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The flavonoid tangeretin inhibits invasion of MO_4 mouse cells into embryonic chick heart *in vitro*

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Tangeretin, a flavonoid from citrus plants, was found to inhibit the invasion of MO_4 cells (Kirsten murine sarcoma virus transformed fetal mouse cells) into embryonic chick heart fragments *in vitro*. The flavonoid appeared to be chemically stable in tissue culture medium, and the anti-invasive effect was reversible on omission of the molecule from the medium. Unlike (+)-catechin, another anti-invasive flavonoid, tangeretin bound poorly to extracellular matrix. It did not alter fucosylated surface glycopeptides of MO_4 cells. Tangeretin seemed not to act as a microtubule inhibitor, as immunocytochemistry revealed no disturbance of the cytoplasmic microtubule complex. However, at anti-invasive concentrations of tangeretin, cell proliferation and thymidine incorporation appeared to be inhibited. When cultured on an artificial substrate, treated MO_4 cells were less elongated, covered a larger surface area and exhibited a slower directional migration than untreated cells. From the decrease in ATP content in MO_4 cells after tangeretin treatment, we deduce that this flavonoid inhibits a number of intracellular processes, which leads to an inhibition of cell motility and hence of invasion.

Introduction

As invasion is now accepted as the common denominator of malignant tumors [10, 15], interest in potentially anti-invasive agents has been growing progressively. Using an assay for invasion *in vitro*, we have found that the flavonoid (+)-catechin inhibits the invasion of a number of cell types [6, 7]. Binding of the flavonoid to the extracellular matrix glycoprotein laminin appears to be responsible for its anti-invasive effect [4]. This interaction with laminin leads to an interference with adhesion and spreading of potentially invasive cells.

The findings with (+)-catechin prompted us to look for other flavonoids with an anti-invasive effect. Confronting cultures of invasive MO_4 mouse cells and embryonic chick heart fragments [25] were used as a screening assay for this purpose. This assay is relevant for a number of aspects of tumor invasion *in vivo* [19, 20], and allows accurate dosage of agents in the culture medium. We report here on the

capability of tangeretin, a flavonoid from citrus plants, to inhibit invasion of MO_4 cells in the assay.

Anti-invasive agents fall into three classes according to the site of their presumed molecular targets: intracellular, at the plasma membrane or in the extracellular matrix [8]. First, to explore possible effects of tangeretin on the intracellular compartment we analysed cell proliferation and motility in their different aspects. DNA synthesis, the cytoplasmic microtubule complex and intracellular ATP content were studied as possible intracellular targets of the flavonoid. Second, the effect of tangeretin on the fucosylated surface glycopeptides was investigated because invasion and metastasis are influenced by glycoproteins at the plasma membrane [3, 24, 32, 36] and because glycosylation of these glycoproteins determines their function [11, 26, 33]. Third, strictly extracellular targets were taken into account in binding experiments of the flavonoid with extracellular matrix homogenates from human amnion. Thus the aim of our study was to gain information concerning the action mechanism of tangeretin on invasion *in vitro* by exploring a number of known molecular targets of anti-invasive agents.

Materials and methods

MO_4 cells

MO_4 cells are Kirsten murine sarcoma virus transformants of MO fetal mouse cells [2]. They produce invasive tumors after inoculation into C_3H/He syngeneic mice [28] and are invasive when confronted with chick heart in organ culture [25]. The cells were maintained in plastic tissue culture flasks (Flow, Irvine, Scotland; Cat. No. 61-450B5) with Eagle's minimum essential medium (Flow; Cat. No. 12.127.54) supplemented with 0·05 per cent (w/v) L-glutamine, 250 IU/ml penicillin and 10 per cent (v/v) fetal bovine serum (Flow; Cat. No. 29.101.54, Lot No. 027012), hereafter called 'culture medium'.

Tangeretin

Tangeretin (figure 1) was a gift from Dr J. Attaway (Department of Citrus, Lakeland, FL, U.S.A.). Stock solutions at 25 mM in dimethyl sulfoxide (DMSO) were kept at −20°C until further dilution in culture medium. In all control experiments without tangeretin, 0·4 per cent DMSO was included in the culture medium. Purity and stability of the flavonoid were tested with reversed-phase high-performance liquid chromatography (HPLC): 0·1-ml samples were separated on an analytical 250 × 4·6 mm i.d. C-18 RSil LL (5 μm) column (Alltech Europe, Eke, Belgium; Cat. No. 8796) with a discontinuous gradient of acetonitrile (LiChrosolv)

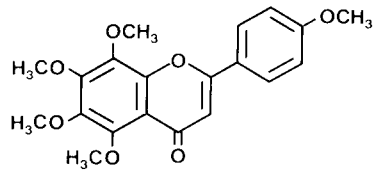


Figure 1. Structure of tangeretin.

(Merck, Darmstadt, F.R.G.; Cat. No. 30) in water from 10 to 100 per cent. At a flow-rate of 1 ml/min and a pressure of 15 MPa, absorption of the effluent was measured at 280 nm on a Series 3 liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with an LC75 spectrophotometric detector.

Invasion in vitro

In accordance with the technique of Mareel *et al.* [25], precultured embryonic chick heart fragments (PHF) were confronted with spheroidal aggregates of MO_4 cells. After 4 days of confronting culture in liquid medium, the tissue fragments were fixed in Bouin–Hollande's solution, processed for paraffin embedding and sectioned serially. Consecutive sections, stained with hematoxylin–eosin [34] or with an immunohistochemical technique for chick heart antigens [22], allowed histological evaluation of the interaction between PHF and MO_4 cells. These interactions were rubricated in accordance with a scale of grades [6]. To examine the effect of tangeretin on the interaction between MO_4 cells and PHF, the flavonoid was dissolved in the medium during the preculture period (4 days) of the heart fragments, during the confronting culture period or during both periods. Concentrations ranged between 0·000 and 0·100 mM and were limited by the solubility of the molecule in the culture medium. Reversibility of a possible anti-invasive effect of tangeretin was tested by changing the medium after 4 days of confronting culture for flavonoid-free medium and further culturing during another 4 days. Transmission electron microscopy (TEM) with a JEM-100B (Jeol, Tokyo, Japan) at 80 kV was performed on specimens fixed with 2·5 per cent glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4) and post-fixed with 1 per cent osmium tetroxide. Embedding, sectioning and contrasting were carried out as described by Van Peteghem and Mareel [42].

