Enhanced sensitivity of human oral tumours to the flavonol, morin, during cancer progression: involvement of the Akt and stress kinase pathways

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Introduction

Animal studies have shown that several flavonoids reduce the incidence of various chemical-induced cancers (reviewed in ref. 1). Quercetin, quantitatively the most important dietary flavonoid for humans, reduces the incidence of 7,12-dimethylbenz[a]anthracene (DMBA)- and N-nitrosomethylurea-induced mammary cancer (2,3), azomethane-induced colon cancers (4), N-nitrosodiethylamine/pHENOBARBITAL-induced liver cancers (5), DMBA-induced oral cancers (6), DMBA-induced skin cancers (7) and benzo[a]pyrene-induced pulmonary tumours in rodents (8). However, there is also limited in vivo evidence that quercetin can be co-carcinogenic in some contexts (9–12). Several other flavonoids are also protective in various chemical-induced models, for example diosmin and hesperidin in relation to 4-nitroquinoline 1-oxide-induced oral cancer incidence (13), apigenin in DMBA-induced skin cancers (14) and robinetin in benzo[a]pyrene-induced pulmonary tumours in newborn mice (8). Thus there is considerable clinical interest in discovering or designing novel flavonoid derivatives for potential use in cancer prevention or treatment. Indeed, flavopiridol, a synthetic chlorinated derivative of a novel naturally occurring flavone, L868276, (−)-cis-5,7-dihydroxyphenyl-8-[4-(3-hydroxy-1-methyl)piperidinyl]-4H-benzyopyran-4-one hydrochloride hemihydrate, is currently in Phase I trials against breast cancer (15–18).

How flavonoids exert their chemopreventive effects is unknown. They are known to have antioxidant properties and reduce DNA damage after carcinogen treatment (reviewed in ref. 19). However, flavonoids can inhibit tumour formation if given after carcinogen, for example in the two-stage skin and liver carcinogenesis models, suggesting that they also act by preventing tumour promotion (5,7,14).

It is clearly important, therefore, to determine whether flavonoids can affect the growth and progression of human cancers and to elucidate the mechanisms involved. In our recent work we have investigated the capacity of various flavonoids to inhibit the growth of primary cultures of biopsies of normal human oral mucosa cells (NOMC) and lesions at various stages of progression from dysplasias to squamous cell carcinomas (SCC). In the course of these studies we discovered that morin (3,5,7,2′,4′-pentahydroxyflavone) shows significant tumour-specificity and we have therefore investigated its mechanism of action.

Materials and methods

Cells and culture conditions

The derivation and characterization of the primary cultures of human NOMC, dysplasias and SCC have been described previously (20,21). All cells were maintained on irradiated 3T3 feeders, either in FAD + medium (1:3 Ham’s F12/Dulbecco MEM with 10% foetal calf serum and insulin, EGF, transferrin, cholera toxin, hydrocortisone and adenine) for NOMC and dysplasia cells, or 10H medium (Dulbecco MEM plus 10% foetal calf serum without added growth factors except hydrocortisone) for SCC. NOMC and the senescent dysplasia cultures were used within the first two passages from frozen stocks, before their growth rate deteriorated significantly. 3T3 cells were maintained in 10C medium (Dulbecco MEM plus 10% foetal calf serum without added growth factors except hydrocortisone) for SCC. NOMC and the senescent dysplasia cultures were used within the first two passages from frozen stocks, before their growth rate deteriorated significantly. 3T3 cells were maintained in 10C medium (Dulbecco MEM plus 10% foetal calf serum without added growth factors except hydrocortisone) for SCC. NOMC and the senescent dysplasia cultures were used within the first two passages from frozen stocks, before their growth rate deteriorated significantly. 3T3 cells were maintained in 10C medium (Dulbecco MEM plus 10% foetal calf serum without added growth factors except hydrocortisone) for SCC. NOMC and the senescent dysplasia cultures were used within the first two passages from frozen stocks, before their growth rate deteriorated significantly. 3T3 cells were maintained in 10C medium (Dulbecco MEM plus 10% foetal calf serum without added growth factors except hydrocortisone) for SCC. NOMC and the senescent dysplasia cultures were used within the first two passages from frozen stocks, before their growth rate deteriorated significantly.

