.76398	
	132.63°	−947.76851	4.84
	180°	−947.76080	
B3LYP/6-31G(d)//B3LYP/6-31G(d)	38.37°	−953.73174 (ZPE = 0.21761)	7.39 (7.74)[b]
	0°	−953.71996 (ZPE = 0.21818)	
	139.56°	−953.73135 (ZPE = 0.21756)	8.76 (9.14)[b]
	180°	−953.71739 (ZPE = 0.21818)	
B3LYP/6-31G(d,p)//B3LYP/6-31G(d,p)	37.79°	−953.75985	7.11
	0°	−953.74851	
	141.27°	−953.75945	8.48
	180°	−953.74594	
MP2/6-31G(d,p)//B3LYP/6-31G(d,p)	37.79°	−951.02030	8.84
	0°	−951.00621	
	141.27°	−951.01998	10.39
	180°	−951.00342	
MP2/6-311G(d,p)//B3LYP/6-31G(d,p)	37.79°	−951.38690	8.74
	0°	−951.37298	
	141.27°	−951.38661	10.28
	180°	−951.37022	

[a] Two dihedral angles are considered for planar and non-planar molecules allowing 4′OH group orientation. [b] Electronic energy with the zero-point energy (ZPE) correction.

slow on the diffusion time scale as the NH signals remain close to DNA signals. In the presence of genistein, the NH signals of C-2 and C-6 remain in their positions, characteristic for a free DNA duplex, but the NH^F and NH^B signals of the C-8 base have much larger diffusion coefficients. The larger diffusion coefficients for C-8 are suggested to originate from hydrogen bonding of the genistein OH phenolic proton and NH^F proton of the C-8 base forming three site exchange as shown in Scheme 2. The C-7-OH phenolic group in **1** is a more likely candidate for this interaction than C-4′-OH as the former is more acidic.

Taking into account the above experiment one can confirm a weak but specific interaction of genistein established here with DNA (*vide infra*). The non-planarity of the phenyl ring and lack of DNA induced shifts also may hint at a different, compared to **2** or **3**, binding mode, likely to be a minor groove positioning of the benzopyran part with the phenyl ring sticking outside. The lack of experimental restraints precludes more detailed geometry design *via* molecular modelling in this case and precludes the possibility of the visualization of a complex.

Therefore we have also carried out calculations of the energy necessary to flatten the genistein structure by rotating along the C3–C1′ bond, because, as established by X-ray[25] and computer calculations, the most stable are conformations twisted from the plane. Our computation at the MP2 and DFT levels estimates this energy as 9 kcal mol^{-1} (Table 3). That is an amount of excess energy of the DNA–genistein molecular complex over the other binding modes, which should be compensated if the genistein were intercalating or stacking in a planar conformation. It is therefore rather unlikely to assume the intercalation as a preferred binding mode in comparison to other geometries which allow the twist along C3–C1′ bond.

Conclusion

In this work we have established a binding affinity of flavonoids, genistein and quercetin, to DNA duplexes by means of studying by NMR the diffusion constants of these compounds with and without the presence of DNA. All studied compounds are shown to bind the DNA oligomer and the DNA affinities are comparable for luteolin (taking into account the literature data) and quercetin, but the affinity of genistein is much smaller than for the other compounds. In the case of genistein a hydrogen bond of the $NH^{F,B}$ protons of the cytidine in the edge base pair to a genistein hydroxyl is proposed, based on the shape of the DOSY spectrum. The MP2 and DFT calculations reveal *ca.* 9 kcal mol^{-1} energy differences between coplanar and twisted genistein forms, the latter being more stable. This amount of energy should be compensated if the genistein were intercalating in a planar conformation. For this reason stacking inside the stem of the base pair is rather unlikely to happen in appreciable population.

Acknowledgements

The authors acknowledge the help of W. Koźmiński, Dr Sc., Warsaw University for the modification of pulse sequences for PGSE experiments performed in H_2O.

