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Specific antitumor activities of natural and oxovanadium(IV) complexed flavonoids in human breast cancer cells†

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The structure–activity relationships of the natural flavonoids quercetin, chrysin and silibinin have revealed that they meet the key structural requirements for killing tumor cells. When the structures of these three flavonoids are modified by complexation using the oxovanadium(IV) cation their cytotoxic properties in human breast cancer cell lines are enhanced. Breast epithelial cells are used to determine the selectivity of these compounds. The mechanisms of action of the flavonoids and the oxovanadium(IV) cation in the MDA-MB231 cell line seem to be different to the apoptotic mechanisms of cell death exerted by the oxovanadium(IV) complexes. These results showed the mechanisms of the antitumoral effect of these complexes, making them promising compounds for cancer treatment.

Introduction

Breast cancer is the leading cause of cancer death among women. Relapses occur in 50% of the patients despite radical surgery and there is no curative therapy for metastatic breast cancer. Current studies have shown that the ingestion of diets high in fruits and vegetables may decrease the risk of cancer, cardiovascular disease, and immunodysfunctions and their health effects have been attributed to flavonoids, a major class of phytochemicals found ubiquitously in them.[1] Therefore, searching for new anticancer flavonoids with higher bioactivities has been a particularly explored area. In this context, it was determined that the flavonoid quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one), Fig. 1, has a strong growth inhibitory effect on several cancer cell lines such as human colon and breast.[2–5] The natural flavonoid silibinin ((2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl]chroman-4-one), Fig. 1, has a wide range of pharmacological effects, such as inhibition of cell proliferation, cell cycle progression, and induction of apoptosis in various cell lines, including fibroblasts, colon, breast and lung cancer cells.[6–9] Moreover, it was reported that chrysin (5,7-dihydroxy-2-phenyl-4*H*-chromen-4-one), Fig. 1, displayed multiple biological activities, such as antiinflammatory and anticancer. Specifically, chrysin has shown anticancer activity in leukemia, anaplastic thyroid and colon cancer cells.[10,11] The inhibition of growth in anaplastic thyroid cancer cells by chrysin was reported to occur *in vitro via* apoptosis.[12,13]

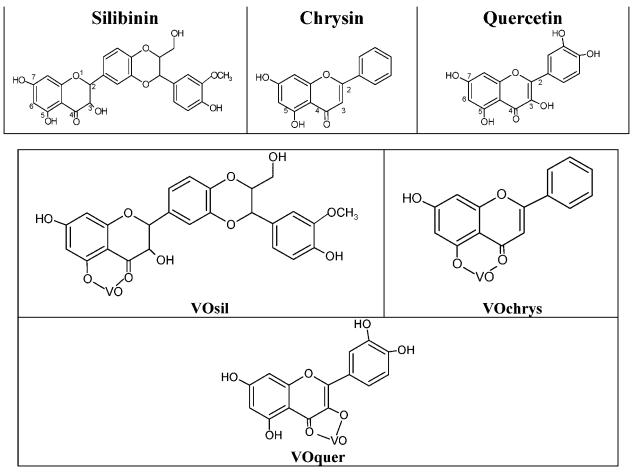

Fig. 1 Schematic structures of the flavonoids silibinin, chrysin and quercetin and schematic representation of the coordination sites of the different flavonoids to the metal.

The bioactivities of the flavonoids are strongly dependent on their molecular structures.[14] On this basis, it is very essential to modify their functional groups to improve their physiological actions such as antitumoral and antioxidant. Taking into

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consideration that to date there has been great interest in the potential antitumoral effects of oxovanadium(IV) ($V(IV)O^{2+}$) compounds,[15,16] we have prepared and studied the antioxidant and antitumoral actions of the oxovanadium(IV) coordination complexes with quercetin (VOquer), silibinin (VOsil) and chrysin (VOchrys) in two osteoblast cell lines (MC3T3E1 and the tumoral line, UMR106) in culture.[17–19] Furthermore, the action of chrysin and VOchrys on cytotoxicity, genotoxicity and oxidative stress in the MG-63 cell line (human osteosarcoma) was evaluated.[20] Considering that metastasis to bone occurs most frequently in breast cancer, the objective of the present work is to assess and compare the effects of each flavonoid and its complexes on the viability of four human cancer cells (MDA-MB231, MDA-MB468, T47D and SKBR3) and the normal breast epithelial cell line for comparative purposes. The present work provides new evidence that these complexes possess superior anticancer effects against those types of breast cancer cells. It is a well known fact that cell death can occur either by apoptosis (a highly regulated pathway involving signal transduction cascades), by necrosis (a more chaotic way of dying, which results from circumstances outside the cell, and is characterized by cellular edema and disruption of the plasma membrane) or by other caspase-independent programmed cell death (PCD) processes like paraptosis, mitotic catastrophe, slow cell death or autophagy.[21] We have studied herein the probable pathway by which the complexes induced cell death in the MDA-MB231 breast cancer cell line using different tests like activation of caspase 3/7, measurement of intracellular ROS content (using the CM-H2DCFDA probe), and mitochondrial membrane potential (using the TMRM probe), lactate dehydrogenase (LDH) release and DNA-damage (histone phosphorylation) by immunohistochemistry determination.

