

Structure-activity Studies of the Binding of the Flavonoid Scaffold to DNA

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Abstract. *Background: Flavonoids have been shown to have a wide variety of biological activities and proven to be good scaffolds for the design of DNA-binding agents as anticancer therapeutics. Materials and Methods: In structure-activity relationship studies, flavonoid derivatives were designed and synthesised through various organic synthesis protocols, resulting in novel or previously described molecules. These were studied by UV-Vis absorbance and fluorescence spectroscopy as well as competition dialysis for their binding to DNA isoforms. Their cytotoxic potential was assessed using MTS assays on MCF-7 breast cancer and CCRFCM leukaemia cell lines. Results and Conclusion: Introduction of moieties such as chloride, nitrogen, acetoxy and methoxy groups did not help to improve binding affinity, but introduction of tertiary amines improved the binding 1,000-fold due to an improved interaction of the compound with the nucleic acid; replacement of oxygen by sulphur increased the binding 7-fold, possibly because sulphur being less electronegative than oxygen would allow the electrons of the molecule to interact more strongly with the nucleic acid. Inhibition of growth by 50% (IG₅₀) values were moderate in breast and leukaemia cancer cell lines possibly due to the flavonoids interacting with other cellular components besides the nucleic acids.*

Three arene rings connected in varying ways can form planar structures that have the potential to intercalate into DNA and form a stable complex. This principle has led to the design of many intercalators such as acridines (1), acridones (2), xanthenes (3) and others.

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A new group of emerging anticancer agents is based on the naturally occurring flavonoids. Research on their biological activity is extensive, and they not only show anticancer properties on various cancer cell lines (4, 5) but they also show chemopreventative properties (6), the potential for protein inhibition (7, 8) and interruption of different pathways in the cell cascade (9, 10). Compounds such as quercetin, baicalein, baicalin and luteolin have also been studied for their DNA interactions (11, 12); they intercalate the double helix, bind preferentially to higher order DNA structures such as triplexes and tetraplexes (human telomeric sequence), and have binding constants of the order of 10⁴ M⁻¹ (see previous article in this volume).

Studies of flavonoids in the presence of metal ions (13) (and previous article in this volume) reported that flavonoids also interact with metals when binding to DNA, with varying effects depending on the compound and the metal present. For some, binding to DNA is reduced, as the flavonoid complexes only with the metal, whereas in other cases an increase in binding by a factor up to a further 104 has been observed (see previous article in this volume). It is thought that metals bind both to the flavonoid and to the DNA, allowing the flavonoid to intercalate and form a stable complex with the double helix (14, 15).

In this paper we describe the design and synthesis of flavonoid derivatives and a study of their interactions with DNA, with the aim of elucidating binding preferences and cytotoxic potential in an attempt to identify a lead compound for ongoing drug development.

Materials and Methods

Compounds. Chrysin, 3',4'-dihydroxyflavone, flavone, flavanone, luteolin, 7-hydroxyflavone and xanthone were purchased from Sigma-Aldrich (Dorset, UK).

Synthesis. Isoflavone (3-phenyl-4H-chromen-4-one) (**3**) was synthesised following the method of Singh *et al.* (16). Thioflavone (2-phenyl-4H-chromene-4-thione) (**5**) was synthesised from flavone using Lawesson's reagent according to the method of Abdou *et al.* (17). 3-Azaflavone (2-phenyl-4H-benzo[e][1,3]oxazin-4-one) (**6**) was synthesised from salicylamide and benzoyl chloride according to

the method of Kemp and Vellacio (18). Flavone, (2-phenyl-4H-chromen-4-one) (**1**), 6-hydroxyflavone (6-hydroxy-2-phenyl-4H-chromen-4-one) (**11**), 5-hydroxyflavone (5-hydroxy-2-phenyl-4H-chromen-4-one) (**12**), and 3',4'-dichloroflavone (3',4'-dichloro-2-phenyl-4H-chromen-4-one) (**23**) were synthesised by the method of Ares *et al.* (19) 3',5'-Dinitroflavone (3',5'-dinitro-2-phenyl-4H-chromen-4-one) (**21**) was synthesised from 2-hydroxyacetophenone and 3,5-dinitrobenzoyl chloride following the method of Marder *et al.* (20). 5-Hydroxy-8-nitroflavone (5-hydroxy-8-nitro-2-phenyl-4H-chromen-4-one) (**17**) was synthesised following the procedure of Cushman *et al.* (21) 7-Methoxyflavone (7-methoxy-2-phenyl-4H-chromen-4-one) (**14**) and 5-hydroxy-7-methoxyflavone (5-hydroxy-7-methoxy-2-phenyl-4H-chromen-4-one) (**16**) were afforded by methylation of 7-hydroxyflavone and chrysin, respectively, using iodomethane according to the method of Shin *et al.* (22). 5,7-Dimethoxyflavone (5,7-dimethoxy-2-phenyl-4H-chromen-4-one) (**13**) was synthesised from the anion of 5-hydroxy-7-methoxyflavone and iodomethane as reported by Shin *et al.* (22) 5,7-Diacetoxyflavone (5,7-diacetoxy-2-phenyl-4H-chromen-4-one) (**15**) was produced by direct diacetylation of chrysin using acetic anhydride (22).