Viability of MO_4 aggregates and PHF

Individual heart fragments and MO_4 aggregates were treated with different tangeretin concentrations for 8 and 4 days, respectively, under similar circumstances as for confronting cultures. They were then explanted in tangeretin-free culture medium on a tissue culture plastic substrate as described previously [6]. The number of explants showing radial outgrowth on the substrate versus the total number of explants was considered to be a measure of tangeretin cytotoxicity on MO_4 cells and on chick heart.

Growth of cell aggregates

Spheroidal MO_4 aggregates with an initial diameter of 0·2 mm were grown in suspension culture for 7 days in the presence or absence of tangeretin. Changes in volume were calculated from daily measurements of aggregate diameters, as described by Storme and Mareel [37].

Colony formation

In accordance with the technique described earlier [6], 1-ml aliquots of solid agar medium containing $1{\cdot}5 \times 10^4$ MO_4 cells in suspension were incubated in 35-mm diameter plastic Petri dishes at 37°C for 7 days. Culture dishes were then individually placed on a cross-hatched plastic matrix. In each surface unit

($1{\cdot}8 \times 1{\cdot}8\,mm^2$) of the matrix all groups of three cells or more were counted with an inverted microscope (magnification $\times 100$) to a total of 100 groups per dish. The diameter of each group was measured with an eyepiece graticule.

Thymidine incorporation

MO_4 cell aggregates with a diameter of 0·2 mm were brought in suspension culture at four different concentrations of tangeretin for 1, 2 or 3 days. In accordance with a method described earlier [24], the medium contained 2 μCi/ml of [6-^{3}H]thymidine (23 Ci/mmol; Amersham, Buckinghamshire, U.K.; Cat. No. TRK 61) during the final 24 h of culture. After suspension culture the aggregates were photographed with a Macroscope (Wild, Heerbrugg, Switzerland) (magnification $\times 50$), washed extensively and solubilized in 1 M NaOH. DNA-bound thymidine was then precipitated with 3 M perchloric acid. Samples were dissolved in Insta-Gel II (Packard, Downers Grove, IL, U.S.A.) and counted in a Tri-Carb liquid scintillation counter (Packard) for 10 min. Thymidine incorporation was calculated as the amount of DNA-associated thymidine per mm^3 MO_4 cells.

Directional migration

The diameters of individual MO_4 cell aggregates explanted on the bottom of Nunclon Delta SI 24-well multidishes (Nunc, Roskilde, Denmark; Cat No. 1-43982A) were measured daily during 7 days, as described by Storme and Mareel [37]. The area covered by the outgrowing cell populations was calculated for cultures containing different tangeretin concentrations in the medium. As directional migration is affected by microtubule inhibitors, we examined whether tangeretin could disturb the cytoplasmic microtubule complex (CMTC). Single MO_4 cells (1×10^4 cells/ml) were allowed to adhere and spread on 15 mm diameter glass coverslips with culture medium (5 per cent CO_2 in air, 100 per cent relative humidity) for 24 h. The cultures were incubated with different tangeretin concentrations for 24 or 48 h, then fixed with 1 per cent glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4) at room temperature for 10 min. Cells were further processed as described by De Mey *et al.* [9]. The CMTC was visualized immunocytochemically with an antiserum against tubulin (kindly provided by Dr M. De Brabander, Janssen Pharmaceutica, Beerse, Belgium) at a dilution of 1 : 200.

Morphology and motility of individual MO_4 cells

Observations were made on cells in 25-cm^2 plastic tissue culture flasks in medium with or without tangeretin.

For observation and image analysis we made video films using a Wild inverted microscope with phase contrast optics ($10\times$ objective) in a thermostated chamber ($37 \pm 0{\cdot}5°C$), a National Panasonic WV-1850C video camera and a Sony Umatic VO-5850P video recorder equipped with an AC-580 animation control unit and with a time–date generator. Analysis of morphology was carried out on real-time video films, whereas analysis of motility was performed on time-lapse video films (two images every 15 s).

For image analysis, paper copies of video images to be analysed were obtained through a Mitsubishi P70B video copy processor. On these video copies cells to be analysed were outlined. During the drawing the corresponding video image was viewed for reference in the pause mode of the video recorder, then the video copies were analysed by automated image analysis using an IBAS 2 system (Kontron,

F.R.G). Images were introduced into the system by a Bosch TV camera with a plumbicon tube and digitized at a resolution of 512 × 512 pixels and 256 grey levels. Binary images were then obtained for each object of interest. Analysis of the morphology of the phase contrast image of individual cells (measurements of area and form parameters) was carried out automatically. The form parameters used were as follows: conv. peri./peri. = the ratio of the convex perimeter on the actual perimeter of the cell (figure 2); D_{min}/D_{max} = the ratio of the smallest to the largest diameter through the geometric center of gravity of the cell under study (figure 2); 4 pi ar/peri^2 = the ratio of 4π times the cell area and the square power of the cell perimeter; b/a = the ratio of two distances perpendicular to each other, and based on the areal moment of inertia; in the case of an ellipse the major (a) and minor (b) axes are exactly reproduced. The IBAS system calculates automatically the ratio of the two values.