Chemicals

All chemicals were obtained from Sigma. An independent batch of morin was also obtained from Apin Chemicals (Abingdon, UK). For cell growth experiments, morin and quercetin were dissolved in ethanol and then diluted to give the required concentration in 1% ethanol. For the assays of kinases, since ethanol alone was found to induce MAP kinase, flavonoids were dissolved in DMSO and then diluted in medium containing <0.1% DMSO. Neither of the solvents, used alone at the concentrations used in the flavonoids-treated cultures, affected the growth of the cells, but in all experiments, control experiments included solvent at the appropriate concentration.
DNA synthesis assay
10^6 irradiated 3T3 cells were plated in 200 µl 10C medium per microtitre well. After 3 days, the medium was removed and oral epithelial cells were added (5000 NOMC or dysplasias; or 2500 SCC) in 150 µl 10H medium. After 18 h, 50 µl of morin at four times the required final concentration in 10H medium was added and incubated for 3 days. Each well was then given 0.5 µCi of tritiated thymidine for 6 h, the medium was removed and the cells were trypsinized and transferred onto a filter paper mat (printed filtermat A: Pharmacia) using a microtitre plate harvester (Skatron Combi Harvester, model 11900, LKB, Skatron, Norway). After adding scintillator, the mat was scanned and counted using a plate counter (model 1205 Betaplate, Pharmacia).

Duplicate wells were used for each condition. Pilot experiments established that the irradiated feeders supported growth of the oral cells for the duration of the experiment, but contributed <5% of the thymidine incorporation of the live oral cells. The extent of thymidine incorporation with increasing concentrations of morin was then expressed as a percentage of the control value and IC50 values were calculated from the dose–response curves obtained.

Typically, the radioactivity incorporated in untreated SCC and NOMC in these experiments were ~22 000 counts per minute (c.p.m.) and 7400 c.p.m., respectively.

Cell growth experiments
5 × 10^4 carcinoma cells or 10^5 normal cells were plated out overnight in 6-well plates together with 2 × 10^5 irradiated 3T3 cells in 10H or FAD + medium, respectively. The required concentration of morin in 1% ethanol was added and the cell counts measured after 3 days. Alternatively, after 3 days, replicate wells were re-fed with fresh medium with morin and the cell number measured after a total of 7 day growth. In each case, feeder cells were removed prior to cell counting by washing with PBS/EDTA. Duplicate wells were set up for each condition.

Cell cycle analysis
Chinese hamster ovary (CHO) cells were plated at a density of 5 × 10^3 cells/10 ml medium in 90 mm dishes and morin or solvent added 1 day later. After a further 2 days, the cells were harvested, washed in cold PBS and fixed in 70% ethanol. Prior to FACS analysis, DNA was stained with propidium iodide (20 µg/ml) and the cell cycle distribution calculated from 10^4 cells with the CellQuest software using a FACScan flow cytometer (Becton Dickinson, Cowley, UK).

