Site-specific DNA cleavage by mammalian DNA topoisomerase II induced by novel flavone and catechin derivatives

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Four naturally occurring flavones (baicalein, quercetin, quercetagenin and myricetin) and two novel catechins [(–)-epicatechin gallate and (–)-epigallocatechin gallate, from the tea plant *Camellia sinensis*], which are known inhibitors of reverse transcriptase, were shown to induce mammalian topoisomerase II-dependent DNA cleavage *in vitro*. The flavones differed from the catechins in causing unwinding of duplex DNA, but both classes of compound induced enzymic DNA breakage at the same sites on DNA. Moreover, the cleavage specificity was the same as that for the known intercalator 4'-(acridin-9-ylamino)methanesulphon-*m*-anisidide, suggesting that these agents trap the same cleavable complex. Analysis of some 30 flavonoid compounds allowed elucidation of the structure–function relationships for topoisomerase II-mediated DNA cleavage. For flavonoid inhibitors an unsaturated double bond between positions 2 and 3 of the pyrone ring and hydroxy groups at the 5, 7, 3' and 4' positions favoured efficient cleavage. Hydroxy substitutions could be tolerated at the 3, 6 and 5' positions. Indeed, the absence of substituents at the 3', 4' and 5' positions could be compensated by a hydroxy group at position 6 (baicalein). Similar requirements have been reported for flavonoid inhibitors of protein kinase C that act competitively with ATP, suggesting interaction with a conserved protein feature. Formation of the cleavable complex is a cytotoxic lesion that may contribute to the growth-inhibitory properties of flavones observed for three human tumour cell lines. These results are discussed in regard to the selectivity of antiviral agents.

INTRODUCTION

Several flavonoid natural products have attracted recent attention as novel inhibitors of virus-associated reverse transcriptase. A number of plant extracts, e.g. the traditional Kampo drug Sho-saiko-to [1], and the plant-derived flavonoids baicalein, quercetin, quercetagenin and myricetin (Fig. 1) have potent inhibitory effects on the reverse transcriptases from Rauscher murine leukaemia virus and human immunodeficiency virus [2,3]. Two catechin compounds, (–)-epicatechin gallate and (–)-epigallocatechin gallate (Fig. 1), isolated from the tea plant *Camellia sinensis* were even more potent [4]. These flavonoid derivatives when tested *in vitro* were also more or less inhibitory to various cellular DNA polymerases and RNA polymerases including eukaryotic DNA polymerases α , β and γ [3–5]. Inhibition of such enzymes *in vivo* could result in toxic side effects. Clearly, an understanding of these interactions with polymerases and other key cellular enzymes will be essential for the design of agents with selective antiretroviral action.

Kinetic studies have provided insight into the mechanisms of enzymic inhibition by flavonoids. The mode of inhibition of flavones and catechins was competitive (Rauscher-murine-leukaemia-virus reverse transcriptase, cellular DNA polymerases) or partially competitive (human-immunodeficiency-virus reverse transcriptase) with respect to the template–primer complex $rA_n \cdot dT_{12-18}$ [3–5]. Thus these compounds could interfere with polymerase activity by binding to the double-stranded nucleic acid template–primer or by competing with it for a site on the enzyme. Baicalein, quercetin, quercetagenin and myricetin are planar aromatic flavone derivatives (confirmed by the crystal structure of quercetin [6]) that could bind DNA by an intercalative mode. These considerations led us to examine the DNA-unwinding properties of flavonoid reverse transcriptase inhibitors.

DNA intercalation could also contribute to the cytotoxicity of

flavonoids. Other intercalators, including antitumour acridines and anthracyclines such as 4'-(acridin-9-ylamino)methanesulphon-*m*-anisidide (*m*-AMSA) are toxic to cells by virtue of their inhibition of DNA topoisomerase II (reviewed in refs. [7] and [8]). This essential replicative enzyme is involved in chromosome segregation via the introduction of transient double-strand breaks in DNA [9,10]. Intercalative and non-intercalative inhibitors of topoisomerase II, e.g. etoposide and teniposide, exert their cytotoxic effects by trapping a topoisomerase II–DNA intermediate, the 'cleavable complex'. Such complexes can be detected *in vitro* by the addition of detergent, thereby inducing cleavage of double-stranded DNA at specific sites [7,8]. We have examined the ability of some 30 flavonoids to induce enzymatic DNA breakage by mammalian topoisomerase II. In addition, the cytotoxic effects of flavonoids have been measured for three different human tumour cell lines.