Measurements of parameters concerning motility were carried out on paired video copies (taken at 14-s intervals). A reference frame was used for a correct location of the video copies, so that an immobile point would occupy an identical position in both of the paired images. The following parameters were measured. Constant area 1 h = that part of the phase contrast image of the cell that was overlapping in video copies taken at 1-h intervals (expressed as a percentage of the mean area of the cell at times 0 and 1 h). Intervals of 2 and 4 h were also used. This parameter is a reflection of both translocation and stationary (on the spot) motility. Corrected constant area 1 h = that part of the phase contrast image of the cell that, after correction for translocation of the geometric center, was overlapping in video copies taken at 1-h intervals (expressed as a percentage of the mean area of the cell at times 0 and 1 h). An interval of 4 h was also used. This parameter is a reflection of the stationary (on the spot) motility, a measure of the extent to which a cell changes shape in a certain time interval. This parameter stands in a simple arithmetic relation to the shape-change factor d shape proposed by Verschueren and Van Larebeke [43]: cor. const. area = 100 × (1 − d shape). Translocation distance 1 h = the distance between the location of the geometric center of the phase contrast images of the cell at times 0

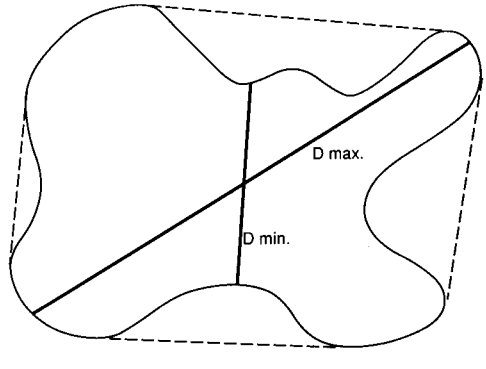


Figure 2. Schematic representation of the perimeter (solid line), the convex perimeter (dashed line), the smallest (D_{min}) and the largest (D_{max}) diameter of a cell.

and 1 h (expressed as micrometer). An interval of 4 h was also used. This parameter reflects the extent to which the cell translocates within a given time interval.

For statistical evaluation the Mann–Whitney U-test was used.

ATP determination [1]

Confluent MO_4 cell cultures in 25-cm^2 plastic tissue culture flasks were treated with tangeretin at 37°C for 24 h. After three washes with phosphate-buffered saline at room temperature, 1 ml of lysis buffer [0·1 M Tris–4 mM EDTA–0·1 per cent (v/v) Triton, pH 7·75] was added and the cultures were immediately placed on ice. ATP was measured in 25-μl samples with a luminometer (LKB, Stockholm, Sweden) using firefly bioluminescence. The results were expressed as mol ATP/μg protein and corrected for quenching of luminescence by tangeretin.

Gel filtration of fucosylated surface glycopeptides

We followed the methods of Van Beek *et al.* [41] and Gilfix and Sanwal [12]. MO_4 cells were grown on plastic tissue culture flasks for 4 days at an initial density of $1{\cdot}5 \times 10^5$ cells per 75 cm^2. Culture medium was refreshed without or with 0·100 mM tangeretin after 2 days and 1 μCi/ml L-[6-^{3}H]fucose (86·3 Ci/mmol; NEN, Boston, MA, U.S.A.) was added during the last 24 h of incubation. Then the cells were washed and trypsinized for 10 min in order to isolate glycoproteins of the plasma membrane. After centrifugation of the cells and exhaustive pronase digestion of the supernatant, the radiolabeled glycopeptides were loaded on a $90 \times 1{\cdot}6$ cm Bio-Gel P10 (200–400 mesh) (Bio-Rad, Richmond, CA, U.S.A.; Cat. No. 150-1050) column. Dextran blue and phenol red were added to the samples in order to determine the void and inner volume of the column, respectively. Radioactivity was measured in both the digested supernatant and the cell pellet. Total radioactivity per milligram of protein, as measured on the cell pellet by the Lowry method, was calculated. These techniques allow a comparison of both the amount of incorporation and the apparent molecular volume of the fucosylated surface glycopeptides.

Binding of tangeretin to extracellular matrix (ECM)

ECM was prepared from human amnion as described by Liotta *et al.* [17]. The complete amniotic ECM from one afterbirth was frozen in liquid nitrogen, pulverized in a mortar and lyophilized. Aliquots (10 mg) of the powder were suspended in 0·5 ml of Tris–HCl (0·01 M) (pH 7·5) for mixing with an equal volume of tangeretin solutions in the same buffer. After an incubation at 37°C for 1 h, the mixtures were centrifuged at 14 500 *g* for 15 min to remove larger ECM particles. The supernatants (0·8 ml) were then filtered through a YMT ultrafiltration membrane (molecular weight cut-off 3×10^4) using MPS-I micropartition systems (Amicon, Danvers, MA; Cat. No. 4010). Structural ECM molecules, having molecular weights between 1×10^5 and 1×10^6 daltons, were expected to be retained by the filter, whereas free flavonoids (molecular weights $< 1 \times 10^3$ daltons) should pass freely. The concentration of free tangeretin in the ultrafiltrates was calculated from absorbance readings of the solutions at 325 nm with a DU-2 spectrophotometer (Beckman Instruments, Munich, F.R.G.). HPLC analysis proved that these absorbances reflected tangeretin concentrations in the ultrafiltrates. Free tangeretin concentrations were compared with total tangeretin concentrations, obtained by omitting ECM from the procedure. Experiments in which tangeretin was replaced with (+)-catechin solutions served as a positive control for ECM binding [4, 5].

Results

Stability of tangeretin in culture media

Tangeretin in water was detectable as a single symmetric peak on the HPLC traces. This peak was discernible after dissolution in culture medium and appeared the same after incubation under culture circumstances for at least 8 days (figure 3).