References

1 (*a*) K. Cheng, N. J. Rahler, B. M. Eisenhauer, R. Gao, S. J. Thomas and S. M. Hecht, *J. Am. Chem. Soc.*, 2005, **127**, 838; (*b*) A. Cagir, S. H. Jones, R. Gao, B. M. Eisenhauer and S. M. Hecht, *J. Am. Chem. Soc.*, 2003, **125**, 13628.
2 P. Pourquier and Y. Pommier, *Adv. Cancer Res.*, 2001, **80** 189–216.

3 G. Capranico, M. Palumbo, S. Tinelli, M. Mabilia, A. Pozzan and F. Zunino, *J. Mol. Biol.*, 1994, **235**, 1218.
4 M. Palumbo, B. Gatto and C. Sissi, in *DNA Topoisomerase-targeted Drugs in DNA and RNA Binders, From Small Molecules to Drugs*, ed. M. Demeunynck, C. Bailly and W. D. Wilson, Wiley, Weinheim, 2002, ch. 19, p. 503.
5 (*a*) F. Zunino and G. Pratesi, *Expert Opin. Invest. Drugs*, 2004, **13**, 269; (*b*) J. F. Pizzolato and L. B. Saltz, *Lancet*, 2003, **361**, 2235; (*c*) H.-K. Wang, S. L. Morris-Natschke and K.-H. Lee, *Med. Res. Rev.*, 1997, **17**, 367.
6 K. Polkowski and A. P. Mazurek, *Acta Pol. Pharm.-Drug Res.*., 2000, **57**, 135.
7 P. Fiedor, L. Kozerski, J. Cz. Dobrowolski, R. Kawęcki, K. Biniecki, J. Pachecka, W. Rowiński and A. P. Mazurek, *Transplant. Proc.*, 1998, **30**, 357.
8 A. P. Mazurek, K. Biniecki and L. Kozerski, *US Pat.*, 5637703, June 10, 1997.
9 F. M. Uckun, W. E. Evans, C. J. Forsyth, K. G. Waddick, L. T. Åhlgren, L. M. Chelström, A. Burkhardt, J. Bolen and D. E. Myers, *Science*, 1995, **267**, 886.
10 R. A. Dixon and D. Ferreira, *Phytochemistry*, 2002, **60**, 205.
11 A. R. Chowdhury, S. Sharma, S. Mandal, A. Goswami, S. Mukhopadhyay and H. K. Majumder, *Biochem. J.*, 2002, **366**, 653.
12 L. F. Bjeldanes and G. W. Chang, *Science*, 1977, **197**, 477.
13 T. Akiyama, J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya and Y. Fukami, *J. Biol. Chem.*, 1987, **262**, 5592.
14 J. Markovits, S. Junqua, F. Goldwasser, A.-M. Venuat, C. Luccioni, J. Beaumatin, J.-M. Saucier, A. Bernheim and A. J. Sablon, *Biochem. Pharmacol.*, 1995, **50**, 177.
15 A. Constantinou, K. Kiguchi and E. Huberman, *Cancer Res.*, 1990, **50**, 2618.
16 P. S. Fiedor, M. Maksymowicz, K. Biniecki, L. Kozerski, R. Kawęcki, J. Cz. Dobrowolski, J. Pachecka, P. Aranjo, W. Rowiński and A. P. Mazurek, *Congress of European Society for Artificial Organs, Warsaw*, October 17–19, 1996, (Abstract no. 196), p. 3.
17 W. Bocian, R. Kawęcki, E. Bednarek, J. Sitkowski, A. Pietrzyk, M. P. Wiliamson, P. E. Hansen and L. Kozerski, *Chem.–Eur. J.*, 2004, **10**, 5776.
18 B. L. Staker, K. Hjerrild, K. M. D. Feese, C. A. Behnke Jr, A. B. Burgin and L. Stewart, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 15387.
19 G. S. Laco, J. R. Collins, B. T. Luke, H. Kroth, J. M. Sayer, D. M. Jerina and Y. Pommier, *Biochemistry*, 2002, **41**, 1428.
20 Y. Yamashita, S. Kawada and H. Nakano, *Biochem. Pharmacol.*, 1990, **39**, 737.
21 R. Solimani, *Int. J. Biol. Macromol.*, 1996, **18**, 287.
22 M. S. Ahmed, V. Ramesh, J. H. Parish and S. M. Hadi, *Mutagenesis*, 1994, **9**, 193.