7-(2-(Dimethylamino)ethoxy)-5-hydroxy-2-phenyl-4H-chromen-4-one (**18**). *N,N*-Dimethylethanolamine (1 equiv) was added to chrysin (6 mmol) in dry THF (30 ml) in the presence of triphenylphosphine (1.1 equiv); sonication at 40 KHz (23) was carried out till the materials were soluble. DIAD (1.3 equivalent) was added dropwise and sonication carried out for 60 min. The solvent was evaporated and the product poured into acidified water (100 ml). Dichloromethane (50 ml) was used to extract the triphenylphosphine oxide and hydrazine-derived by-products. The aqueous layer was neutralized (pH 7), extracted with ethyl acetate (3×20 ml), the extract washed with a saturated solution of NaHCO₃ (3×25 ml), dried with MgSO₄ and the solvent evaporated. The product was purified by silica column using a gradient of hexane:ethyl acetate:isopropanolamine as eluent. ES-MS *m/z* (relative intensity): 325.48 (M+H⁺ 4). ¹H NMR (300 MHz, CDCl₃): 12.5 (s, 1H), 7.8 (m, 2H), 7.4 (m, 3H), 6.6 (s, 1H), 6.4 (d, J 2.2, 1H), 6.3 (d, J 2.2, 1H), 4.07 (t, J 5.6, 2H), 2.7 (t, J 5.7, 2H), 2.3 (s, 6H). ¹³C NMR (300 MHz, CDCl₃): 182.4, 164.7, 164, 162, 158, 132, 131.3, 129, 126.3, 106, 98.6, 93.2, 66.7, 58.0, 45.9. Melting Point (Electrothermal Digital MP apparatus, uncorrected): 126-126.8°C.

7-(3-(Dimethylamino)propoxy)-5-hydroxy-2-phenyl-4H-chromen-4-one (**19**). The above method for compound (**18**) was followed, using *N,N*-dimethylpropanolamine in place of *N,N*-dimethylethanolamine. HRMS MH⁺ found 340.1543, calculated 340.1549. ¹H NMR (300 MHz, CDCl₃): 7.8 (m, 2H), 7.4 (m, 3H), 6.6 (s, 1H), 6.4 (d, J 2.2, 1H), 6.3 (d, J 2.2, 1H), 4.07 (t, J 5.6, 2H), 2.7 (t, J 5.7, 2H), 2.3 (s, 6H), 1.03 (quin., J 5.6, 2H). ¹³C NMR (300 MHz, CDCl₃): 187, 169.7, 168.4, 162.4, 136.5, 133.7, 130.8, 128.7, 126.4, 118.4, 110.2, 103.3, 97.63, 71.53, 60.7, 37.2, 31.9. Elemental analysis (Medac Ltd. Brunel Science Centre, Cooper's Hill Lane, Englefield Green, Egham, Surrey, TW20 0JZ, UK) found: C 69.66%, H 6.15%, N 4.12%, calculated for C₂₀H₂₁NO₄·0.3H₂O: C 69.67%, H 6.31%, N 4.06%.

7-(2-(Morpholinoethoxy)-5-hydroxy-3-phenyl-4H-chromen-4-one (**20**). The method for compound (**18**) was followed, using morpholine in place of *N,N*-dimethylethanolamine. HRMS MH⁺ found 368.1492 calculated 368.1498. ¹H NMR (300 MHz, CDCl₃):

7.8 (dd, J 1.65/2.2, 2H), 7.4 (m, 3H), 6.6 (s, 1H), 6.4 (d, J 2.2, 1H), 6.3 (d, J 2.2, 1H), 4.09 (t, J 5.6, 2H), 3.64 (t, 4H), 2.7 (t, J 5.7, 2H), 2.5 (t, 4.74, 4H). ¹³C NMR (300 MHz, CDCl₃): 182, 164.3, 163.6, 161.6, 157.3, 131.7, 131.5, 130.7, 128.7, 128.3, 128.1, 125.9, 105.4, 98.3, 92.8, 66.4, 53.6, 53.3, 39.9. Elemental analysis (Medac Ltd.) found: C 65.62%, H 5.53%, N 3.82%, calculated for C₂₁H₂₁NO₅·H₂O: C 65.44%, H 6.02%, N 3.63%.