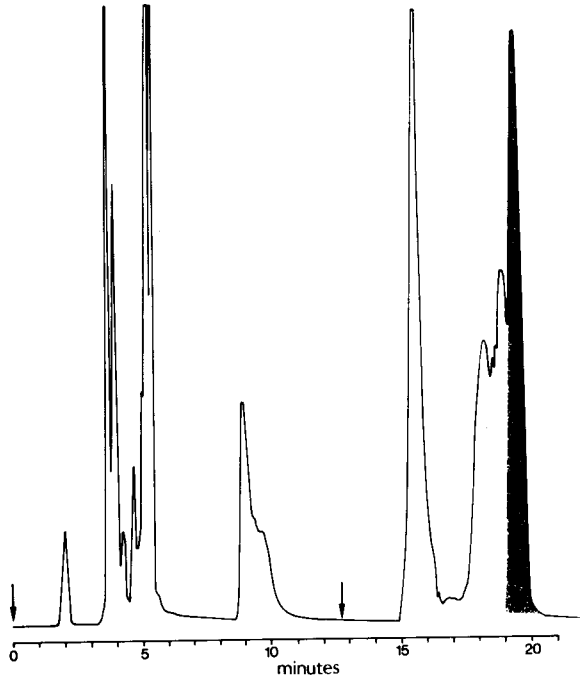
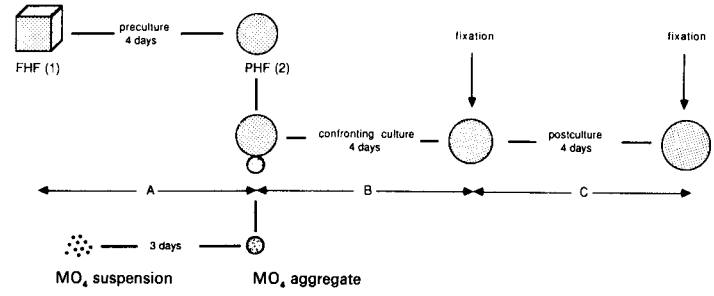


Figure 3. HPLC trace of tangeretin in culture medium; 0·002 mM tangeretin in culture medium was eluted on a C18 reversed-phase HPLC column. A discontinuous gradient of acetonitrile in water (at first arrow = 10 : 90; at second arrow = 100 : 0) was used. The tangeretin peak (shaded area) was detected at 280 nm and inferred from chromatograms of the flavonoid in water.

Invasion in vitro

The results of our invasion experiments *in vitro* are summarized in table 1. Tangeretin inhibited the invasion of MO_4 cells provided that the flavonoid was present in the culture medium both during the preculture period of the heart fragments and during the confronting culture period with MO_4 cells. Inhibition of invasion was noticeable at 0·010 mM and maximal at 0·100 mM tangeretin, as judged from serial histological sections (figure 4). Postculture in the absence of tangeretin indicated that the inhibitory effect on invasion was completely reversible after omission of the flavonoid. Histological sections showed that anti-invasive concentrations of tangeretin appeared to inhibit the growth of the MO_4 cells also, and that they produced morphological changes in the PHF. Peripheral heart cells tended to be less cohesive and to be separated from each other by translucent spaces. Transmission electron micrographs of PHF treated with 0·100 mM tangeretin for 4

Table 1. Effect of tangeretin on invasion of MO_4 cells in embryonic chick heart *in vitro*.



Treatment during period			mM tangeretin in culture medium	Grading (3)		
A	B	C		II	III	IV
+	−	n.a. (4)	0.000 0.001 0.010 0.100			
−	+	n.a. (4)	0.000 0.001 0.010 0.100			
+	+	n.a. (4)	0.000 0.001 0.010 0.100			
+	+	−	0.000 0.001 0.010 0.100			

(1) FHF: freshly prepared heat fragment.
(2) PHF: precultured heart fragment.
(3) Grade II: MO_4 cells occupy the outer cell layers of PHF only. Grade III: MO_4 cells occupy the PHF so that more than half is left. Grade IV: as grade III, but less than half of the PHF is left. One grey square represents one culture.
(4) n.a.: not applicable, cultures were fixed after period B.

days revealed few morphological differences from untreated PHF. In a number of heart cells dilatations of the rough endoplasmic reticulum, of the Golgi apparatus and of mitochondria were perceptible (figure 5).

Viability of MO_4 aggregates and PHF

Solitary PHF ($n=4\times2$) and MO_4 aggregates ($n=4\times2$), treated with different tangeretin concentrations up to 0·100 mM under analogous circumstances as in the assay for invasion, adhered and grew out without exception after explantation without tangeretin on a tissue culture plastic substrate.

Growth, colony formation and thymidine incorporation of MO_4 cells

Growth of MO_4 cell aggregates in suspension culture ($n=4\times6$) was inhibited by increasing the tangeretin concentration in the medium (figure 6A). Student's *t*-test