While our work was in progress, two reports appeared showing that the isoflavone genistein interacts with topoisomerase II [11] and is the active constituent of a *Penicillium* extract that mediated topoisomerase II cleavage of DNA [12].

MATERIALS AND METHODS

Materials

The sources of flavonoid compounds used in this study have been described previously [2–4]. Sho-saiko-to was obtained from Tsumura and Co. (Tokyo, Japan). (–)-Epicatechin gallate and (–)-epigallocatechin gallate were obtained from the Mitsui Norin Food Co. Food Research Laboratories (Fujieda, Japan). *m*-AMSA was from the Drug Synthesis Branch, National Institutes of Health (Bethesda, MD, U.S.A.). Compounds were dissolved in dimethyl sulphoxide (Me_2SO) at 1 mg/ml, stored at –20 °C and diluted in Me_2SO immediately before use. Supercoiled plasmid pBR322 DNA was purified from *Escherichia coli*

Abbreviations used: Me_2SO , dimethyl sulphoxide; *m*-AMSA, 4'-(acridin-9-ylamino)methanesulphon-*m*-anisidide.

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Fig. 1. Structures of flavonoids and related compounds

strain MG1182 and banded twice by CsCl/ethidium bromide density centrifugation as previously described [13]. Kinetoplast DNA from *Crithidia fasciculata* was generously provided by Dr. G. Richie of I.C.I. Pharmaceuticals (Alderley Edge, Cheshire, U.K.). Agarose type II and low-gelling agarose (type VII) were from Sigma Chemical Co. (Poole, Dorset, U.K.). DE81 paper was purchased from Whatman (Maidstone, Kent, U.K.).

Human leukaemic CCRF-CEM cells were provided by Dr. A. P. Johnstone, and Daudi Burkitt lymphoma and HeLa cell lines were from Professor M. J. Clemens, both of the Department of Cellular and Molecular Sciences, St. George's Hospital Medical School (London, U.K.). Non-essential amino acids, glutamine, trypsin and RPMI 1640 medium containing 2 mM-glutamine were purchased from Gibco-BRL (Paisley, Strathclyde, Scotland, U.K.). Minimal Essential Medium with Earle's salts was obtained from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Fetal bovine serum was from Imperial Laboratories (Andover, Hampshire, U.K.).

Restriction enzymes, T4 polynucleotide kinase, Klenow fragment and [γ - 32 P]ATP (3000 Ci/mmol) were from Amersham International (Little Chalfont, Bucks., U.K.). DNA topoisomerases I and II were purified from calf thymus tissue in

St. George's Hospital Medical School [14]; C. A. Austin & L. M. Fisher, unpublished work). Topoisomerase II migrated as a single 140 kDa band on SDS/PAGE gels [15]. One unit of topoisomerase II activity decatenates 0.5 μ g of kinetoplast DNA at 37 °C in 30 min under standard conditions. Calf intestine alkaline phosphatase was from Boehringer (Mannheim, Germany).

Methods

DNA unwinding. Supercoiled pBR322 DNA (2 μ g) was incubated with calf thymus DNA topoisomerase I (20 units) at 37 °C for 30 min in relaxation buffer (100 μ l) containing 50 mM-Tris/HCl buffer, pH 7.5, 120 mM-KCl, 5 mM-dithiothreitol, 0.5 mM- Na_3EDTA , 30 μ g of BSA/ml, various concentrations of the flavonoid compounds and 5% (v/v) Me_2SO . (Before addition of the compounds, relaxation solutions were preincubated at 37 °C for 30 min and a DNA sample was withdrawn to demonstrate full relaxation of plasmid DNA by the topoisomerase.) Reactions were quenched by the addition of 20% (w/v) SDS (5 μ l), extracted three times with an equal volume of phenol and twice with an equal volume of chloroform and electrophoresed at 3 V/cm in a 0.8% agarose gel at room temperature. The electrophoresis buffer contained 40 mM-Tris/HCl buffer, pH 8.0, 5 mM-magnesium acetate and 1 mM- Na_3EDTA , and was circulated while the gel was running. Gels were stained in several changes of the TBE buffer (89 mM-Tris/borate buffer, pH 8.3, 2 mM- Na_3EDTA) containing ethidium bromide (1 μ g/ml) over 3 h, destained in TBE buffer and photographed under u.v. illumination [13].