Nucleic acids. Salmon testes DNA (ST-DNA) was purchased from Sigma-Aldrich (Dorset, UK) and used in filtered phosphate buffer (10 mM), EDTA (1 mM), pH 7.4 solutions. The molar concentration, expressed in phosphate base pairs, was determined spectrophotometrically, using an extinction coefficient (M⁻¹ cm⁻¹) of ε_{260 nm}=13200. The polynucleotides poly(dAdT)-poly(dAdT) [or poly(dAdT)₂], poly(dGdC)-poly(dGdC) [or poly(dGdC)₂], poly(dA)-poly(dT) [or poly(dAdT)], poly(dG)-poly(dC) [or poly(dGdC)], and poly(dT) were purchased from Sigma-Aldrich (Dorset, UK) and dissolved in water to generate stock solutions. The molar concentrations were determined spectrophotometrically, using the following extinction coefficients (M⁻¹ cm⁻¹): poly(dAdT)₂ ε_{260 nm}=13100; poly(dGdC)₂ ε_{260 nm}=16800; poly(dAdT) ε_{260 nm}=12000; and poly(dGdC) ε_{253 nm}=14800 (all concentrations expressed in phosphate base pairs); and for polydT ε_{264 nm}=8500 (expressed in single strands). Triplex DNA poly(dA)-poly(dT)-poly(dT) [or polydAdTdT] was prepared using a ratio of 1:1 poly(dA)-poly(dT):poly(dT) in Na₂HPO₄ (20 mM), NaH₂PO₄ (80 mM), NaCl (300 mM), EDTA (0.1 mM). The resulting solution was heated to 95°C, allowed to cool to room temperature over 18 h, and stored at 4°C. The molar concentration, expressed in triplets, was determined spectrophotometrically, using an extinction coefficient (M⁻¹ cm⁻¹) of ε_{260 nm}=17200. Triplex purine G-A-G (G₃A₄G₃; C₃T₄C₃:G₃A₄G₃) was prepared in cacodylate buffer (50 mM) containing MgCl₂ (50 mM), EDTA (0.1 mM) at pH 6.5 from G₃A₄G₃ and C₃T₄C₃ oligonucleotides (purchased from MWG Biotech Ltd., Milton Keynes, UK) using a ratio of 2:1. The solution was heated to 95°C, allowed to cool to room temperature over 18 h, and stored at 4°C. The molar concentration, expressed in bases, was determined spectrophotometrically, using the following extinction coefficients (M⁻¹ cm⁻¹): ε_{255 nm}=11500 for G₃A₄G₃ and ε_{271 nm}=8300 for C₃T₄G₃. Human telomeric sequence A-G DNA (AG₃T₂AG₃T₂AG₃T₂AG₃) was purchased from MWG-Biotech Ltd. The oligonucleotide was dissolved in pH 7.4 buffer containing Na₂HPO₄ (6 mM), NaH₂PO₄ (2 mM), NaCl (185 mM), EDTA (0.1 mM); the solution was heated to 95°C for 10 min, allowed to cool to room temperature over 18 h, and stored at 4°C. The molar concentration, expressed in strands, was determined spectrophotometrically using an extinction coefficient (M⁻¹ cm⁻¹) of ε₂₆₀=73000.

UV-Vis absorption and fluorescence spectroscopy. Stock solutions of flavone derivatives were prepared by dissolving the compounds in DMSO to achieve a final concentration of 10 mM and stored at 4°C. Extinction coefficients for each compound were determined in pH 7.4 phosphate buffer (10 mM) containing EDTA (1 mM).

Absorption and fluorescence spectra were obtained using a UVIKON UV/Visible spectrophotometer (NorthStar Scientific Ltd, UK) and a Fluoromax P fluorimeter (HORIBA, Jovin Yvon, UK). The Beer-Lambert law was obeyed within the concentration ranges used for both drug and DNA solutions. Changes in the UV-Vis absorption spectra during the drug-DNA interaction studies were measured as follows: drug concentrations were adjusted to give an

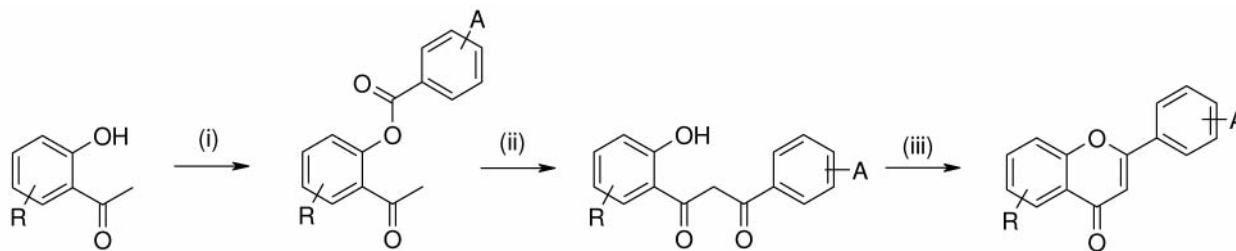


Figure 1. General synthesis of flavones: (i) $ArCOCl$, base; (ii) $KOBu^t$, reflux; (iii) H^+ .

absorbance ~ 0.5 (1 cm pathlength quartz cell), nucleic acid concentrations were increased by adding 2 μ l of the appropriate nucleic acid stock solution. As the concentration of the drug in the cuvette therefore varied, the absorbance was corrected using the following formula: $Abs = Abs_{orig}(1000+V)/1000$; where V is the volume of nucleic acid added. The initial titration point was the UV spectrum of the free drug; experimentally, the final titration point corresponding to the completely bound ligand was obtained by adding volumes of nucleic acid till either no further change in the absorbance was observed or any increase in the absorbance was due solely to the absorbance of the nucleic acids. Generally, absorbance measurements were made at the wavelength maximum of each flavone. For fluorescence measurements the excitation and emission slits were 5 and 20 nm respectively. To obtain binding constants, absorbance or fluorescence data obtained by this method were fitted to the equation (24):

$$Abs_c = \frac{(Abs_f - Abs_o) \left\{ 1 + KnD + KAbs - \sqrt{(1 + KnD + KAbs)^2 - 4KKnDAbs} \right\}}{2KnD} + AbsnD$$