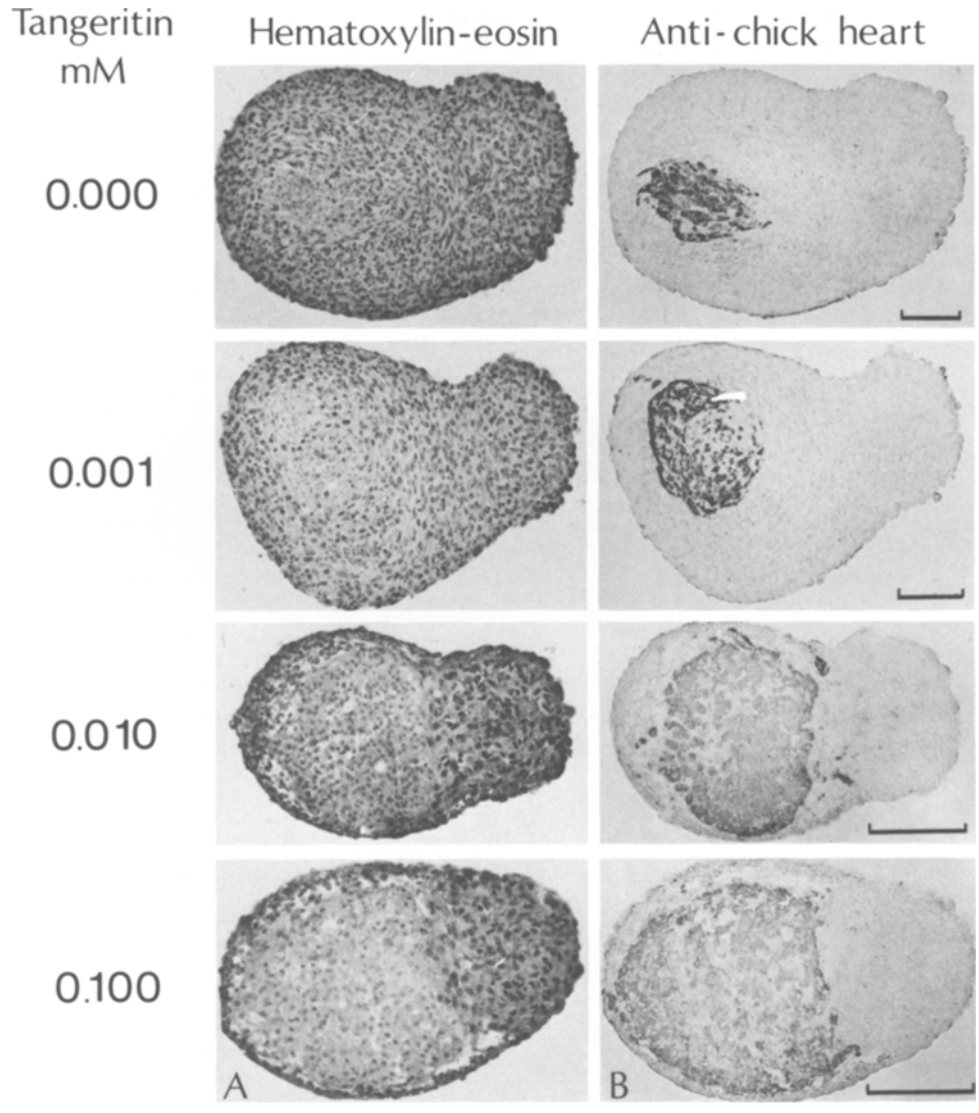


Figure 4. Light micrographs of sections from confronting cultures between PHF and MO_4 cells treated with different concentrations of tangeretin, and stained with hematoxylin–eosin (A) or with an immunospecific technique for chick heart antigens (B). Tangeretin had been present in the culture medium both during preculture of the heart fragments and during confronting culture with MO_4 cells. Examples are shown of grade II (0·100 and 0·010 mM), grade III (0·001 mM) and grade IV (0·000 mM). Scale bar: 100 μm.

proved the inhibition to be significant at 0·100 mM ($p < 0·001$). Colony formation of MO_4 cells in soft agar was also inhibited by the flavonoid (four Petri dishes for each concentration of tangeretin). Both the number of cells producing groups (figure 6B) and the mean diameter of these groups (figure 6C) were affected by tangeretin in the soft agar medium ($p < 0·001$ at 0·100 mM). Thymidine incorporation into MO_4 cell aggregates treated with tangeretin decreased as a function of time and of flavonoid

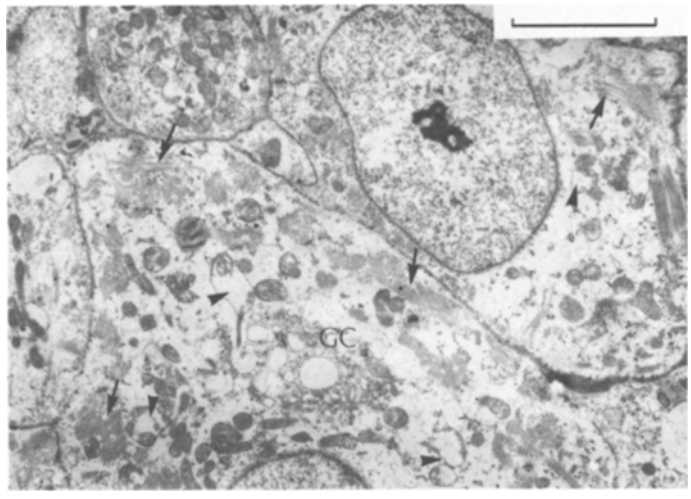


Figure 5. Transmission electron micrograph of PHF treated with 0·100 mM tangeretin for 4 days. Numerous myofilaments (arrows) are present throughout the cytoplasm. In a number of cells a dilated Golgi complex (GC) and swollen mitochondria (arrowheads) were observed. Scale bar = 10 μm.

concentration ($n = 3 \times 4 \times 5$) (figure 6D). After 3 days of suspension culture the thymidine incorporation into DNA had been inhibited by tangeretin in a dose-dependent way and the inhibition appeared to be significant ($p < 0{\cdot}001$ at 0·010 mM).

Directional migration of MO_4 cells

Tangeretin inhibited the directional migration of MO_4 cell populations on tissue culture plastic substrates, as shown in figure 7 ($n = 4 \times 6$). Inhibition was significant ($p < 0{\cdot}001$) at 0·100 mM after 7 days. This inhibition was not associated with a morphological disruption of the CMTC. Even at the highest tangeretin concentration (0·100 mM) the CMTC of MO_4 cells was unaltered in comparison with control cells (figure 8).