End-labelled linear DNA. Plasmid pBR322 DNA was linearized with *Eco*RI, incubated with calf intestinal alkaline phosphatase and then radiolabelled at its 5'-ends with the use of [γ - 32 P]ATP and polynucleotide kinase [16]. The DNA was then digested with *Hind*III and the 4333 bp *Eco*RI-*Hind*III fragment was separated from a 31 bp fragment by electrophoresis in 1% low-gelling agarose in TBE buffer. The larger fragment, radiolabelled uniquely at its *Eco*RI end, was isolated from the gel and purified as described previously [16].

DNA cleavage. Cleavage reaction mixtures contained 40 mM-Tris/HCl buffer, pH 7.5, 100 mM-KCl, 10 mM- MgCl_2 , 0.1 mM-dithiothreitol, 0.5 mM-EDTA, 5% (v/v) Me_2SO , 30 μ g of BSA/ml, 40 units of calf thymus DNA topoisomerase II, 32 P-end-labelled *Eco*RI-*Hind*III pBR322 DNA (2000 c.p.m. Čerenkov) and flavonoid compound at 0–50 μ g/ml or *m*-AMSA at 4–40 μ g/ml (total volume 20 μ l). Reactions were incubated at 37 °C for 30 min and then treated with 1 μ l of 10% (w/v) SDS and 1 μ l of 1.5 mg/ml proteinase K at 50 °C for 30 min. DNA samples were analysed by electrophoresis in 1% agarose in TBE buffer. Gels were dried by blotting on to DE81 paper before autoradiography.

Decatenation of kinetoplast DNA by topoisomerase II. Reaction mixtures contained 50 mM-Tris/HCl buffer, pH 7.5, 150 mM-KCl, 10 mM- MgCl_2 , 1 mM-dithiothreitol, 1 mM- Na_3EDTA , 1 mM-ATP, 1 unit of calf thymus topoisomerase II and 0.5 μ g of kinetoplast DNA (total volume 20 μ l). Flavonoids were added in Me_2SO to a final Me_2SO concentration of 5% (w/v). Samples were incubated at 37 °C for 30 min and the DNA was analysed by electrophoresis in 0.8% agarose in TBE buffer.

Cell culture. CCRF-CEM and Daudi cells were grown as stationary suspension cultures in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 2 mM-glutamine, 100 units of penicillin/ml and 100 μ g of streptomycin/ml. CEM and Daudi cells were seeded at densities of 2.5×10^5 cells/ml and incubated at 37 °C in a humidified chamber containing an atmosphere of 5% CO_2 in air, subculturing every 2–3 days. HeLa cells were grown as monolayer cultures in Minimum

Fig. 2. Flavonoid-induced unwinding of closed circular DNA

Supercoiled pBR322 plasmid DNA (S) was relaxed with calf thymus DNA topoisomerase I in the absence (R) or presence of flavonoid compounds F1–F4, C1 and C2 at the indicated concentrations ($\mu\text{g/ml}$). Reactions were stopped by addition of SDS, flavonoids were removed by phenol extraction and the DNA was examined by electrophoresis in a 0.8% agarose gel.

Essential Medium with Earle's salts supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM-glutamine, 1% non-essential amino acids, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cells were incubated under the same conditions as the suspension cells and were routinely subcultured every 3–4 days by detaching with trypsin/EDTA.