The results are indicative of a probable mechanism of action with DNA injury for VOquer and the activation of apoptotic processes in various steps along the death signaling pathway for the other complexes. On the other hand, the mechanisms of action of the flavonoids and the oxovanadium(IV) cation seem to be different to the mitochondrial apoptotic pathway.

Results and discussion

Cell viability: WST-8 assay

The cytotoxic effect on the MDA-MB231, MDA-MB468, T47D, SKBR3 cell lines and breast epithelial cells treated with different concentrations of oxovanadium(IV), the flavonoids or their complexes was determined (Fig. S1, ESI†). The effect of the compounds at 10 μM and 100 μM concentrations in the MDA-MB231 cell line is displayed in Fig. 2 and Table 1. Quercetin exerts a deleterious effect at 10 μM concentration inhibiting *ca.* 25% of cell viability (except SKBR3 with 15% inhibition) and in a dose–response manner (see Fig. 2) in accordance with previous reports for some of these cell lines.[2,5] A little cytotoxic effect was observed for the two tested concentrations in the normal cells. Silibinin at 10 μM concentration displays a different behavior (cytotoxic for MDA-MB468, MDA-MB231 and SKBR3 lines and proliferative for normal and T47D lines). At 100 μM concentration the flavonoid exerts a cytotoxic effect manifested by total cell killing for the first three cell lines and *ca.* 30% effect for normal and T47D lines. The cytotoxic effect on MDA-MB231 breast cancer cells was reported previously[9] and the mechanism of action was explained by the enhancement of the G2/M phase of the cell cycle through the down-regulation of cyclin B1 and cdc2 expression and the up-regulation of p21 expression in breast cancer cells. In contrast, S. Kim *et al.* found that the viabilities of MCF7 and MDA-MB231 cells were not affected by silibinin at various concentrations.[22]

Chrysin behaves as a specific and selective flavonoid. It stimulates survival of T47D and breast epithelial cell lines at both concentrations. At 10 μM concentration it produces cell death in the other tumor cell lines. Chrysin showed dose-dependent inhibition on U87-MG, MDA-MB-231, U-251 and PC3 proliferation, and displayed apoptotic activity in U87-MG cells.[23,24] However, these studies did not report details about the apoptotic activity of chrysin in U-251, MDA-MB-231 and PC3 cells. Besides, the flavonoid behaved as an antiproliferative agent in MG-63 cell viability, inhibiting about 50% at 25 μM.[20] The IC_{50} values obtained for the three flavonoids are displayed in Table 2 (see Fig. S1 in ESI†).

The modified quercetin molecule, VOquer, produces a better antitumor effect in all the tested breast cell lines. At 10 μM concentration it stimulates the viability of the normal cell line and behaves as a cytotoxic agent against the cancer cells, improving then the antitumoral effect of quercetin. Both the flavonoid and the complex inhibit the cell growth in a dose-dependent manner (Fig. S1, ESI†) for most of the tumor cell lines. The VOsil complex affects the cellular viability of all tested tumor lines. In the normal cell line it displays a proliferative effect at low concentrations and has no effect at 100 μM concentration. A more deleterious behavior was observed for the VOchrys complex. It inhibits almost completely the viability at both concentrations in the MDA-MB231 cells and the total inhibition in the other breast cancer cell lines is observed at 100 μM concentration. In the breast epithelial cells it displays a proliferative effect at low concentrations and a small deleterious effect at higher concentrations. The complex VOchrys also displayed a strong deleterious effect on MG-63 osteosarcoma cells but the effect is somewhat less than in breast cancer cells (*ca.* 80% inhibition).[20]

In conclusion, all the tested compounds behave as selective anticancer agents. They are active against human cancer cells but practically do not affect non-malignant epithelial cells.

High content assay

Taking into account that the cytotoxic behaviour of all the three complexes is maximum in the MDA-MB231 cell line in most of the tested compounds we have focused on the study of the mechanism of action of the flavonoids, the oxovanadium(IV) cation and their complexes in this cell line, working at 25 μM concentrations and 24 h of incubation in order to obtain a reasonable number of viable cells. First the cells were stained with the different fluorescent probes for 30 min (TMRM for the measurement of mitochondrial depolarization, CM-H2DCFDA for the detection of reactive oxygen species (ROS) production and CellEvent™ Caspase 3/7 Green Detection Reagent for the determination of the activation of