Western blotting for ERKs1/2, ERK5, AKT, and GADD45
To prepare whole cell protein extracts, after removal of 3T3 feeders, the cells were washed twice with ice-cold PBS and then scraped off in 0.2 ml of buffer (20 mM HEPES, pH 6.8, 5 mM EDTA, 10 mM EGTA, 5 mM NaF, 0.1 µg/ml leupeptin, 1 mM DTT, 0.4 M KCl, 0.4% Triton X-100, 10% glycerol, 5 µg/ml leupeptin, 50 µg/ml PMSF, 1 mM benzamidine, 5 mg/ml aprotinin, 1 mM Na orthovanadate) and incubated on ice for 20 min, followed by centrifugation at 13 000 r.p.m. in a Microfuge for 10 min. The supernatant was stored at −70°C. Up to 50 µg of protein sample in 40 µl of buffer was mixed with 20 µl loading buffer (187.5 mM Tris–HCl, pH 6.8, 30% glycerol, 6.9% SDS, 2.1 M β-mercaptoethanol, 0.1% bromophenol blue) and electrophoresed on a SDS/8%PAGE gel. The proteins were then blotted onto nitrocellulose (Amersham, Little Chalfont, UK) using a Camlab (UK) semi-dry blotter, following the manufacturer’s protocol. Western blots were incubated in the presence of TBS-T (50 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1% Tween-20) and 5% dried milk. The amounts of activated kinases were measured, as well as the total amounts of kinase as loading controls, using the following antibodies: activated Jun N-terminal kinase (JNK), monoclonal antibody p-JNK (G-7) (cat. no. sc-6254, Santa Cruz Biotechnology, Santa Cruz, CA); total JNK1, monoclonal antibody JNK1 (F-3) (cat. no. sc-1648, Santa Cruz Biotechnology); activated and total p38 kinase, PhosphoPlus p38 mitogen-activated protein (MAP) kinase (tyr182) polyclonal antibody kit (cat. no. 9210, New England Biolabs, Hitchin, UK); activated MAP kinases (extracellular regulated kinase (ERK) 1/2), polyclonal antibody (cat. no. V667, Promega, Southampton, UK); and total MAP kinase, ERK1 (C-16)-G polyclonal antibody (cat. no. sc-93-G, Santa Cruz Biotechnology). ERK5 was detected as described in (22) using polyclonal antibody ERK5 (C-20) (cat. no. sc-1284, Santa Cruz Biotechnology); activated protein kinase (AKT/PKB) and total AKT, PhosphoPlus Akt (ser 473) polyclonal antibody kit (cat. no. 9270, New England Biolabs); tyrosine-15 phosphorylated cdc2, polyclonal antibody Phospho-cdc2 (Tyr15) (cat. no. 9111, New England Biolabs); total cdc2, polyclonal antibody (cat. no. 9112, New England Biolabs); GADD45, polyclonal antibody GADD45 (C-20) (cat. no. sc-792, Santa Cruz Biotechnology). In all cases bound primary antibody was detected using ECL chemiluminescent methodology (Amersham). Controls omitting the primary antibody were used to establish specificity.

Results
Effects of morin on oral cell growth
The panel of primary cultures derived from biopsies of oral SCC, dysplasias, and NOMC have been described previously (20,21). Our preliminary investigations of the effects of morin (3,5,7,2’,4’-pentahydroxyflavone) and other naturally occurring flavonoids on the growth of SCC and NOMC cultures revealed that higher concentrations of morin were required to inhibit oral cell growth than the other flavonoids, but morin showed significant tumour selectivity in contrast to the other flavonoids tested. A comparison of morin with the closely related flavonoid, quercetin (3,5,7,3’,4’-pentahydroxyflavone) is illustrated in Figure 1: this showed a highly significant difference in the extent of growth inhibition of a SCC culture and a NOMC culture by morin, especially concentrations that inhibit growth of SCC substantially; whereas no significant tumour selectivity was found with quercetin. This difference
in sensitivity between the B56 SCC culture and NOMC culture used in Figure 1 was not a peculiarity of these particular cultures since similar differences in sensitivity to morin were found with cultures of four other, independently derived, SCC biopsies and six NOMC biopsies, using a DNA synthesis assay (see Materials and methods section for details): average IC$_{50}$ values for SSC: 115 ± 21 µM (SEM 10 µM) compared with 173 ± 29 µM (SEM 12 µM) for NOMC (P = 0.009, Student’s t-test), whereas the sensitivities to quercetin were identical (average IC$_{50}$ values for NOMC and SCC being 29 ± 1.7 µM [SEM 0.9 µM] and 28 ± 5 µM [SEM 2.6 µM], respectively P = 0.664, Student’s t test). The difference in sensitivity between SCC and NOMC was also observed when comparing SCC with adjacent apparently normal buccal mucosa taken at the same time from the same patient (Figure 2); for example, compare NOMC NB5 (IC$_{50}$ ~200 µM) and F5, a stage 2 SCC, (IC$_{50}$ = 90 µM) (P for difference <0.001 at both 100 µM and 200 µM morin, by ANOVA); or NOMC NB9 (IC$_{50}$ = 165 µM) and an immortal dysplasia/carcinoma in situ, D19 (IC$_{50}$ = 75 µM) (P for difference <0.002 at both 100 µM and 200 µM morin, by ANOVA).