Cytotoxicity assays. The cytotoxicity of the flavonoid compounds towards CEM and Daudi cells was assessed by a 72 h growth-inhibition assay as described by Conter & Beck [17]. Exponentially growing cells were seeded at a density of $2 \times 10^5/\text{ml}$ in six well plates and exposed to flavonoids or *m*-AMSA added in Me_2SO [final Me_2SO concentration was 0.8% (v/v) in each well]. Cell numbers were determined after 72 h with a Coulter counter. Control experiments showed that 0.8% (v/v) Me_2SO had no measurable cytotoxicity. The toxicities were expressed as IC_{50} values (i.e. the concentration of compound that inhibited cell growth by 50% with respect to the solvent control).

Cytotoxicities against monolayer HeLa cells were determined by a protein–dye binding method [18]. Exponentially growing HeLa cells were trypsinized and seeded in 1 ml portions at a density of 1×10^4 cells/ml in 24-well multiwell dishes. After overnight attachment, fresh medium containing different concentrations of the flavonoid compounds was substituted and cells were incubated for 72 h. Triplicate wells were set up for each drug concentration. In each case the final Me_2SO concentration was 0.8% (v/v), which in control experiments showed no measurable cytotoxicity.

After the 72 h incubation period, the total protein content of each well was determined as described by Knox *et al.* [18]. Briefly, each well was rinsed three times with 12 mM-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl, and cells were fixed with 1 ml of 3% (v/v) glutaraldehyde for 20 min and then stained with Kenacid Blue R stain for 30 min. The wells were then destained and the remaining dye attached to the cells was desorbed by agitation in precisely 1 ml of 1 M-potassium acetate in 70% (v/v) ethanol for 15 min. The absorbances of solutions from each well were measured at 570 nm. The method gives relative values for test wells compared with control wells, rather than an absolute measurement of protein content. The average value for the test wells was expressed as a percentage of the average value for the solvent control wells. The IC_{50} values obtained represent the concentration of compound that inhibits the growth of the cells by 50% with respect to the untreated control.

RESULTS

Baicalein and related flavone derivatives bind and induce unwinding of duplex DNA

Interaction of the flavonoid inhibitors of human-immuno-

Fig. 3. Site-specific DNA cleavage by DNA topoisomerase II promoted by flavonoids

A 4333 bp *EcoRI*–*HindIII* fragment from plasmid pBR322 ($\blacktriangleright$) ^{32}P -labelled at its 5' *EcoRI* end was incubated with calf thymus topoisomerase II in the absence (Tp) or in the presence of flavonoids (C1, C2 and F1–29), *m*-AMSA (mA) or the oriental drug Sho-saiko-to (TJ9). DNA cleavage was induced by the addition of SDS and proteinase K and the DNA was analysed by agarose-gel electrophoresis and autoradiography. Flavonoids F5–29 were present at 50 $\mu\text{g/ml}$: concentrations of the other compounds ($\mu\text{g/ml}$) are shown at the top of the Figure. M and $\blacktriangleright$ indicate a radiolabelled 652 bp *EcoRI*–*PstI* DNA marker. Sites of DNA cleavage were mapped by running size markers alongside (not shown).

deficiency-virus reverse transcriptase with DNA was investigated by using a DNA unwinding assay (Fig. 2). Closed circular pBR322 DNA was relaxed with calf thymus topoisomerase I, an enzyme that removes both positive and negative supercoils. Flavonoids were added and after further incubation the topoisomerase I was inactivated and the flavonoid removed by phenol extraction. The flavone derivatives quercetin (F1), quercetagetin

Table 1. Activity of flavonoid compounds in promoting DNA breakage by mammalian DNA topoisomerase II

Abbreviations: Glu, glucuronide; Me, methyl; Rh, rhamnoside; Ru, rutinose. Levels of DNA cleavage judged from Fig. 3: —, no cleavage above enzyme alone control; —/+, weakly enhanced DNA breakage; +, 5% cleavage; ++, substantial (10–50%) cleavage.