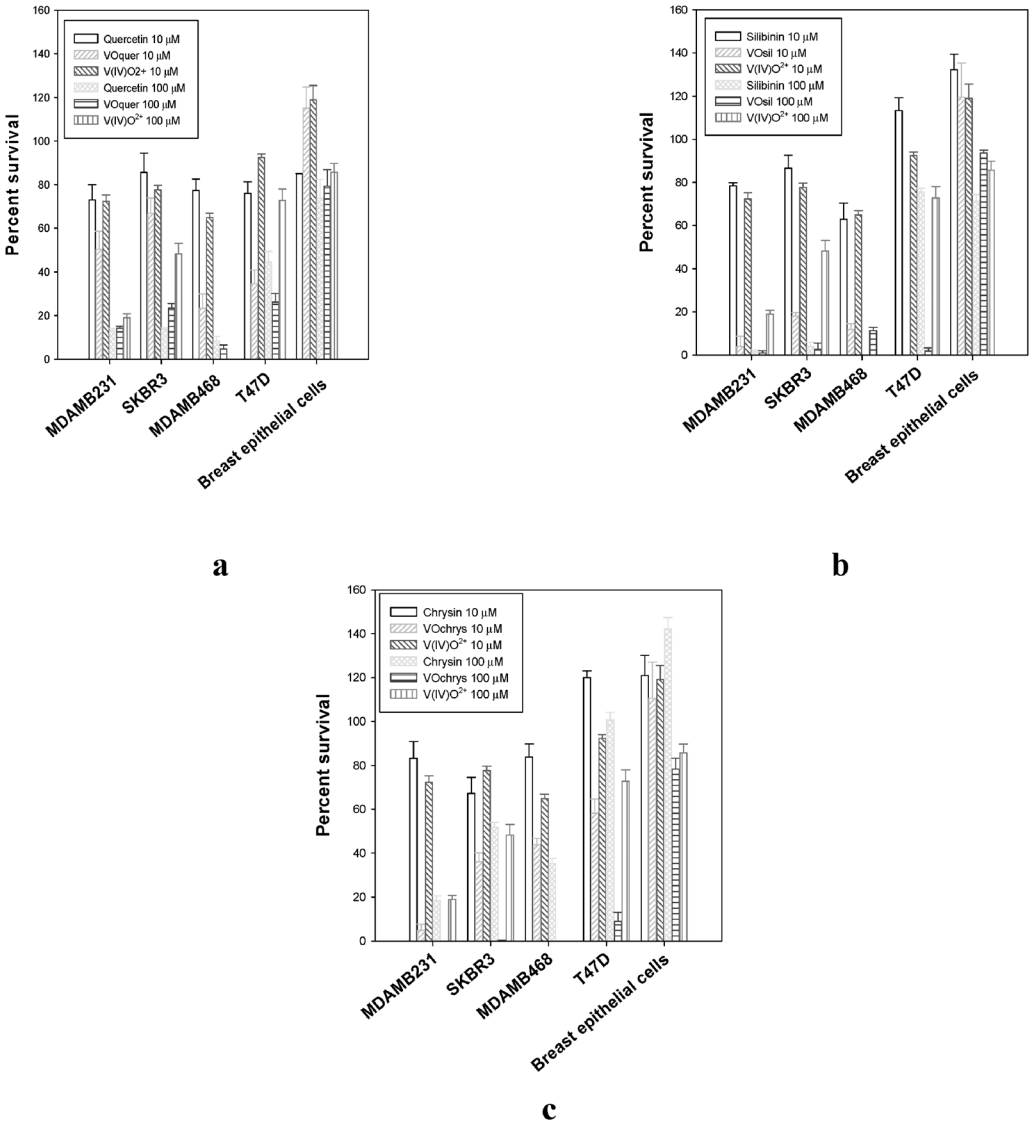

Fig. 2 Effects of the oxovanadium(IV) cation ($V(IV)O^{2+}$), quercetin and VO/quercetin (a), silibinin and VO/silibinin (b) and chrysin and VO/chrysin (c) on MDA-MB231, MDA-MB468, T47D, SKBR3 and breast epithelial cell line viability. Cells were incubated alone (basal) or with 10 μM and 100 μM of the compounds at 37 °C for 48 h. The number of basal cells is 5000 cells per well. The results are expressed as the percentage of the basal level and represent the mean ± the standard error of the mean (SEM) from three separate experiments.

Table 1 Effects of quercetin, silibinin, chrysin, the oxovanadium(IV) cation, and their complexes on the viability of human MDA-MB231 breast cancer and normal breast epithelial cell lines

% Survival (10 μM)		% Survival (100 μM)		
MDA-MB231	Breast epithelial cells	MDA-MB231	Breast epithelial cells	Compound
73.0 ± 7.0	84.9 ± 0.2	13.2 ± 0.9	73.8 ± 8.6	Quercetin
50.5 ± 8.1	115.1 ± 9.8	14.2 ± 0.9	79.4 ± 7.5	VOquer
78.4 ± 1.5	132.2 ± 7.1	1.4 ± 0.9	71.3 ± 3.1	Silibinin
3.9 ± 4.7	119.6 ± 15.8	0.9 ± 1.1	93.6 ± 1.3	VOsil
83.3 ± 7.6	120.9 ± 9.2	18.5 ± 2.1	142.2 ± 5.2	Chrysin
4.9 ± 2.7	110.6 ± 16.5	0	78.4 ± 5.0	VOchrys
72.3 ± 2.9	119.0 ± 6.5	18.9 ± 1.9	85.7 ± 4.0	$V(IV)O^{2+}$

caspase 3/7). After that, the cells were washed three times and treated with the different compounds.