Morphology and motility of individual MO_4 cells

As shown in table 2, 0·050 mM tangeretin caused MO_4 cells to occupy a larger surface on tissue culture plastic. Also, as seen by phase contrast, tangeretin-treated cells tended to be less elongated and to have a smaller perimeter relative to the surface they occupied. The latter change could reflect the fact that the cells have a less irregular form (with less pseudopods extending from the cell body) or, alternatively, it could result from the fact that the overall shape of the area occupied by the cells comes closer to a circle. The length of the convex perimeter of the phase contrast image relative to its perimeter was, however, not affected by treatment with tangeretin. This parameter reflects the regularity of the contours of the cell (as seen by phase contrast), taking a lower value for cells with more or longer pseudopods or more or deeper indentations. As direct observation of the cells permitted it to be ruled out that tangeretin caused cells to have fewer but longer pseudopods or indentations, it can be concluded that tangeretin treatment did not result in changes in the number or dimensions of pseudopods or indentations. Taken together, the observed changes in form parameters point to the fact that tangeretin caused MO_4

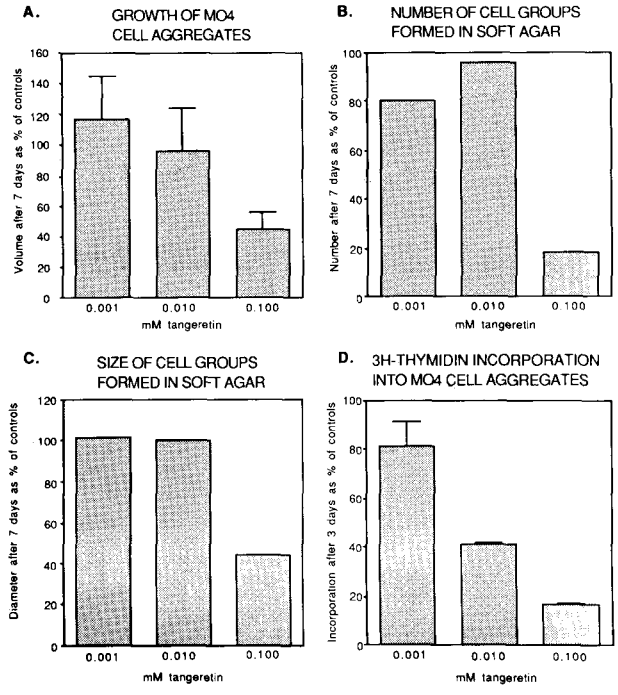


Figure 6. Effect of tangeretin on growth and proliferation of MO_4 cells. (A) Growth of MO_4 cell aggregates. Aggregates were incubated individually in culture medium at four different concentrations of tangeretin. Growth of the treated aggregates in suspension is expressed as their volume after 7 days of incubation divided by the mean volume of untreated aggregates, and multiplied by 100 to represent a percentage. Mean ± standard deviation. (B) Number of MO_4 cell groups formed in soft agar. Single cells were incubated in soft agar at four different concentrations of tangeretin. After 7 days the number of cell groups (>3 cells) within a standard surface area were counted and measured. This number at different tangeretin concentrations is expressed as a percentage of the number found in the absence of the flavonoid. (C) Size of MO_4 cell groups formed in soft agar. Same experiment as in B. Here the mean diameter of the same cell groups is compared with the mean diameter of control cultures. (D) Thymidine incorporation into MO_4 cell aggregates. MO_4 cell aggregates were kept in suspension culture for 3 days at four different concentrations of tangeretin. [6-^{3}H]Thymidine incorporation during the final 24 h of culture was calculated per unit volume and expressed as a percentage of incorporation into tangeretin-free cultures. Mean ± standard deviation.

cells to become less elongated, showing a projection on the substrate that became more circular. The 'corrected constant area' parameter reflects, as a percentage of the mean surface of the projection of the cell on the substrate at the limits of the time interval considered, the extent to which the projection on the substrate of the cell, after correction for movement of the geometric centre of the cell, still occupies the same position after a certain time interval. As table 3 shows, tangeretin, at a concentration of 0·050 mM, slowed the rate at which MO_4 cells on tissue culture plastic change their shape or their orientation on the substrate (as judged from their phase contrast image). The flavonoid also diminished the speed of translocation of individual MO_4 cells on tissue culture plastic.

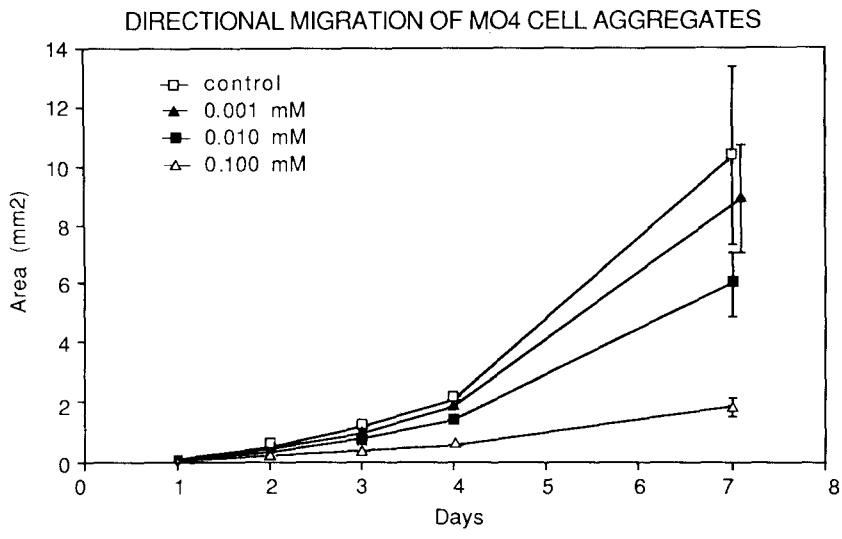


Figure 7. Effect of tangeretin on directional migration of MO_4 cell populations. MO_4 cell aggregates were explanted on a tissue culture plastic substrate at different concentrations of tangeretin. From daily measurements of the largest and the smallest diameter, the area covered by the explants was calculated. Mean ± standard deviation.