Compound		Position of OH substitution					Topoisomerase II cleavage
Flavone and flavonols							
F1	Quercetin	3	5	7	3'	4'	++
F2	Quercetagetin	3	5	6 7	3'	4'	++
F3	Myricetin	3	5	7	3'	4' 5'	++
F4	Baicalein		5	6 7			++
F5	Flavone	—					—
F6		3					—
F7			5				—
F8				6			—
F9				7			—
F10	Chrysin		5	7			—
F11				6 7			—/+
F12				7 8			—/+
F13	Apigenin		5	7		4'	++
F14	Baicalin		5	6 7-OGlu			++
F15	Wogonin		5	7-8-OMe			—
F16	Kaempferol	3	5	7		4'	++
F17	3,4'-Dimethylkaempferol	3-OMe	5	7		4'-OMe	—
F18	Luteolin		5	7	3'	4'	++
F19	Morin	3	5	7	2'	4'	+
F20	Quercitrin	3-ORh	5	7	3'	4'	—/+
F21	Rutin	3-ORu	5	7	3'	4'	—
Flavanones							
F22	Gardenin		5,	6 7 8-triOMe	3'	4' 5'-triOMe	—
F23	Hesperetin		5	7	3'	4'-OMe	—
F24	Hesperidin		5	7-ORu	3'	4'-OMe	—/+
F25	Eriodictyol		5	7	3'	4'	—/+
F26	Homoeriodictyol		5	7	3'-OMe		—
F27	Fustin	3		7	3'	4'	—/+
F28	Naringenin	3	5	7		4'	—
Catechins							
F29	(+)-Catechin	3	5	7	3'	4'	—/+
C1							+
C2							++

(F2), myricetin (F3) and baicalein (F4) altered the linking number of the DNA compared with the relaxed DNA control (lane R). Supercoiling was evident at 50 µg of flavonoid/ml and there was a slight but detectable change in supercoiling at 2.5 µg/ml. All four flavonoids resulted in broadly similar levels of DNA supercoiling, although quercetin was the most efficient and baicalein the last efficient agent. In contrast, the catechin-based reverse transcriptase inhibitors (–)-epicatechin gallate (C1) and (–)-epigallocatechin gallate (C2) had no effect on DNA topology even at 50 µg/ml (Fig. 2).

To establish whether flavonoids induce positive or negative supercoiling, DNA samples in Fig. 2 were electrophoresed at 4 °C rather than room temperature. The effect of lowering temperature on an underwound (i.e. negatively supercoiled) DNA is to alter the helix pitch such that the DNA becomes more negatively supercoiled and runs further from the position of nicked DNA [13]. Comparison of DNA mobilities established that compounds F1–F4 generate negatively supercoiled DNA consistent with an intercalative mode of DNA binding (results not shown).

Flavone and catechin derivatives promote the formation of a cleavable complex between topoisomerase II and DNA

The ability of flavonoids to induce topoisomerase II-mediated DNA cleavage was assessed as follows (Fig. 3). A 4333 bp

EcoRI–*HindIII* restriction fragment from pBR322, uniquely labelled with ³²P at its *EcoRI* 5'-end, was incubated with calf thymus topoisomerase II and flavonoid compounds. DNA cleavage was induced by the addition of detergent followed by proteinase K and the DNA products were separated according to size by agarose-gel electrophoresis. Autoradiography of the gel revealed the extent of DNA cleavage and allowed mapping of cleavage sites on pBR322 DNA. Some 30 flavonoid compounds were tested (Fig. 3) and the results are summarized in Table 1.

Reverse transcriptase inhibitors F1–F4 and C1 and C2 were all proficient in promoting enzymic DNA breakage though less so than *m*-AMSA (mA) (Fig. 3a). DNA breakage was seen at flavonoid concentrations of 5 µg/ml and was pronounced at 50 µg/ml. C1 was the least active of this group. Interestingly, the oriental drug Sho-saiko-to (TJ9), made from various plant roots [1] and which contains flavonoid compounds, was also able to induce DNA breakage by topoisomerase II, though at 10-fold higher concentrations (Fig. 3a).

Of the other flavone derivatives (F5–F21), only apigenin (F13), baicalin (F14), kaempferol (F16) and luteolin (F18) produced cleavage with an efficiency comparable with that of compounds F1–F4, C1 and C2 (Figs. 3b and 3c). Morin (F19) was somewhat less effective: F11, F12 and quercitrin (F20) also generated weak DNA cleavage. None of the flavanone derivatives (F22–F28), which have a saturated 2,3-bond in the pyrone ring

(Fig. 1) were efficient promoters of enzymatic cleavage of DNA (Fig. 3c). However, hesperidin (F24) and eriodictyol (F25) were weakly active.