Caspases (cysteine-aspartic proteases) are a family of cysteine proteases that play key roles in initiating and executing apoptosis. Members of the caspase family are activated sequentially following a wide variety of apoptosis-triggering events such as binding of ligands to a cell surface death receptor or the release of cytochrome *c* from mitochondria. In particular, caspases 3/7 are active effector caspases that proteolytically degrade a host of intracellular proteins to carry out the cell death program. Caspase activation is characteristic for the apoptotic process. It has previously been observed for the MDA-MB453 cell line that the mechanism by which quercetin induces apoptosis is either by the induction of the expression of cytochrome *c* or the cleavage of caspase-3.[3] It was also pointed out that quercetin may also inhibit cancer-cell proliferation at concentrations higher than 10 μM by inhibiting a variety of cellular enzymes.[5] In addition, it was determined that

Table 2 Half maximal inhibitory concentration (concentration able to inhibit 50% cell viability, IC_{50}) of quercetin, silibinin, chrysin, the oxovanadium(IV) cation ($V(IV)O^{2+}$), and their complexes in human breast cancer cells. Cells were incubated in basal medium alone or with increasing concentrations of flavonoids, $V(IV)O^{2+}$ or VO/flavonoids at 37 °C for 48 h. The IC_{50} calculation was performed using Sigma plot 10.2.2 software. Results are expressed as mean values ± SEM from three separate experiments

IC_{50} (μM)	MDA-MB231	SKBR3	MDA-MB468	T47D
Quercetin	49.6 ± 6.0	25.8 ± 4.5	23.8 ± 5.2	81.5 ± 4.8
VOquer	10.2 ± 8.0	22.8 ± 7.6	7.4 ± 5.4	4.8 ± 7.6
Silibinin	20.8 ± 2.5	35.8 ± 6.0	25.0 ± 7.5	>100
VOsil	6.3 ± 2.5	4.6 ± 5.0	5.1 ± 2.5	5.6 ± 2.5
Chrysin	58.3 ± 2.1	>100	62.0 ± 2.5	>100
VOchrys	3.0 ± 4.0	6.4 ± 3.0	8.7 ± 3.0	14.8 ± 6.5
$V(IV)O^{2+}$	49.0 ± 2.5	95.7 ± 4.8	19.4 ± 2.0	>100

chrysin induced apoptosis through caspase activation in U937 leukemia, HCT116 human colorectal cancer cells, the HepG2 human liver cancer cell line, and CNE-1 human nasopharyngeal carcinoma cells.[10,11,25] Studies in renal cancer Caki-1 and human prostate DU145 carcinoma cells showed that silibinin inhibits cell proliferation triggering the cascades of caspase pathways, leading to strong caspase-3 activation.[26,27] In disagreement with the observations in other cancer cell lines, we have not detected activation of caspase 3/7 with these flavonoids in the MDA-MB231 cell line at 24 h (Table 3 and Fig. 3).

The modified flavonoids induced activation of caspase 3/7. The higher effect was achieved by VOchrys being similar to the effect observed in MG-63 cells.[20] VOquer and VOsil produced the activation to a lesser extent (Table 3 and Fig. 3).

The membrane potential $\Delta\Psi_m$ is a key marker of mitochondrial function, generated by the pumping of protons across the inner mitochondrial membrane in association with electron transport. Upon induction of apoptosis, mitochondrial membrane potential is perturbed, which results in the release of cytochrome *c* from the mitochondria to cytosol, which ultimately leads to caspase 9 activation followed by caspase 3/7 activation and apoptotic cell death. Assessment of mitochondrial membrane potential ($\Delta\Psi_m$) represents one such index of mitochondrial function and a key process controlling the ultimate fate of a cell.[28] TMRM (tetramethylrhodamine methyl ester) has been used for labeling and measuring the membrane potential ($\Delta\Psi_m$) of mitochondria in living cells. The resulting signal of TMRM-stained cells thus represents only the probe accumulated in mitochondria and the cell retains the probe when the membrane potential remains intact. When there has been any damage in the mitochondrial membrane the amount of the probe diminishes.

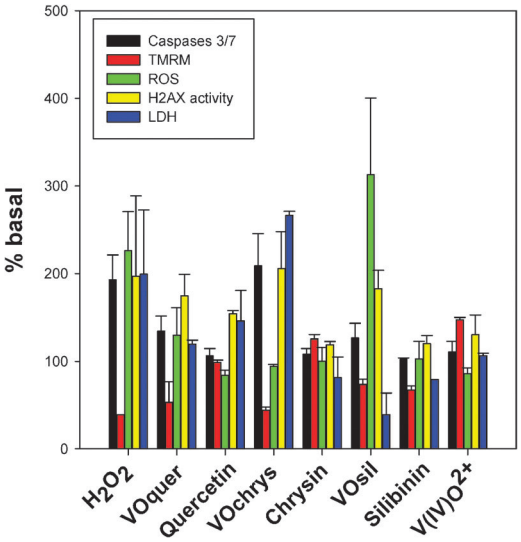

Fig. 3 Representative results for the MDA-MB231 cell line incubated with 25 μM flavonoids, the oxovanadium(IV) cation ($V(IV)O^{2+}$) and VO/flavonoids at 37 °C. Positive control: 100 μM H_2O_2 for caspase 3/7 activation, disruption of the mitochondrial membrane potential (TMRM), ROS generation, DNA damage (H2AX) and damage of the plasmatic membrane (LDH) measurements. Results are the percentage of the basal level and represent the mean values ± the standard error of the mean (SEM) from three separate experiments.