where: Abs_c is the absorbance calculated, Abs_f is absorbance of the fully bound ligand, Abs_o is the absorbance of the free ligand, Abs is the observed absorbance, K is the association binding constant, D is the drug concentration and n is the binding sites per drug molecule. When fluorescence titration data are used, Abs_f represents the value of Fo/F corresponding to the fluorescence (F) of the flavone at the maximum STDNA concentration (drug fully bound), Abs_o corresponds to the value of Fo/F=1 for the free drug. In both situations, the Abs_c , K and n values were obtained by iterative adjustment using the program Origin 6.0 (Microcal, Inc), with the observed UV/fluorescence experimental data plotted *versus* the molar concentration of the nucleic acid.

Competition dialysis. The reservoir contained a pH 7.4 buffer comprising 200 ml of 6 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 185 mM NaCl, 0.1 mM EDTA, and 1 μ M of the test compound. A volume of 0.5 ml of each nucleic acid was pipetted into separate 0.5 ml DispoDialyser® units (SpectraPor, USA), which were placed in the reservoir. All nucleic acid samples were of identical concentration (75 μ M), expressed in terms of the monomeric unit (base pairs for duplex DNA, triplets for triplex DNA and tetrads for tetraplex DNA). The beaker was covered with parafilm, wrapped in foil, and its contents allowed to equilibrate with continuous stirring at 4°C for 24 h. At the end of the equilibration period, nucleic acid samples were carefully removed to microfuge tubes, and were made up to a final concentration of 1% (w/v) sodium dodecyl sulfate by the

addition of appropriate volumes of a 10% (w/v) stock solution. The total concentration of each test compound (Ct) within each dialysis unit was then determined spectrophotometrically using the appropriate extinction coefficient. The free ligand concentration was determined spectrophotometrically using an aliquot of the dialysate solution from the reservoir.

Cells. Clonal populations of MCF-7 and CCRFCM cells were used in these experiments. The cells were routinely grown in RPMI-glutamax (SIGMA) supplemented with 10% and 5%, for each cell type respectively, heat-inactivated foetal calf serum (GIBCO, Invitrogen) at 37°C, and 6% CO_2 in air. The cells were subcultured twice a week.

MTS cell proliferation assay. The Cell Titer 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, cat# G1112) is a colorimetric method for determining the number of viable cells in proliferation. The tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) (MTS) was dissolved in PBS buffer and electron coupling reagent (phenazine methosulfate) (PMS) was added according to the protocol supplied. Cells were grown in 96 well-plates (0.2 ml) to an initial concentration of 1×10^4 cells/ml in the appropriate medium for 24 h at 37°C and 6% CO_2 in air. Incubation periods of 24, 48 and 72 h with 0, 1, 3, 10, 30, 100, 300 and 1,000 μ M of the test compounds then followed. Following the prescribed protocol, 40 μ l of MTS-PMS solution were added to each incubation solution and further incubated at 37°C and 6% CO_2 in air for 2 h prior to reading. Readings were recorded at 490 nm.

Results and Discussion

Chemistry. Figure 1 shows the general pathway followed in the current study for the synthesis of the flavones, while Figure 2 illustrates the chemistry employed to synthesise derivatives of chrysin (for a complete list of compounds see Table I). Synthesis of the flavones by the general method proved unremarkable, except for the systems bearing additional hydroxyl groups. For these, the main product isolated from the first step is an over-benzoylated derivative that interferes with the subsequent rearrangement process in step (ii), and yields of the desired product are typically in the range of 1-2%, even using Jain and Makrandi's approach (25). This problem was resolved, in part, when using hydroxylated 2-hydroxyacetophenones as starting materials,

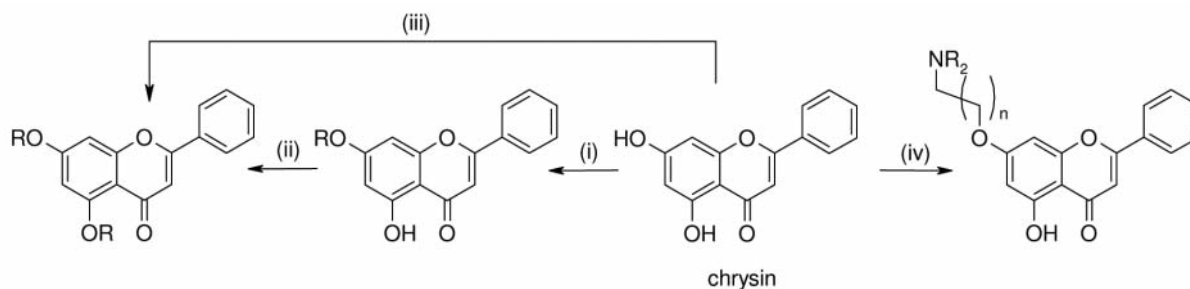


Figure 2. Chemistry employed to synthesise chrysin derivatives: (i) K_2CO_3 , MeI ($R=Me$); (ii) NaH, MeI ($R=Me$); (iii) Ac_2O ($R=Ac$); (iv) $Ph_3P/DIAD$, R_2NH .