It is interesting that flavonoids active in the topoisomerase II cleavage assay all induce the same pattern of cleavage sites on DNA (Fig. 3). The agents appear to enhance cleavage at sites attacked by topoisomerase II in the absence of any added drug (and which are weakly discernible in lanes Tp of Fig. 3). It is also noteworthy that the cleavage sites revealed by flavonoids appear to be identical with those induced by *m*-AMSA, a structurally different compound (Fig. 3).

Flavonoids inhibit the catalytic activity of DNA topoisomerase II *in vitro*

The effect of flavonoids on the DNA strand passage activity of topoisomerase II was determined with kinetoplast DNA as substrate (Fig. 4). Kinetoplast DNA consists of topologically interlocked DNA circles (catenated) that can be released by topoisomerase II in an ATP-dependent reaction (lane c). Myricetin (F3), one of the strongest inducers of DNA strand breakage by topoisomerase II, was inhibitory in the decatenation assay even at 1.25 $\mu\text{g}/\text{ml}$ (Fig. 3, lanes d–f). Compound C1 was much less inhibitory, again paralleling the DNA cleavage results (lanes g–i).

Cytotoxicity of flavonoids against human tumour cell lines

Two different methods were used to measure the cytotoxicities of flavonoids against leukaemic CCRF-CEM and Daudi Burkitt lymphoma lines, which grow in suspension culture, and HeLa cervical carcinoma cells, which grow in monolayer culture. Fig. 5 shows results obtained for CCRF-CEM cells. Increasing concentrations of quercetin (F1), quercetagenin (F2), myricetin (F3) or baicalein (F4) led to a decrease in cell growth compared with the untreated controls. The IC_{50} determinations show that the order of cytotoxicity was: baicalein, 17 μM (5 $\mu\text{g}/\text{ml}$); quercetin, 21.3 μM (7.2 $\mu\text{g}/\text{ml}$); quercetagenin, 22.5 μM (7.2 $\mu\text{g}/\text{ml}$); myricetin, > 25 μM (> 7.9 $\mu\text{g}/\text{ml}$). In contrast, compounds C1 and C2 were non-cytotoxic in this range and indeed appeared to give some stimulation of cell growth (Fig. 5). The same order of IC_{50} values was seen for Daudi cells: baicalein, 13.3 μM (3.5 $\mu\text{g}/\text{ml}$); myricetin, > 25 μM (IC_{60} value 25 μM) (results not shown).

Cytotoxicities towards HeLa cells were determined by a protein-dye binding assay in which the level of binding of Kenacid Blue dye to total cell protein in fixed monolayer cells is directly proportional to cell number (Fig. 6). Description of bound dye and quantitative determination by measurement of A_{570} gave the following IC_{50} values: baicalein, 22.5 μM (6.35 $\mu\text{g}/\text{ml}$); myricetin, > 24 μM (IC_{80} value 25 μM). Compound C1 showed no cytotoxicity towards Daudi cells and appeared stimulatory towards HeLa cells.

Thus baicalein and related flavone derivatives each exhibited broadly similar cytotoxicities to the three cell lines but were much less toxic than *m*-AMSA, whose IC_{50} for CCRF-CEM cells was 0.03 μM (Fig. 5).