The results observed for the flavonoids and the complexes were compared with the positive control (H_2O_2). From Table 3 and Fig. 3 it can be seen that silibinin induced the activation of the mitochondrial death pathway in the tested cell line, this effect being similar to that found for the silibinin-treated MCF-7 cells.[29] The loss of the mitochondrial membrane potential produced by the oxovanadium(IV) complexes is putatively the initial event leading to apoptosis. A similar effect was observed for VOchrys in the MG-63 cell line.[20] No changes were observed when the cell line was treated with chrysin and quercetin.

A main feature of cancer cells, when compared to normal ones, is a persistent pro-oxidative state that leads to an intrinsic oxidative stress. Cancer cells have higher levels of reactive oxygen species (ROS) than normal cells, and ROS are, in turn, responsible for the maintenance of the cancer phenotype.[30] Non-transformed cells have a lower basal intracellular ROS level, and have a full antioxidant capacity, being less vulnerable

Table 3 High content cytotoxicity assay. Effect of flavonoids, the oxovanadium(IV) cation ($V(IV)O^{2+}$) and their complexes (25 μM, 24 h) on caspase 3/7 activation, disruption of the mitochondrial membrane potential (TMRM), ROS production, DNA damage (histone phosphorylation, H2AX) and damage of the plasmatic membrane (LDH) measurements. NO indicates no effect. Results are expressed as the percentage of the measured basal level and represent the mean values ± the standard error of the mean (SEM) from three separate experiments

MDA-MB231	VOquer (%)	Quercetin (%)	VOchrys (%)	Chrysin	VOsil (%)	Silibinin (%)	$V(IV)O^{2+}$
CASP 3/7	+34.2	NO	+108.9	NO	+26.4	NO	NO
TMRM	−47.1	NO	−55.9	NO	−26.6	−32.7	NO
ROS	+29.6	NO	NO	NO	+212.0	NO	NO
H2AX	+74.0	+54.0	+105.0	NO	+82.0	NO	NO
LDH	NO	+47.0	+167.0	NO	NO	NO	NO

to the oxidative stress induced by flavonoids and modified flavonoids.[30,31]

Given the role of antioxidants in cancer prevention and treatment it seems contradictory that a pro-oxidant therapy, which is based on the generation of oxygen radicals, could be used for cancer annihilation.[32] Although oxidative stress caused by ROS accumulation promotes tumor growth, high concentrations of ROS can also induce growth arrest or cell death and can therefore function as anti-tumorigenic species.[33]

The antioxidant flavonoids may function as terminators of free radical chains by interacting with other free radicals. Flavonoids are ideal scavengers of peroxyl radicals and in principle they are effective inhibitors of lipid peroxidation (eqn (1)).

$$\text{Flavonoid-OH} + \text{ROO}^{\bullet} \rightarrow \text{Flavonoid-O}^{\bullet} + \text{ROOH} \quad (1)$$

But if they act under certain conditions provided by the concentration and environment (oxygen pressure) they may produce oxidative stress behaving as pro-oxidants (eqn (2)).

$$\text{Flavonoid-O}^{\bullet} + \text{RH} \rightarrow \text{Flavonoid-OH} + \text{R}^{\bullet} \quad (2)$$

Likely, the generation of ROS may produce DNA damage as a consequence of the peroxide activation of flavonoids caused by one-electron oxidation catalyzed by peroxidase.[34,35]

On the other hand, the oxovanadium(IV) cation may also produce free radicals. It has been proposed that the interaction of oxovanadium(IV) with H_2O_2 and lipid hydroperoxide generates reactive species *via* a Fenton-like reaction (eqn (3)).[36]

$$\text{VO(IV)} + \text{H}_2\text{O}_2 \rightarrow \text{V(V)} + \text{OH}^{-} + \text{OH}^{\bullet} \quad (3)$$

The effects of flavonoids on ROS production in the MDA-MB231 cancer breast cell line and at 24 h incubation are shown in Table 3 and Fig. 3. No appreciable changes in ROS are observed. It was previously reported that quercetin strongly increased intracellular ROS levels in cancer cells[4,30] but at least in the MDA-MB231 line this effect was not observed by Akbas *et al.*[2] confirming our observations. Although multiple activities of quercetin were believed to arise from its antioxidant properties, it was recently suggested that quercetin behaves as a cytotoxic agent and as a mutagen which might affect cytotoxicity directly.[37]

When the cells were incubated with VOsil a higher production of ROS was observed. The production of ROS by VOquer was less significant. The reactive oxygen species have been implicated as a second messenger in multiple signaling pathways and can also play an important role in apoptosis by regulating the activity of certain enzymes involved in the cell death pathway. The growth inhibition and ROS generation caused by VOsil and VOquer indicates an apoptotic cell-death mechanism *via* the mitochondrial pathway.