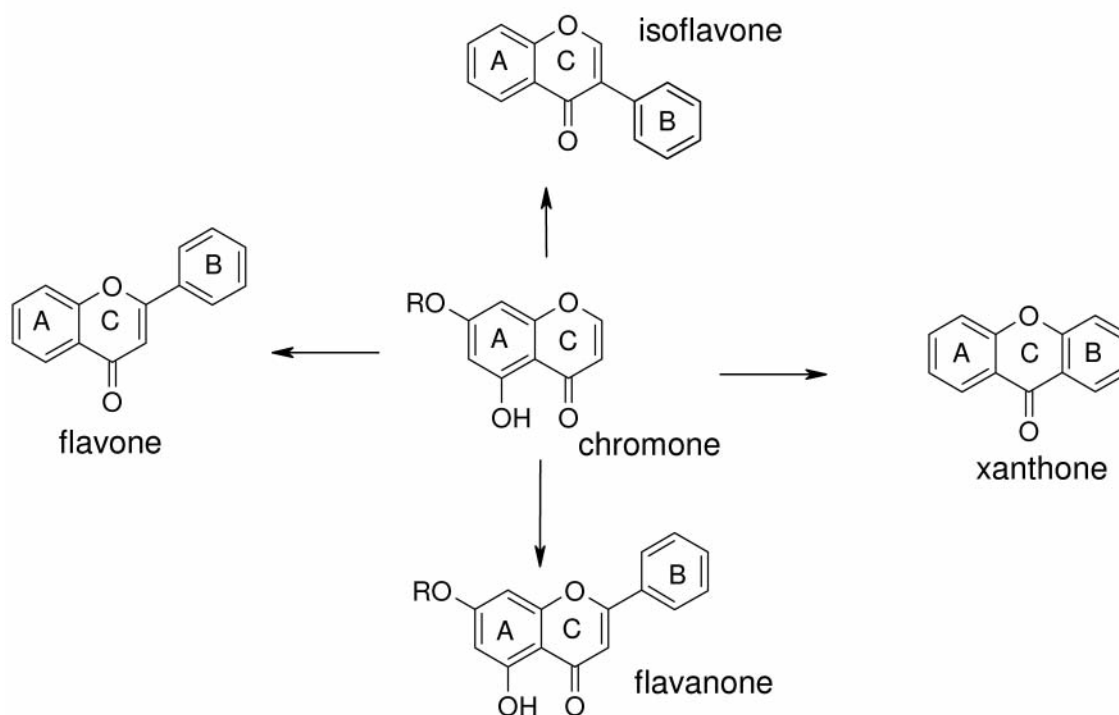


Figure 3. Different chromone possibilities.

by employing 4 equivalents of lithium bis(trimethylsilyl)amide (21). This forms a lithium enolate from the acetyl group in the starting 2-hydroxyacetophenone and treatment of this enolate with 1 equivalent of aroyl chloride directly affords a 1,3-diketone that is subsequently cyclised in acidic conditions. Unfortunately, this methodology proved inconvenient when scaling up, but the use of potassium tert-butoxide at $-78^\circ C$ in place of lithium bis(trimethylsilyl)amide resolved this problem(19) and the procedure was used for synthesizing compounds **11**, **12**, **17**, **22** and **23**.

Alkylation and acylation of chrysin (5,7-dihydroxyflavone), **10**, and 7-hydroxyflavone, **9**, were realized according to Figure 2. The 7-position proved to be easily alkylated, either

using simple methylation conditions or the Mitsunobu reaction; presumably hydrogen-bonding of an OH proton in the 5-position with the carbonyl oxygen atom at position 4 renders this proton less acidic. To alkylate the 5-OH we employed a second step, using the strong base sodium hydride (NaH) in tetrahydrofuran (THF) followed by methylation by MeI. Acetylation of chrysin, using the more reactive acetic anhydride, afforded the disubstituted product directly.

DNA binding properties. To establish whether the compounds are able to intercalate into the double helix, we performed association binding studies of compounds **1** to **23** with Salmon Testes DNA (STDNA) using a variety of