DISCUSSION

Baicalein, quercetin, quercetagenin and myricetin are newly described natural product inhibitors of reverse transcriptase [2,3]. Here we show that the four compounds unwind DNA and promote site-specific DNA cleavage mediated by mammalian DNA topoisomerase II. In contrast, two novel catechin derivatives, (–)-epicatechin gallate and (–)-epigallocatechin gallate, which are even more potent inhibitors of reverse transcriptase *in vitro* [4], also promoted DNA breakage by topoisomerase II but

Fig. 4. Effect of flavonoids on DNA strand passage by DNA topoisomerase II

Kinetoplast DNA was incubated with calf thymus topoisomerase II in the absence (lanes b and c) or in the presence (lanes d–i) of flavonoids F3 and C1 (see Table 1) and the DNA was analysed by electrophoresis in a 0.8% agarose gel. Flavonoids were included at 50 $\mu\text{g}/\text{ml}$ (lanes d and g), 5 $\mu\text{g}/\text{ml}$ (lanes e and h) and 1.25 $\mu\text{g}/\text{ml}$ (lanes f and i). Lane a is untreated kinetoplast DNA. M denotes 1 kb markers; Mc, DNA minicircles released from kDNA. All reaction mixes contained 5% Me_2SO except lane b.

Fig. 5. Cytotoxic effects of flavonoids and *m*-AMSA on the human leukaemic CCRF-CEM cell line

Exponentially growing cells were seeded at 2×10^5 per ml in wells of a tissue-culture plate and exposed to compounds over a 72 h period at 37 °C. Cell numbers determined with a Coulter counter are expressed as percentages of that determined for cells grown in the absence of added inhibitor. All measurements were made in triplicate. Note the difference in abscissa for *m*-AMSA.

did not unwind DNA. These observations have implications for understanding the inhibitory and cytotoxic properties of these compounds.

Flavone inhibitors induced relatively modest extents of DNA unwinding: even at 50 $\mu\text{g}/\text{ml}$ only a small linking difference was observed and unwinding was barely detectable at lower concentrations (Fig. 2). Similar low-level DNA unwinding has been reported recently for the flavone derivatives fisetin (3,7,3',4'-tetrahydroxyflavone) and quercetin [12]. DNA unwinding is consistent with an intercalative mode of DNA binding, as suggested by Yamashita *et al.* [12]. DNA intercalation would also be favoured by the planar structure of flavones, as

Fig. 6. Flavonoid cytotoxicity against HeLa cells determined by a protein-dye binding method

HeLa cells (10^4) were seeded in triplicate into compartments of a multiwell dish and incubated overnight to allow attachment. Cells were then exposed over 72 h to medium containing flavonoid compounds. Cells were fixed with glutaraldehyde and then stained for protein with Kenacid Blue R dye. The Figure shows stained cells before desorption and spectrophotometric quantitative determination of released dye. C denotes untreated cells; 0, solvent controls; numbers indicate concentration of compound added in μM .

confirmed by the X-ray structure of quercetin [6]. However, the low degree of unwinding by flavones compared with known intercalators such as ethidium bromide (results not shown) and *m*-AMSA [12] suggests that if intercalation occurs the compounds must either have a low DNA affinity or undergo only partial intercalation. Alternatively, DNA binding by flavones could be non-intercalative with minimal effects on DNA helix pitch evident only at high ligand concentrations.

The broadly similar levels of DNA unwinding induced by the four flavone inhibitors (Fig. 2) contrast with their markedly different inhibitory effects on reverse transcriptase and cellular polymerases assayed with the same $\text{rA}_n \cdot \text{dT}_{12-18}$ template primer [3–5]. Thus the K_i values for baicalein, quercetin, quercetagenin and myricetin measured for human-immunodeficiency-virus reverse transcriptase were 2.53, 0.52, 0.46 and 0.08 μM respectively, i.e. a 30-fold range (approx 0.025–0.7 $\mu\text{g}/\text{ml}$). Moreover, the four flavones exhibited different selectivities in their inhibition of

cellular DNA polymerases *in vitro*. Baicalein was inhibitory only to DNA polymerase γ (K_i 0.9 μM) whereas quercetagenin inhibited DNA polymerases α , β and γ (K_i 3.97, 0.56 and 1.19 μM). Thus there is no correlation between the enzyme-inhibitory effects of flavones and DNA binding measured by the unwinding assay. Indeed, the extremely potent reverse transcriptase inhibitors (–)-epicatechin gallate (K_i 7.2 nM) and (–)-epigallocatechin gallate (K_i 2.7 nM) did not generate observable unwinding at 50 $\mu\text{g}/\text{ml}$ (110 μM). Taken together, the data suggest that the differential inhibitory effects on reverse transcriptase and on polymerase observed at low flavone concentrations arise from binding to a site in the enzyme complex in competition with the template primer rather than by binding to the template-primer itself.