The chrysin complex (VOchrys) behaved in a similar manner to the flavonoids. The schematic representation of the coordination sites of the different flavonoids to the metal ion is displayed in Fig. 1. In the three cases the pi bond of the V=O moiety is involved in electron delocalization along with the flavonoid ring. In the case of VOchrys the C2=C3 bond is next to a single bond and then the pi electrons of the system will in addition undergo resonance with the V=O atom. According to the structure–activity relationship it may be postulated that the presence of the free C2=C3 bond makes the VOchrys complex a better antioxidant compound. The images obtained from these assays are shown in Fig. 4.

Taking into account that the production of ROS is a dynamic effect, the generation of ROS *vs.* time was measured and compared with the effect of the positive control (hydrogen peroxide, Fig. 5). It can be observed that the different compounds stimulated ROS generations peaking at 5 h but VOsil maintained the generation with a maximum ROS production at 24 h. The mechanism of action of these compounds was not studied in the normal breast epithelial cells because the viability of this cell line was practically not affected by 10 μM of compounds. Considering the increase in the ROS level in the first 5 h of the assay, it could be postulated that ROS production may be the first step involved in the process of the cell death.

One of the first cellular responses to the introduction of DNA double-strand breaks is the phosphorylation of H2AX histone. H2AX is phosphorylated within 1 to 3 min after damage, and the number of H2AX molecules phosphorylated increases linearly with the severity of the damage.[38] Immunohistochemistry for H2AX protein was performed and the number of cells with DNA damage was measured using a BD Pathway™ Bioimager. From Table 3 it can be seen that quercetin produces

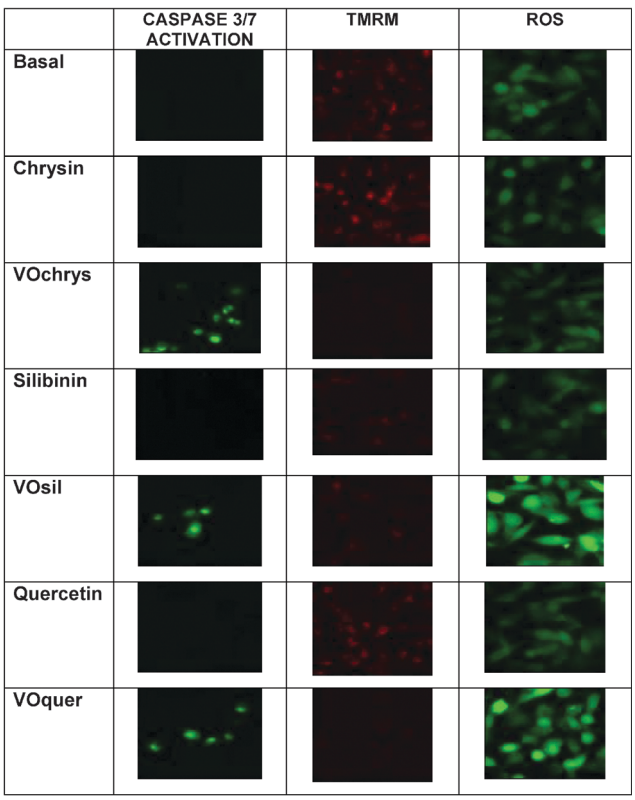

Fig. 4 Mechanisms of action of flavonoids and VOflavonoids in the MDA-MB231 breast cancer cell line. Cells were treated with 25 μM of chrysin, silibinin, quercetin, VOchrys, VOsil and VOquer for 24 h. Then the cells were incubated with CellEvent™ Caspase 3/7 Green Detection Reagent (for the assessment of caspase 3/7 activation), TMRM (for the measurement of mitochondrial depolarization) and CM-H2DCFDA (for the determination of reactive oxygen species production) for 30 min at 37 °C.

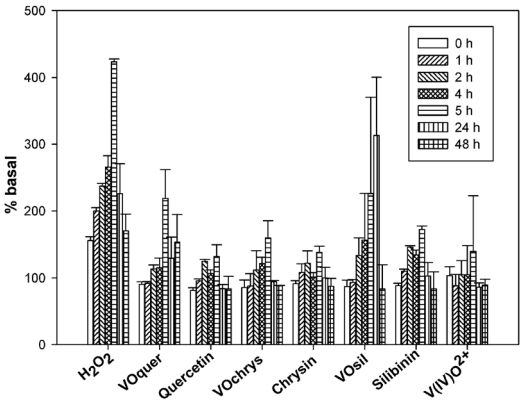

Fig. 5 Representative results for ROS measurement *vs.* time for MDA-MB231 incubated with 25 μM flavonoids, oxovanadium(IV) and VO/flavonoids at 37 °C. The results are expressed as the percentage of the basal level and represent the mean values ± the standard error of the mean (SEM) from three separate experiments.