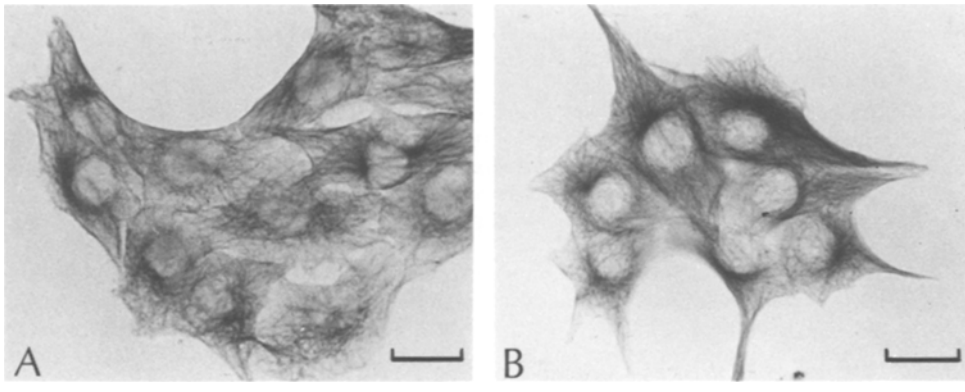


Figure 8. Effect of tangeretin on the cytoplasmic microtubule complex (CMTC) of MO_4 cells. MO_4 cells were cultured on glass cover-slips in the presence of different tangeretin concentrations up to 0·100 mM for 24 h. Immunocytochemical staining for tubulin revealed no CMTC differences between untreated cultures (A) and cultures treated with 0·100 mM tangeretin (B). Scale bar: 20 μm.

ATP content of MO_4 cells

Untreated MO_4 cells contained $5{\cdot}06 \times 10^{-9}$ mol ATP/μg protein. After correction for quenching of ATP determination due to tangeretin (23 per cent at 0·100 mM), the ATP content in treated (0·100 mM tangeretin for 24 h) MO_4 cultures was $1{\cdot}57 \times 10^{-9}$ mol ATP/μg protein. Hence 0·100 mM tangeretin reduced the ATP content of MO_4 cells to 31 per cent.

Table 2. Effect of tangeretin on morphology of MO_4 cells on tissue culture plastic.

	mM tangeretin (mean ± standard deviation)		
Parameter[a]	0·000	0·050	p[b]
Area	568 ± 204	835 ± 502	< 0·001
Conv. peri./peri.	0·845 ± 0·057	0·848 ± 0·058	> 0·1
D_{min}/D_{max}	0·406 ± 0·179	0·522 ± 0·182	< 0·001
4 pi ar/peri^2	0·361 ± 0·195	0·507 ± 0·210	< 0·001
b/a	0·335 ± 0·164	0·407 ± 0·144	< 0·005

[a] see Materials and methods section for explanation of parameters.
[b] in the Mann–Whitney U-test.

Table 3. Effect of tangeretin on motility of MO_4 cells.

		mM tangeretin (mean ± standard deviation)		
Parameter[a]		0·000	0·050	p[b]
Constant area	1 h (per cent)	40·14 ± 20·12	55·81 ± 17·19	0·007
	2 h (per cent)	35·89 ± 18·61	50·94 ± 15·00	0·009
	4 h (per cent)	1·77 ± 3·67	34·8 ± 24·77	< 0·001
Corrected constant area	1 h (per cent)	69·07 ± 13·37	75·20 ± 11·51	0·051
	4 h (per cent)	50·91 ± 15·69	64·59 ± 16·75	0·008
Translocation distance	1 h (μm)	15·25 ± 6·76	6·18 ± 5·38	< 0·001
	4 h (μm)	29·78 ± 19·54	15·88 ± 10·6	0·003

[a] see Materials and methods section for explanation of parameters.
[b] in the Mann–Whitney U-test.

Gel filtration profile of fucosylated surface glycopeptides

Tangeretin treatment of MO_4 cells only altered the relative proportions of the peak areas, but did not induce shifts of the peaks towards different apparent molecular volumes (figure 9). The amount of L-[6-^{3}H]fucose incorporation into MO_4 cells treated with 0·100 mM tangeretin during 24 h was 40 663 dpm/mg protein. This was 45 per cent of the amount incorporated in untreated cultures (90 218 dpm/mg protein). Taken together, these data indicate that tangeretin slowed the incorporation of fucose into glycopeptides, but did not alter the profile of the glycan moieties.

Binding of tangeretin to ECM

In the presence of an excess of human amniotic ECM, binding of tangeretin was tested at concentrations ranging from 0·005 to 0·050 mM ($n = 16$). Tangeretin binding to the ECM ranged from 0 to 12 per cent. The mean (6 per cent) was at least ten times lower than that found for (+)-catechin solutions in the same assay.

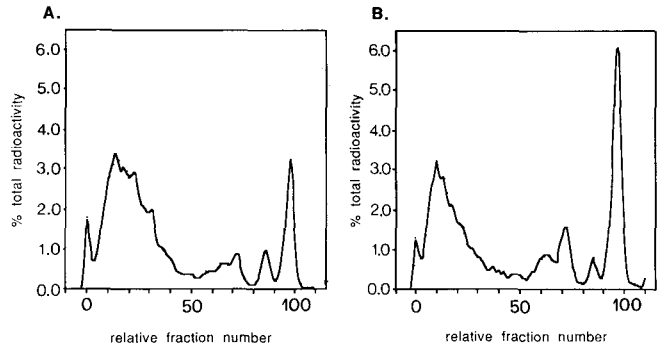


Figure 9. Effect of tangeretin on the gel filtration profile of fucosylated surface glycopeptides. MO_4 cells were treated with 0·000 mM (A) or 0·100 mM (B) tangeretin for 48 h and labeled with L-[6-^{3}H]fucose during the last 24 h of culture. After trypsinization and exhaustive pronase digestion the fucosylated glycopeptides were filtered on Bio-Gel P10. Abscissa: relative fraction number; 0 and 100 correspond to the fractions containing dextran blue (void volume = 37 ml) and phenol red (inner volume = 180 ml), respectively. Ordinate: percentage of total radioactivity.