Previous studies on the non-intercalative isoflavone genistein showed that this tyrosine kinase inhibitor induces formation of the cleavable complex in cells and promotes DNA cleavage by topoisomerase II *in vitro* [11,19]. In common with genistein, we find that several flavones as well as catechins promote topoisomerase II-mediated DNA breakage. Flavonoids active in the DNA breakage assay each generated cleavage at the same sites and with similar relative intensities. Moreover, the pattern of cleavage sites was the same as that induced at much lower concentrations by the anticancer drug *m*-AMSA, suggesting that they trap the same cleavable complex. Genistein caused cleavage at two additional sites to that of *m*-AMSA, perhaps reflecting its structural difference from flavones or that ATP was included in the genistein experiments [11]. Our results are the first comparison of the site-specificity of DNA cleavage induced by flavonoids.

Extensive cleavage by topoisomerase II was observed only with quercetin, quercetagenin, myricetin, baicalein, apigenin, baicalin, kaempferol, luteolin and less so morin (Table 1). {A recent study using a different assay, namely topoisomerase II-mediated linearization of pBR322 DNA, also demonstrated breakage with apigenin, fisetin (3,7,3',4'-tetrahydroxyflavone), quercetin, myricetin and morin [12].} These active compounds carry invariant hydroxy substituents at the 5 and 7 positions. Several have hydroxy groups at the 3' or 4' positions. Moreover, whereas a 3-hydroxy substitution did not interfere with activity, a rhamnosylglucoside moiety did (compare quercetin with rutin, Table 1). None of the flavanone derivatives, which have a saturated 2,3-bond, was particularly active. On the basis of these results, we propose a minimal flavone structure for DNA cleavage by topoisomerase II as shown in Fig. 7.

Interestingly, the same minimal structure has been determined for flavone inhibition of protein kinase C, another ATP-dependent enzyme [20]. Inhibition of protein kinase C by fisetin is competitive with the binding of ATP [20]. It seems possible therefore the flavones recognize and bind a conserved structural feature present in protein kinase C and in the topoisomerase

Fig. 7. Structural requirements for flavone-promoted DNA breakage by DNA topoisomerase II

Lines indicate positions at which substitution with hydroxy groups does not adversely affect DNA breakage.

II-DNA complex. A similar idea has been proposed to explain the observation that genistein is an inhibitor of topoisomerase II and of tyrosine kinases [11]. Thus genistein and quercetin have been shown to inhibit the tyrosine kinase activities of the epidermal-growth-factor receptor and the Rous-sarcoma-virus transforming protein pp60^{src}, in each case competitively with ATP [21,22]. However, it is not clear whether any of these flavonoid inhibitors act at the ATP site of topoisomerase II. In the case of genistein, addition of ATP augmented DNA cleavage by topoisomerase II, i.e. the opposite of that expected for competitive inhibition [11]. Currently, the mechanism of enzymic DNA cleavage has not been elucidated for any inhibitor of DNA topoisomerase II. The availability of a number of flavone derivatives with known differential effects on topoisomerase II may allow this question to be approached.

Stabilization of the cleavable complex of topoisomerase II *in vivo* is a known cytotoxic lesion [7,8]. Baicalein, quercetin, quercetagenin and myricetin were cytotoxic to human CEM, Daudi and HeLa cells with IC₅₀ values in the range 13–25 μ M (5–10 μ g/ml). Our studies show that flavonoids inhibit topoisomerase II at low concentration *in vitro*: myricetin completely inhibited DNA strand passage by topoisomerase II at 1.25 μ g/ml (Fig. 4). As the level of formation of the cleavable complex compatible with cell survival is not easily measured, DNA topoisomerase II may be considered a potential cytotoxic target for flavonoids, where it is accessible. The absence of cytotoxicity of the catechins may arise from lack of uptake or metabolic inactivation within the tumour cells tested. Further studies on molecular and enzymic aspects of flavonoid action will be essential in designing agents with antineoplastic or selective antiretroviral activity.

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