DNA damage as previously observed in human lymphoblastoid cell lines.[39] On the other hand, the three flavonoid complexes induced a breakage of DNA with the consequent histone phosphorylation. It was previously observed that in the MG-63 cell line VOchrys induced single and double strand DNA breaks, leading to a positive result in the Comet assay and induction in micronuclei frequency.[20] Lactate dehydrogenase (LDH) is a soluble enzyme located in the cytosol. The enzyme is released into the surrounding culture medium upon cell damage or lysis, processes that occur during both apoptosis and necrosis. LDH activity in the culture medium can be used as an indicator of cell membrane integrity and for the measurement of cytotoxicity. The release of LDH from cells with damaged plasma membranes into the culture medium was assayed by incubating the clarified culture medium with sodium pyruvate and measuring the rate of oxidation of NADH to NAD+ at 340 nm.[40] Among the three tested flavonoids quercetin produced the release of LDH and this effect was also observed in other tumor cancer cells (colon, LoVo and breast, and MCF-7).[4] From Table 3 it can be observed that VOchrys produced the higher damage in the plasma membrane releasing the enzyme into the supernatant and displaying the higher enzymatic activity of LDH.

The biological effects of the oxovanadium(IV) cation were compared with the effects of the flavonoids and their complexes, under the same experimental conditions (Fig. 3). It can be seen that the cytotoxic effect of the oxovanadium(IV) cation (shown in Fig. 2 and Fig. S1, ESI†) in this breast cancer cell line is not related to the mitochondrial apoptotic pathway.

Conclusions

The structurally related flavonoids selected herein exerted deleterious effects on the breast cancer cell lines and these effects were in general improved when the structure of each polyphenol was modified by coordination. Different pathways were measured to determine the mechanism of action of cell death produced by the compounds.

These compounds alter the viability of the breast cancer cells in a dose–response manner selectively killing the tumor cells without affecting the viability of normal cells that have a lower basal intracellular ROS level and a full antioxidant capacity. According to our observations the mechanism of cell death exerted by the flavonoids seems to be different than those of the complexes. Quercetin produced histone phosphorylation and LDH release without caspase activation through a non-apoptotic pathway of cellular death. The flavonoid chrysin did not alter the parameters determined at 24 h but the inhibitory effect of cell survival at 25 μM concentration could be due to the generation of ROS that were detected at 5 h incubation. The other tested flavonoid, silibinin, produced a decrease in the membrane potential through a caspase-independent cell death pathway like quercetin. The cytotoxic effect of the oxovanadium(IV) ion on this cell line was exerted without mitochondrial membrane damage.

By the structural modification of the tested flavonoids their inhibitory effects on cell survival could be enhanced. The improved cytotoxic ability of VOchrys correlated with caspase 3/7 activation and the same effect was observed to a lesser extent for VOquer and VOsil. Besides, VOchrys produced higher damage in DNA and the mitochondrial and plasmatic membranes with activation of the intrinsic pathway of apoptosis (mitochondrial pathway). The VOsil complex generated the higher ROS production in the breast cancer cell line and an increase of histone phosphorylation indicating that the free radicals damaged DNA causing cell death by activation of the caspase pathway. When the cells were incubated with VOquer a slight increase in ROS production, caspase activation and histone phosphorylation and a low decrease in the membrane potential were observed indicating that the mitochondrial pathway was activated accompanied by increased DNA damage, probably by an intercalation mechanism.

We can conclude that:

– The modification of the structure of the selected flavonoids upon complexation with the oxovanadium(IV) cation improved their anticancer effects on the MDA-MB231 breast cancer cell line through different mechanisms of action.

– Owing to the biological and biochemical differences between cancerous and normal cells, these substances can achieve selectivity of treatment, while maintaining a weak or null impact on normal cells.

In a continuous effort to identify chemotherapeutic drugs that selectively kill tumor cells without side effects on normal cells, VO/flavonoids and flavonoids certainly remain attractive compounds for the treatment of primary breast cancer, even if further studies are needed.

Experimental

Materials

Human MDA-MB231, MDA-MB468, T47D, and SKBR3 breast cancer cell lines were obtained from HPA Culture Collection (Salisbury, United Kingdom). The first three cell lines were cultured in endotoxin-free RPMI medium supplemented with 10% FBS, 1% non-essential amino acids and 100 U mL^{-1} penicillin and

100 μg mL^{-1} streptomycin. SKBR3 cells were cultured in endotoxin-free McCoy medium supplemented with 10% FBS and 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. All reagents were from Sigma-Aldrich (St Louis, MO). Primary human mammary epithelial cells were obtained from ScienCell Research Laboratories and were grown in mammary epithelial medium supplemented with a 1% mammary epithelial growth supplement and 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and passaged according to the manufacturer's instructions. The complexes $[VO(Quer)_2EtOH]_n$, $[VO(chrysin)_2EtOH]_2$ and $Na_2[VO(silibinin)_2]\cdot 6H_2O$ were prepared and purified according to published procedures.[17–19]