To determine the stage during oral cancer progression when sensitivity to morin was acquired we also measured the sensitivities of dysplasias to morin. We have previously shown that about half of oral dysplasia biopsies senesce in culture after 4–45 population doublings, whereas the remainder are immortal in culture (20,21). One immortal dysplasia, D9, proved to have a IC$_{50}$ value (145 µM) intermediate between the IC$_{50}$ values of normal and carcinoma cells, whereas the IC$_{50}$ concentration (75 µM) for another immortal dysplasia (D19) was significantly different from the normal cultures, NB5 and NB9 (IC$_{50}$ concentrations >200 µM and 150 µM, respectively) (P <0.002, by ANOVA) but not significantly different from SCC F5 (IC$_{50}$ 85 µM) (P = 1.0, by ANOVA). (Note, D19 was significantly different from another BICR56 at 100 µM only.) In contrast, the mortal dysplasia, D6 (IC$_{50}$ value 175 µM) was significantly different from SCC, B56 and F5, and the immortal dysplasias, D19 (IC$_{50}$ concentrations 110, 85 and 75 µM, respectively) (P <0.001, by ANOVA), but not significantly different from the normal cell cultures NB5 or NB9, at either 100 µM or 200 µM morin (P = 1.0, by ANOVA; Figure 2). This suggests that the increased sensitivity to morin is acquired during the mortal to immortal transition at the dysplasia stage.

Table I. Inhibition of a normal oral mucosa culture or a stage 4 SCC culture (B56) by different flavonoids

<table>
<thead>
<tr>
<th>IC50 (µM)</th>
<th>OH substitutions</th>
<th>Normal</th>
<th>SCC</th>
<th>Significance (Student’s t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morin</td>
<td>3, 5, 7, 2', 4'</td>
<td>180</td>
<td>110</td>
<td>0.003 (n = 6)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3, 5, 7, 3', 4'</td>
<td>31</td>
<td>30</td>
<td>0.807 (n = 3)</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>3, 5, 7, 4'</td>
<td>37</td>
<td>35</td>
<td>0.142 (n = 4)</td>
</tr>
<tr>
<td>Datsicetin</td>
<td>3, 5, 7, 2'</td>
<td>54</td>
<td>54</td>
<td>0.957 (n = 4)</td>
</tr>
<tr>
<td>Galangin</td>
<td>3, 5, 7</td>
<td>54</td>
<td>50</td>
<td>0.243 (n = 3)</td>
</tr>
</tbody>
</table>

n = Number of experiments performed.

Comparison of the structures of morin (3,5,7,2',4'-penta-hydroxy flavone) with quercetin (3,5,7,3',4'-pentahydroxy flavone) or kaempferol (3,5,7,4'-tetrahydroxyflavone) suggested that the tumour specificity of morin requires either the 2' hydroxyl group, or both the 2' and 4' hydroxyl groups. We therefore tested this by further experiments with other related flavonoids that lack the 2' or 2',4' hydroxyl groups, i.e. galangin (3,5,7,hydroxyflavone), datsicetin (3,5,7,2'-hydroxyflavone). Since neither of these flavonoids showed any tumour selectivity (Table I), this indicates clearly that both the 2’ and 4’ hydroxy groups in morin are required for its tumour specificity.

Effect of morin on signal transduction pathways

Cell cycle analysis demonstrated that morin arrested NOMC and SCC in G2/M (data not shown). There was no evidence of induction of apoptosis by several criteria at growth inhibitory concentrations of morin: no significant increase in cells with sub-G1 DNA content, DNA fragmentation resulting in the formation of DNA ‘ladders’ after polyacrylamide gel electrophoresis, or poly-ADP ribose polymerase (PARP) cleavage (data not shown).