23 A. Plaper, M. Golob, I. Hafner, M. Oblak, T. Šolmajer and R. Jerala, *Biochem. Biophys. Res. Commun.*, 2003, **306**, 530.
24 T. Walle, T. S. Vincent and U. K. Walle, *Biochem. Pharmacol.*, 2003, **65**, 1603.
25 A. P. Mazurek, L. Kozerski, J. Sadlej, R. Kawęcki, E. Bednarek, J. Sitkowski, J. Cz. Dobrowolski, J. K. Maurin, K. Biniecki, J. Witowska, P. Fiedor and J. Pachecka, *J. Chem. Soc., Perkin Trans. 2*, 1998, 1223.
26 A. P. Mazurek, J. Cz. Dobrowolski, J. Sadlej, E. Bednarek and L. Kozerski, *J. Mol. Struct.*, 2000, **520**, 45.
27 L. Kozerski, B. Kamieński, R. Kawęcki, Z. Urbańczyk-Lipkowska, W. Bocian, E. Bednarek, J. Sitkowski, K. Zakrzewska, K. Nielsen and P. E. Hansen, *Org. Biomol. Chem.*, 2003, **1**, 3578.
28 J. Jeener, B. H. Meier, P. Bachmann and R. R. Ernst, *J. Chem. Phys.*, 1979, **71**, 4546.
29 G. Bodenhausen, H. Kogler and R. R. Ernst, *J. Magn. Reson.*, 1984, **58**, 370.
30 D. J. States, R. A. Haberkorn and D. J. Ruben, *J. Magn. Reson.*, 1982, **48**, 286.
31 D. Neuhaus and M. P. Williamson, *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, VCH Publishers, New York, 1989, ch. 8.4.
32 J. Cavanagh and M. Rance, *J. Magn. Reson.*, 1992, **96**, 670.
33 G. Wider, V. Dötsch and K. Wüthrich, *J. Magn. Reson., Ser. A*, 1994, **108**, 255.
34 D. Wu, A. Chen and C. S. Johnson Jr., *J. Magn. Reson., Ser. A*, 1995, **115**, 260.
35 C. S. Johnson, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1999, **34** 203.
36 B. Antalek and W. Windig, *J. Am. Chem. Soc.*, 1996, **118**, 10331.
37 W. Windig and B. Antalek, *Chemom. Intell. Lab. Syst.*, 1997, **37**, 241.
38 M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, V. G. Zakrzewski, J. A. Montgomery, Jr., R. E. Stratmann, J. C. Burant, S. Dapprich, J. M. Millam, A. D. Daniels, K. N. Kudin, M. C. Strain, O. Farkas, J. Tomasi, V. Barone, M. Cossi, R. Cammi, B. Mennucci, C. Pomelli, C. Adamo, S. Clifford, J. Ochterski, G. A. Petersson, P. Y. Ayala, Q. Cui, K. Morokuma, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. Cioslowski, J. V. Ortiz, A. G. Baboul, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. Gomperts, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, C. Gonzalez, M. Challacombe, P. M. W. Gill, B. G. Johnson, W. Chen, M. W. Wong, J. L. Andres, M. Head-Gordon, E. S. Replogle and J. A. Pople, *GAUSSIAN 98 (Revision A.7)*, Gaussian, Inc., Pittsburgh, PA, 1998.
39 A. R. Waldeck, P. W. Kuchel, A. J. Lennon and B. E. Chapman, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1997, **30**, 39.
40 E. J. Cabrita and S. Berger, *Magn. Reson. Chem.*, 2002, **40**, S122.
41 A. Gafni and Y. Cohen, *J. Org. Chem.*, 1997, **62**, 120.
42 O. Mayzel and Y. Cohen, *J. Chem. Soc., Chem. Commun.*, 1994, 1901.
43 M. Breton, G. Precigoux, Ch. Courseille and M. Hospital, *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.*, 1975, **31**, 921.
44 G.-Z. Jin, Y. Yamagata and K.-I. Tomita, . *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.*, 1990, **46**, 310.