Discussion

In addition to the demonstration of an anti-invasive effect on MO_4 invasion *in vitro*, our confronting cultures treated with tangeretin gave at least two hints concerning the possible mechanism of action of the flavonoid. First, treatment of either the heart fragments during the preculture period or of the confronting cultures separately had no inhibitory effect on MO_4 invasion, while treatment during both the preculture and confronting culture periods did inhibit invasion. This indicates that the tangeretin target cannot be localized in MO_4 cells either or in PHF exclusively. With another anti-invasive flavonoid, (+)-catechin, the major impact of the flavonoid could be explained through an interaction with PHF [6]. Second, postculture without tangeretin showed that its anti-invasive activity cannot be ascribed to an irreversible cytotoxic effect on the MO_4 cell population. This was confirmed by explantation experiments showing that solitary PHF and MO_4 aggregates were able to adhere to and migrate on tissue culture plastic after treatment with tangeretin.

We were unable to find indications that tangeretin would act via a binding to ECM or the plasma membrane of MO_4 cells. Binding to human amnion ECM appears to be a useful screening method for interactions with ECM molecules that may be relevant to tumor invasion. The amnion ECM contains, in addition to fibronectin and collagen (types I, III, IV and V), also laminin [35]. To these molecules a role during invasion has been ascribed [16, 27]. Only poor binding, however, between tangeretin and amnion ECM could be demonstrated. This was confirmed by another set of experiments in which MO_4 cells were seeded on amnion basement membrane; pretreatment of the membrane with tangeretin did not alter MO_4 cell adhesion or spreading (unpublished results). Fucosylation of plasma membrane glycoproteins was not altered qualitatively by tangeretin, but the amount of the incorporation was lower, probably owing to a slower metabolism of the cells. These fucose incorporation experiments indicate that the anti-invasive activity of the flavonoid can hardly be the result of a change in the 'malignant' phenotype [3] of the MO_4 plasma membrane glycosylation profile.

As both growth and locomotion of the MO_4 cells are inhibited by the flavonoid, the targets of tangeretin are presumably intracellular. The effect on growth of MO_4 cell aggregates was not a result of increased cell shedding from the aggregate into the liquid culture medium. Colony formation experiments showed that, even when a soft agar medium prevented cell shedding, tangeretin still inhibited growth of the cell groups. The evidence that tangeretin inhibits MO_4 cell proliferation was supported by our thymidine incorporation experiments (the flavonoid blocked DNA synthesis). Growth inhibition, however, cannot be held responsible for the lack of invasion in confronting cultures treated with tangeretin. Our studies on the effect of flavonoids on MO_4 cell invasion provided us with arguments to support the claims of other authors that growth and invasion are basically independent activities of tumor cells at least *in vitro* [21, 39]. Three congeners of tangeretin (hesperetin, nobiletin and naringin, kindly provided by Dr J. Attaway) were potent growth inhibitors at 0·100 mM, but they did not block invasion of MO_4 cells. Other flavonoids such as (+)-catechin [6] and troxerutin (unpublished results) did not block cell proliferation but were able to inhibit invasion at approximately the same concentrations.

One possible clue to explain the anti-invasive activity of tangeretin is the inhibition of MO_4 cell motility. A number of arguments suggested that tangeretin might act as a microtubule inhibitor [23]: first, directional migration of MO_4 cells was inhibited; second, image analysis of treated cells indicated that the cells tended to occupy a larger surface area on tissue culture plastic and to become less elongated; third, these phenomena were accompanied by inhibition of proliferation of the cells. However, further analysis of cell translocation indicated that tangeretin did not inhibit direction finding *per se*, but slowed both stationary and translocative cell motility on tissue culture plastic. As immunocytochemistry of the CMTC in MO_4 cells treated with tangeretin showed no disturbed complex and as no accumulation of mitotic figures, typical of microtubule inhibitors, could be observed on histological sections from tangeretin-treated cultures, no evidence exists to suggest that tangeretin is a microtubule inhibitor, and we consider that the flavonoid interacts with cell translocation in a less specific way. The low ATP content in cells treated with tangeretin also indicates that the flavonoid does not affect a unique cell function. A general inhibition of cell motility could well be invoked to explain the anti-invasive activity of tangeretin and this would be in accordance with the vast body of evidence showing that cell motility is necessary for invasion [38].

Few data in the literature are available concerning biological effects of tangeretin. This flavonoid, which appears to possess a potent antifungal activity [40], inhibits the release of histamine from basophils [29]. In contrast with other agents that inhibit histamine release, tangeretin would not act via protein kinase C inhibition [30]. The flavonoid induces the cytochrome P450-dependent monooxygenase system [44], inhibits lymphocyte responses [31] enhances Ca^{2+}–ATPase activity of female erythrocyte membranes [14], inhibits smooth muscle contraction [18] and inhibits iodothyronine deiodinase [13]. Tangeretin shares its anti-invasive activity with (+)-catechin, but the mechanisms of action of (+)-catechin appear to be different. (+)-Catechin binds to the ECM glycoprotein laminin, interferes with MO_4 cell adhesion and spreading on laminin containing substrates *in vitro*, and thus prohibits one of the earliest steps in invasion, i.e. adhesion to the ECM of the host tissues [4].

In conclusion, tangeretin inhibits invasion of MO_4 cells into embryonic chick heart *in vitro*, possibly by interfering with cell motility. Its mechanism of action

certainly differs from that of another anti-invasive flavonoid, (+)-catechin. This indicates that flavonoids may be new tools for studying mechanisms of tumor invasion.

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