As in other contexts (see refs 23 and 24) for recent reviews), arrest of SCC in G2 is due to induction of GADD45 and inactivation of the cell cycle kinase cdc-2 by phosphorylation on tyrosine 15 (Figure 3A,B). Signalling pathways regulated by the EGF receptor play an important role in control of growth of epithelial cells and the EGF receptor is amplified or over-expressed in most oral SCC (25–30). The mitogen activated protein kinase (MAPK) signalling pathway resulting in activation of ERK1/2 is one of the important regulatory pathways controlling cell growth in response to EGF receptor activation. We therefore tested whether morin inhibited activation of ERK1/2 in response to EGF receptor activation. SCC...
Fig. 3. Effects of morin on induction of (A) GADD45, (B) cdc2 phosphorylation and (C) activation of ERK1/2. (A, B) SCC were grown for 24 h in medium with or without 220 µM morin. (C) SCC were starved in medium containing 0.25% serum with or without 220 µM morin for 18 h, and were stimulated by addition of 10 ng/ml EGF or serum for 15 min as indicated. Whole cell extracts were prepared and western blots performed as described in Materials and methods. Key: p-cdc2: phosphorylated cdc2. Loading control in (A): total p38 kinase.

were treated with two times the IC50 concentrations of morin for 24 h, starved in 0.25% serum for 18 h in the same concentration of flavonoid, and then stimulated by addition of EGF for 15 min. Activation of ERK1/2 was then measured in cell lysates by western blotting with antibodies that recognize either the total amount of ERK1/2 or the amount of activated (phosphorylated) ERK1/2. In fact, pre-treatment with morin did not reduce the activation of ERK1/2 by EGF treatment (Figure 3C), even though by this time the cells were becoming growth arrested, indicating that in these cells morin does not inhibit any of the kinases upstream of ERK1/2 at growth inhibitory concentrations. The activation of stress kinases was therefore measured in cell lysates by western blotting with antibodies that recognize either the total enzyme or specifically only the activated (phosphorylated) form. The results show clearly that growth inhibitory concentrations of morin activate both JNK and p38 kinases after 12–24 h in SCC and NOMC (Figure 4B). Comparison of the dose–response curves for the effects of morin on AKT, GADD45, p38 and JNK activation in SCC and NOMC reveals that JNK, p38 kinase and GADD45 were all induced with very similar dose–response curves in SCC and NOMC (Figure 4B). However, the extent of activation of p38 in SCC may be greater than in NOMC at the same concentration of morin (Figure 4B).

SSC become partially arrested in G2 after morin treatment (Table II) and this also occurs with other cell lines, such as rapidly dividing CHO cells in which the arrest is very dramatic (Table II). We have confirmed that growth-inhibitory concentrations of morin induce p38 and JNK and inhibit AKT activation in CHO cells in a similar manner to SCC (data not shown). To assess whether induction of JNK or p38 kinases (or both) was functionally important for growth inhibition by morin, we determined whether cell cycle arrest by morin in CHO and SCC cells was prevented by pre-treatment with the p38 stress kinase-specific inhibitor, SB202190 (33–35): we have confirmed previously that SB202190 selectively inhibits activation of p38 kinase at 10 µM, whereas 30 µM is required to inhibit JNK activation (at which concentration activation of ERK1/2 is still not affected) (36). These experiments showed that pre-treatment with the p38-specific inhibitory concentration of SB202190 (10 µM) was sufficient to almost completely

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Table II. p38 kinase-dependence of induction of G2 cell cycle arrest by morin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% sub G0</th>
<th>% G0/G1</th>
<th>% S</th>
<th>% G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>50</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>SB</td>
<td>3</td>
<td>50</td>
<td>20</td>
<td>27</td>
</tr>
<tr>
<td>Morin</td>
<td>5</td>
<td>36</td>
<td>16</td>
<td>43</td>
</tr>
<tr>
<td>Morin + SB</td>
<td>6</td>
<td>52</td>
<td>13</td>
<td>29</td>
</tr>
<tr>
<td>SCC cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>70</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>SB</td>
<td>7</td>
<td>73</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Morin</td>
<td>8</td>
<td>47</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>Morin + SB</td>
<td>8</td>
<td>52</td>
<td>18</td>
<td>22</td>
</tr>
</tbody>
</table>

CHO or SCC cells were treated with two times the IC_{50} concentration of morin for 24 h with (SB) or without 10 µM SB202190 and the cell cycle profile determined by FACS analysis. Cells were fixed and DNA content determined by flow cytometric analysis as described in the Materials and methods section. A total of 20 000 events were measured. The percentage of cells in sub-G0, G0/G1, S, and G2/M are shown (averages of three experiments).

reverse the morin-induced arrest of CHO cells in S/G0, though the reversal was less marked, but consistent, in SCC (Table II). Identical data were also obtained using the alternative p38 kinase inhibitor, SB203580 (data not shown).