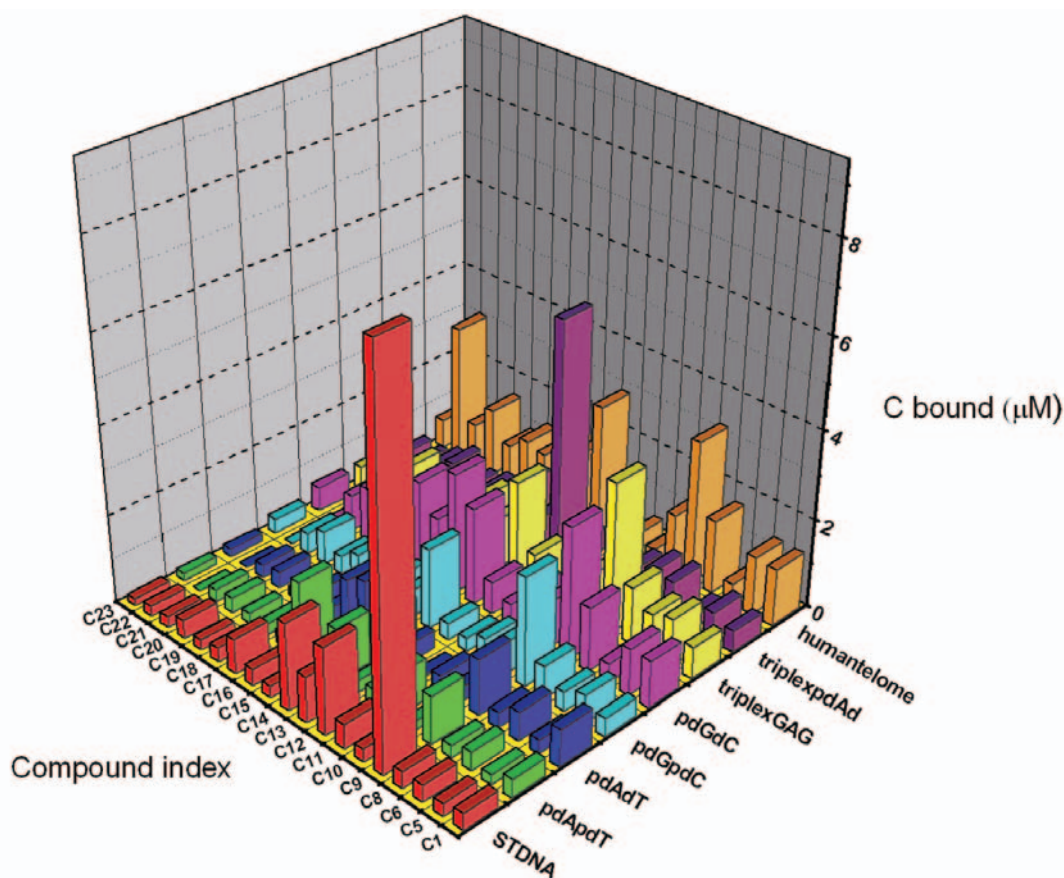


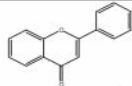
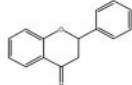
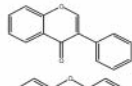
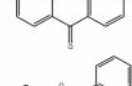
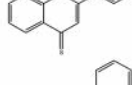
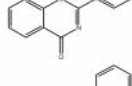
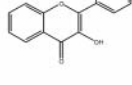
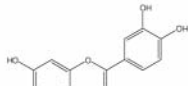
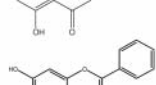
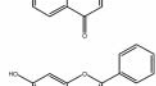
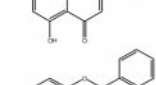
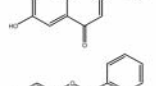
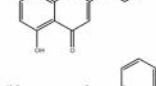
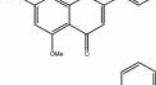
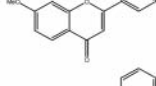
Figure 4. Competition dialysis results, indicating the preference of various compounds for different DNA isoforms.

techniques, such as UV and fluorescence spectroscopy and differential dialysis; the results are reported in Table I. In order to study which flavonoid structure is most suitable for intercalating into the DNA duplex, we first envisaged a model composed by the parent chromone (A and C rings) in which the B ring is appended in different positions and in which the level of saturation of the chromone is varied; see Figure 3. Rings A and C are fused to provide a planar molecule that could intercalate between the stacked nucleic acid bases. Flavone, **1**, has the B ring located at C-2, while isoflavone, **3**, has the B ring at C-3. Xanthone, **4**, has an additional ring fusion at C-2 / C-3, of the chromone system, which maintains a planar system, whereas in the case of flavanone, **2**, a distortion appears as a result of the saturation of the C ring. The binding constants obtained by these four compounds were quite similar and of the order of 10^3 M^{-1} . Flavone was chosen as an appropriate scaffold for further structure-activity related studies. Hydroxyl substituents were found to be important for DNA binding, with the 7-position, as in 7-hydroxyflavone, **9**, and 5,7-dihydroxyflavone, **10**, identified as the most relevant for binding to the double

helix. This was confirmed by the reduction in the binding constant when these hydroxyl groups were substituted, as in 7-methoxyflavone, **14**, 5-hydroxy-7-methoxyflavone, **15**, 5,7-dimethoxyflavone, **13**, and 5,7-diacetoxyflavone, **16**. Other positions for the hydroxyl group were found not to enhance DNA binding, as indicated by the results obtained for 3-hydroxyflavone, **7**, 6-hydroxyflavone, **11**, 5-hydroxyflavone, **12**, 3',4'-dihydroxyflavone, **22**, and luteolin, **8**. The presence of a nitrogen atom at the 3- position, as in compound **6**, appears to have a deleterious affect on DNA binding. Chlorine and nitro substitutions were also tested by using 5-hydroxy-8-nitroflavone, **17**, 3',5'-dinitroflavone, **21**, and 3',4'-dichloroflavone, **23**; these were found not to enhance binding either. However, replacement of the carbonyl oxygen atom with a sulfur atom enhanced the association binding constant by 10-fold, from $1.2 \times 10^3 \text{ M}^{-1}$ for flavone, **1**, to $1.5 \times 10^4 \text{ M}^{-1}$ for thioflavone, **5**.