Methods

WST-8 assay. Breast cancer cells and primary human mammary epithelial cells were seeded at a density of 5000 per well in 96 well plates, grown overnight and treated with either vehicle, flavonoids, VOflavonoids and $VOSO_4\cdot 5H_2O$ of different concentrations (1×10^{-8} to 100 μM) in FBS free medium. The dissolution vehicle was dimethyl sulfoxide to yield a maximum final concentration of 0.5% in the treated well (Sigma-Aldrich, St Louis, MO). The solid complexes $[VO(Quer)_2EtOH]_n$, $[VO(chrysin)_2EtOH]_2$ and $Na_2[VO(silibinin)_2]\cdot 6H_2O$ (VOflavonoids) remain stable in a DMSO solution (no changes were observed in the UV-vis spectra) for at least 30 min during their manipulation time for biological studies (15 min).

After 48 h of incubation at 37 °C, {2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt} (WST-8) was added at 100 μg per well for 2 h (Sigma-Aldrich, St Louis, MO). Reduction of WST-8 by the viable cells produces a water-soluble formazan which dissolves directly into the culture medium, eliminating the need for an additional solubilization step. Then, optical density was measured at 450 nm using a Synergy 2 Multi-Mode Microplate reader (BioTek, Winooski, USA). All experiments were done in triplicate.

High content analysis image assay. For the high content analysis image assay the MDA-MB231 cells were seeded at a density of 5000 per well in a collagen-coated 96 well plate and stained with fluorescent probes (Invitrogen, Life Technologies, Madrid, Spain) for 30 min: 50 μM tetramethylrhodamine methyl ester (TMRM) for the measurement of mitochondrial depolarization related to cytosolic Ca^{2+} transients, 1 μM 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) for the measurement of reactive oxygen species (ROS) production and 5 μM CellEvent™ Caspase 3/7 Green Detection Reagent for the measurement of caspase 3/7 activation. After 30 min cells were washed three times and treated with 25 μM concentration of flavonoids, VOflavonoids and oxovanadium(IV) for 24 h. A positive control of 100 μM H_2O_2 was used. The three probes were measured at 24 h and ROS localization was identified with an absorption/emission maxima of 475/515 nm, TMRM at 549/600 nm and CellEvent™ Caspase 3/7 Green Detection Reagent at 502/530 nm using the BD Pathway™ 20× Olympus Objective. Data were analyzed choosing a region of interest (Text Overlay: ROI (regions-of-interest)) tool from the AttoVision program to select the areas. Then, ROIs from the entire cell body, ROIs from mitochondrial regions or ROIs from nuclear regions in imaged cells were selected to measure the fluorescence intensities (Arbitrary Units – AU) of ROS, TMRM and Caspase 3/7 activation, respectively. The average fluorescence intensities were calculated from all ROIs of each cell for each time point subtracting the background fluorescence intensity (regions next to cells). After the measurements with the probes, conditioned medium from each well was collected for LDH assay and cells were fixed with 3% paraformaldehyde for 30 min at room temperature and permeabilized in 1% SDS for 10 min. Non-specific binding was blocked for 1 h with 4% BSA in PBS buffer and then the cells were incubated with 2 μg mL^{-1} mouse monoclonal H2AX (Abcam, Cambridge, United Kingdom) for 1 h at room temperature. Further wash steps removed unbound antibody prior to the addition of the secondary Alexa 633 goat anti-mouse antibody. Images of the number of cells with DNA damage were acquired on a BD Pathway™ Bioimager.

LDH assay. The LDH assay was performed according to the manufacturer's protocol. Fully automated 'Cytotoxicity detection kit (LDH)' (Roche, Mannheim, FR, Germany) was used to measure LDH release in milliunits per liter (mU L^{-1}) in culture media obtained from the breast cancer cell line subjected to antioxidant treatment described above for 24 h. The release of LDH from cells with the damaged plasma membrane into the culture medium was assayed by incubating the clarified culture medium with sodium pyruvate in the presence of NADH. Pyruvic acid is catalyzed into lactic acid by free LDH along with a simultaneous oxidation of NADH to NAD+. The rate of oxidation of NADH to NAD+ was measured at 340 nm using the Synergy 2 Multi-Mode Microplate reader (BioTek, Winooski, USA).

At least three independent experiments were performed for each experimental condition in all the biological assays. The results are expressed as the mean ± the standard error of the mean.

Abbreviations

CM-H2DCFDA	5-(and-6)-Chloromethyl-2,7,dichlorodihydrofluorescein diacetate acetyl ester
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
H2AX	Subtype of H2A histone
LDH	Lactate dehydrogenase
PBS	Phosphate-buffered saline
ROI	Regions-of-interest
ROS	Reactive oxygen species
RPMI medium	Roswell Park Memorial Institute culture medium
TMRM	Tetramethylrhodamine methyl ester
VOchrys	$[VO(chrysin)_2EtOH]_2$
VOquer	$[VO(Quer)_2EtOH]_n$
VOsil	$Na_2[VO(silibinin)_2]\cdot 6H_2O$
WST-8	{2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt}

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