Discussion

The main conclusion from this study is that morin inhibits growth of oral carcinomas more effectively than normal oral mucosa cells. Growth inhibition by morin does not seem to be associated with induction of apoptosis in such oral cultures. Structure–function experiments have demonstrated that this tumour specificity absolutely requires the 2′,4′ hydroxyl configuration in the B ring. Because 2′ hydroxylated flavonoids lacking hydroxylation in the A ring are not very common in nature, we have not yet been able to test whether hydroxylation at the 3, 5, or 7 positions of the A ring in morin are also required for its tumour specificity. Our studies have also identified the main kinase signalling pathways affected by morin at the growth inhibitory concentrations: in oral cells, activation of the JNK and p38 stress kinase pathways (implicated in growth arrest of cells in response to stress signals of various kinds (32)), and inhibition of the PK–B/AKT pathway (considered to be mainly involved with regulating cell survival (31)) seem to be mainly involved. Dose–response analysis shows that SCC are significantly more sensitive to inhibition of the PK–B/AKT signalling pathway than NOMC, and SCC show a greater degree of induction of p38 kinase, whereas SCC and NOMC show similar dose–response curves for induction of JNK. This suggests that the differential sensitivities of the AKT and p38 pathways to inhibition by morin in SCC and NOMC may be a factor explaining why SCC are more sensitive to growth inhibition by morin. Functional intervention experiments with the chemical inhibitor, SB202190, also support the conclusion that the p38 kinase pathway is important, since concentrations of SB202190 that inhibit p38 kinase but not JNK (or ERK1/2) partially protect SSC from cell cycle arrest by morin. However, morin does not appear to interfere with the Ras/Raf/ERK1/2 pathway. ERK5 is another recently discovered member of the MAPK family implicated in growth regulation by the EGF receptor, as well as in oxidative stress responses in several cell types, including HeLa cells (21,37,38).

However, we have shown previously that ERK5 is not activated by EGF in SCC or NOMC (36). The difference in sensitivity between SCC and NOMC may also reflect the different genetic changes in the carcinomas, for example p53 mutations (39,40) and loss of the cell cycle regulator p16^{ink4a} (41), leading to loss of the G1 checkpoint.

Other flavonoids have been found to inhibit various signalling enzymes involved in growth control, at least in cell-free assays, including various types of kinases (42–51), lipoxygenases or cyclooxygenases (52–55). Another possible mechanism, at least for oestrogen-dependent cancers, is the ability of flavonoids to inhibit enzymes involved in estrogen metabolism, such as aromatase (56) and 17β steroid reductases (57), at sub-micromolar levels. We have recently demonstrated that activation of the p38 kinase pathway is responsible for the effects of kaempferol, luteolin and apigenin on oral cells, whereas lipoxygenase pathways mediate the growth-inhibitory effects of quercetin (J.O’Prey et al., submitted for publication).

There is considerable clinical interest in novel flavonoid derivatives in cancer prevention or treatment, particularly flavopiridol, which is currently in Phase I trials against breast cancer (15–18). Flavopiridol inhibits cell growth and induces apoptosis, both in vitro and in vivo, by specifically inhibiting cyclin-dependent cell cycle kinases (15,58–61) and this specificity is due to the interactions between these kinases and the phenyl ring which do not occur with other kinases such as protein kinase A (15). The information about the structure–function relationships of the tumour-specificity of morin and its mechanism of action may therefore have potential for devising flavonoid derivatives which could be useful as chemopreventive dietary supplements, for example in protecting high-risk groups.

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Tumour-specific effect of morin


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