We envisaged that the presence of a tertiary amine could provide an additional binding environment, as at pH 7.4 such a group would be protonated thereby rendering it more suitable for binding to the negatively charged phosphate

Table I. DNA-binding constants (27) and *n* (number of base pair binding sites per bound molecule) for ST DNA and cytotoxic activities for compounds 1 to 23.

Compound	Structural formula	$K_{\text{ass}}/10^3\text{M}^{-1}$ by UV-Vis	Binding site <i>n</i>	$K_{\text{ass}}/10^3\text{M}^{-1}$ by fluorescence (a)	Binding site <i>n</i>	IG ₅₀ (μM) at 72 hours for MCF7 cell line	IG ₅₀ (μM) at 72 hours for CCRFCEM cell line
1		1.2	2.6	Nio (b)		Promotes growth	> 100
2		1.2	2.5	1.2 (300, 375)	2.04	Not tested	Not tested
3		1.7	10	Nio		Not tested	Not tested
4		2.3	2	2.2 (340, 390)	2.1	Not tested	Not tested
5		Not defined		15.5 (420, 464)	9.3	>100	>100
6		Nio		0.8 (340, 422)	2.1	>100	>100
7		Not soluble in buffer A at a 10% DMSO		Not soluble in buffer A at a 10% DMSO		Not tested	Not tested
8		11.2	1.3	2.2 (340, 383)	4	>100	>100
9		8.13	3.4	Nio		Not tested	Not tested
10		28.5	4	7.5 (340, 442)	3	Not tested	Not tested
11		1.1	2.6	1.2 (420, 481)	2	Not tested	Not tested
12		Nio		Nio		Not tested	Not tested
13		1.4	2.3	1.9 (340, 442)	2	Not tested	Not tested
14		1.5	2	1.6 (340, 534)	2.6	Not tested	Not tested
15		3.2	2.7	1.7 (340, 528)	2	Not tested	Not tested

Compound	Structural formula	$K_{\text{ass}}/10^3\text{M}^{-1}$ by UV-Vis	Binding site n	$K_{\text{ass}}/10^3\text{M}^{-1}$ by fluorescence (a)	Binding site n	IG ₅₀ (μM) at 72 hours for MCF7 cell line	IG ₅₀ (μM) at 72 hours for CCRFCEM cell line
16		0.7	1.7	5.8 (340, 442)	2	Not tested	Not tested
17		Nio		7.84 (340, 383)	4	Promotes growth	>100
18		189.7	2.3	Nio		>100	69.9
19		2.2	1.5	1.8 (420, 490)	3.3	88.6	65.9
20		110.6	0.85	Nio		>100	>100
21		1.1	2.2	1.21 (340, 384)	2	Promotes growth	Promotes growth
22		1.9	7.2	1.2 (320, 467)	3	>100	>100
23		0.7	1.9	1.3 (420, 464)	5.4	>100	>100

(a): Excitation and emission wavelengths (expressed in nm) are shown respectively between brackets. (b): Nio: no interaction observed.

groups of the DNA. Compounds **18–20**, in which such a group is attached *via* an oxygen atom of the 7- position, reveal that the association binding constant can be enhanced by two orders of magnitude, bringing it in the range of 10^5 M^{-1} , provided the tertiary nitrogen atom is only 2 carbon atoms away from the oxygen at position 7 (compounds **18** and **20**), but when the intervening chain is 3 carbon atoms long (compound **19**) the association binding constant drops below that of the parent flavone, *i.e.* ca. $2 \times 10^3\text{ M}^{-1}$.

Cell viability studies. To evaluate the anticancer activity of these compounds, we tested the most relevant against MCF-7 breast cancer and CCRFCM leukaemia cell lines. We studied

Table II. Binding association constants for STDNA and number of base pair binding sites per bound molecule (*n*) for compounds **8**, **9** and **10** in the presence of different cations.

Cation	Luteolin, 8 1:1, $K_{\text{ass}}/10^3\text{M}^{-1}$	7-Hydroxyflavone, 9 1:1, $K_{\text{ass}}/10^3\text{M}^{-1}$	Chrysin, 10 1:1, $K_{\text{ass}}/10^3\text{M}^{-1}$
None	11.2 (1.3)	8.13 (3.4)	28.5 (4)
Cu ²⁺	1.4 (2)	10.5 (2)	137 (2)
Fe ²⁺	1.4 (2.4)	10.9 (2)	590 (4)
Fe ³⁺	1.3 (3)	201 (2)	448 (2.5)
Mg ²⁺	1.5 (3)	1030 (1.4)	1180 (1.5)
Mn ²⁺	1.3 (2)	108 (2)	252 (3)
Zn ²⁺	1.4 (3)	9.9 (2)	74.9 (5)

the concentration causing growth inhibition of 50% of the population of cells (IG_{50}) at 24, 48 and 72 h of incubation with the test compounds at 0, 1, 5, 10, 50, 100, 500 and 1000 μ M. Unfortunately, most of the compounds proved to be ineffective, the majority exhibiting an $IG_{50} > 100$ μ M. Only for 7-dimethylaminoethoxy-5-hydroxyflavone, **18**, and 7-dimethylaminopropoxy-5-hydroxyflavone, **19**, were the IG_{50} values in the order of 65-85 μ M. It is particularly interesting to note that the nitro-substituted compounds **17** and **21** actually promoted growth in these two cell lines (Table I).

Selectivity for DNA isoforms. Using competition dialysis experiments, we studied the binding selectivity of our set of compounds for eight different isoforms of nucleic acids: STDNA, poly(dA)-poly(dT), poly(dAdT)-poly(dAdT), poly(dG)-poly(dC), poly(dGdC)-poly(dGdC), poly(dA)-poly(dT)-poly(dT), purine triplex and G-tetraplex (Figure 4). Flavone, **1**, and thioflavone, **5**, showed preference for G or GC rich sequences, including G-quadruplex, poly(dGdC)-poly(dGdC) and purine triplex (GCG). Similar preferences were observed with 3',4'-dichloroflavone, **23**, 3',4'-dihydroxyflavone, **22**, 3',5'-dinitroflavone, **21**, chrysin, **10**, and luteolin, **8**. Methyl and acetyl derivatives of chrysin exhibited an increased preference for poly(dGdC)-poly(dGdC), poly(dA)-poly(dT) and STDNA over G-quadruplex. In contrast, the addition of a tertiary aminoalkyl group to the oxygen atom at the 7-position in chrysin resulted in retention of the preference for G-quadruplex, poly(dGdC)-poly(dGdC) and purine triplex (GCG). From the competition dialysis experiments, 7-hydroxyflavone, **9**, 7-methoxyflavone, **14**, and 5-hydroxyflavone, **12**, showed a clear preference for STDNA, which presumably implies these compounds do not have a clear selectivity for any base pair sequence in a double helix. 6-Hydroxyflavone, **11**, showed preference for poly(dGdC)-poly(dGdC) and poly(dA)-poly(dT).

Drug-DNA-metal ion interactions. Metal cations have preferences for the bases in the DNA and can modify the nucleic acid conformation from B- to Z- or A-DNA (14). Moreover, drugs can bind to metal ions either directly or indirectly through the molecules of hydration. Thus metal ions may affect the selectivity of the binding molecule for the nucleic acid structure. Yamashita (13) postulated that flavones containing hydroxyl groups in *ortho* positions, or containing a hydroxyl in position 5- together with the carbonyl oxygen at position 4-, can form complexes with copper (II), and this complex would then interact with DNA. Luteolin, **8**, and chrysin, **10**, have hydroxyl groups adjacent to the carbonyl, whereas 7-hydroxyflavone, **9**, does not, which makes it possible to use the latter as a negative control. We analysed the binding associations of these 3 compounds with STDNA in the presence of Fe^{2+} , Fe^{3+} , Cu^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} (as they are the main intracellular cations (26-30)), using a

1:1 ratio of compound:metal ion. Table II shows the results for this study, which indicate that Fe^{3+} , Mg^{2+} and Mn^{2+} increase the binding constants of 7-hydroxyflavone, **9**, for DNA; by one to two orders of magnitude (up to 100-fold) this clearly shows the metals bind to the drug and allow it to interact more strongly with DNA. For chrysin, **10**, Cu^{2+} , Fe^{2+} , Fe^{3+} , Mn^{2+} and Mg^{2+} increase binding by up to two orders of magnitude. In the case of luteolin, **8**, no metal improved the binding of the molecule to DNA. Indeed, the presence of any of the metal ions examined appears to decrease the ability of luteolin to bind to STDNA; by a factor of 10 presumably the presence of the *ortho* hydroxyl groups in the 2-aryl ring is the source of this effect. Taken together, these results indicate that flavones bind preferentially using the 7-hydroxy position and metal cations can markedly enhance this process. The fact that chrysin, **10**, generally exhibits stronger binding than 7-hydroxyflavone, **9**, is probably due to the presence of the 5-hydroxyl in the former, which, together with the carbonyl in the 4-position, allows it to form a bidentate complex with a metal ion

Discussion

Drug design can be employed to understand analogues of drugs which best use a particular feature of the DNA structure to maximise interactions and increase specificity. Rational drug design approaches has allowed us to introduce different moieties into a flavone skeleton, synthesise and study the affinity of these derivatives to DNA. Results have suggested that tertiary amines and hydroxyl groups are the most appropriate groups for improving DNA binding, raising the binding constants to the order of 10^6 M^{-1} . Introduction of chloro- and nitro-groups did not improve the binding to the duplex helix of STDNA, whereas substitutions with methoxy and acetoxy groups reduced the binding constants obtained. Replacement of the carbonyl group by sulfur increased affinity of flavone for DNA by a 7-fold. Competition dialysis experiments showed a remarkable preference for higher order structures of DNA (such as G-quadruplex and triplexes) as the 3 ring structure is probably better accommodated in these structures. Metal cations assist on improving the binding of the drugs to DNA, especially when the later bear hydroxyl groups in position 7. The results of the cell viability assay showed that anticancer activity was not significantly improved by the various substitutions, even though the binding was improved by 1000-fold, suggesting that DNA-binding is not directly related to anticancer activity for these molecules. However, other factors such as metabolism, cellular uptake, drug persistency, cytotoxicity, and possible reactions with cellular components could also be responsible for the apparent lack of anticancer activity.

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