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Biochemical Pharmacology 61 (2001) 1205–1215

Biochemical Pharmacology

# Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1

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Received 10 July 2000; accepted 25 September 2000

**Abstract**

We have investigated the effects of a series of flavonoids on cell proliferation and cell cycle distribution in human melanoma cells OCM-1. Among the compounds that potently inhibited OCM-1 cell proliferation, we show that the presence of a hydroxyl group at the 3′-position of the ring B in quercetin and luteolin, correlated to a G1 cell cycle arrest while its absence in kaempferol and apigenin correlated to a G2 block. Genistein with a hydroxyl at 5-position of the ring A arrested cells in G2 while daidzein which lacks it, induced an accumulation of cells in G1. We demonstrate that flavonoids, which induced a cell cycle block in G1, inhibited the activity of CDK2 by 40–60%. By contrast, those which caused an accumulation of cells in G2/M were without effect. On the other hand, while quercetin, daidzein and luteolin did not alter the activity of CDK1, kaempferol, apigenin and genistein inhibited this kinase by 50–70%. We demonstrate that the up-regulation of the CDK inhibitors $p27^{KIP1}$ and $p21^{CIP1}$ is likely responsible for the inhibition of CDK2 while inhibition of CDK1 was rather due to the phosphorylation of the kinase on Tyr15 residue. 

*Keywords:* Flavonoids; Cell cycle; Proliferation; Cyclin-dependent kinase; Melanoma cells

## 1. Introduction

Flavonoids are polyphenolic compounds naturally present in vegetables, fruits and beverages such as tea and wine [1]. People from European countries consume several mg of flavonoids per day in the common diet. A large number of epidemiological studies [2–4] as well as experiments performed on animal models [5–8] suggest that these compounds can prevent or inhibit cancer development. They are described as anti-oxydant [9,10] and anti-angiogenic [7,11] agents and they have been found to inhibit cell growth *in vitro* [12,13].

More than 4,000 different flavonoids have been categorized into flavones, flavonols, flavanones, flavanonols, flavan-3-ols and isoflavones (Fig. 1). They all consist of a benzene ring (A) fused with a pyrone ring (C) that in position 2 or 3 carries a phenyl ring (B) as a substituent. Flavonoids have been shown to inhibit several kinases involved in signal transduction, such as protein kinases C [14,15], tyrosine kinases [16,17], PI 3-kinases [15,18], or S6 kinase [19]. They can also interact with estrogen type II binding sites [20]. However, the precise mechanism responsible for the antitumoral effects of these compounds is not yet clearly understood.

Flavonoids have been found to arrest cell cycle progression either at G1/S or at G2/M boundaries. While flavone, luteolin, or daidzein have been shown to arrest human gastric cancer cells in G1, the isoflavone genistein has been found to block these cells at the G2/M transition [21]. This might be dependent on the flavonoid structure. However, conflicting results have been reported with regard to the stage-specific cell cycle arrest caused by the same compound. For example, quercetin has been shown to block the cell cycle at the G1/S transition in colon and gastric cancer cells as well as in leukemic cells [22–24]. By contrast, it has been found to cause a G2/M block in breast and laryngeal cancer cell lines or in non-oncogenic fibroblasts [25–27].

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*Abbreviations:* CDK, cyclin-dependent kinase; CKI, CDK inhibitor; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; DTT, dithiothreitol; RIPA, radioimmunoprecipitation assay buffer.

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PII: S0006-2952(01)00583-4

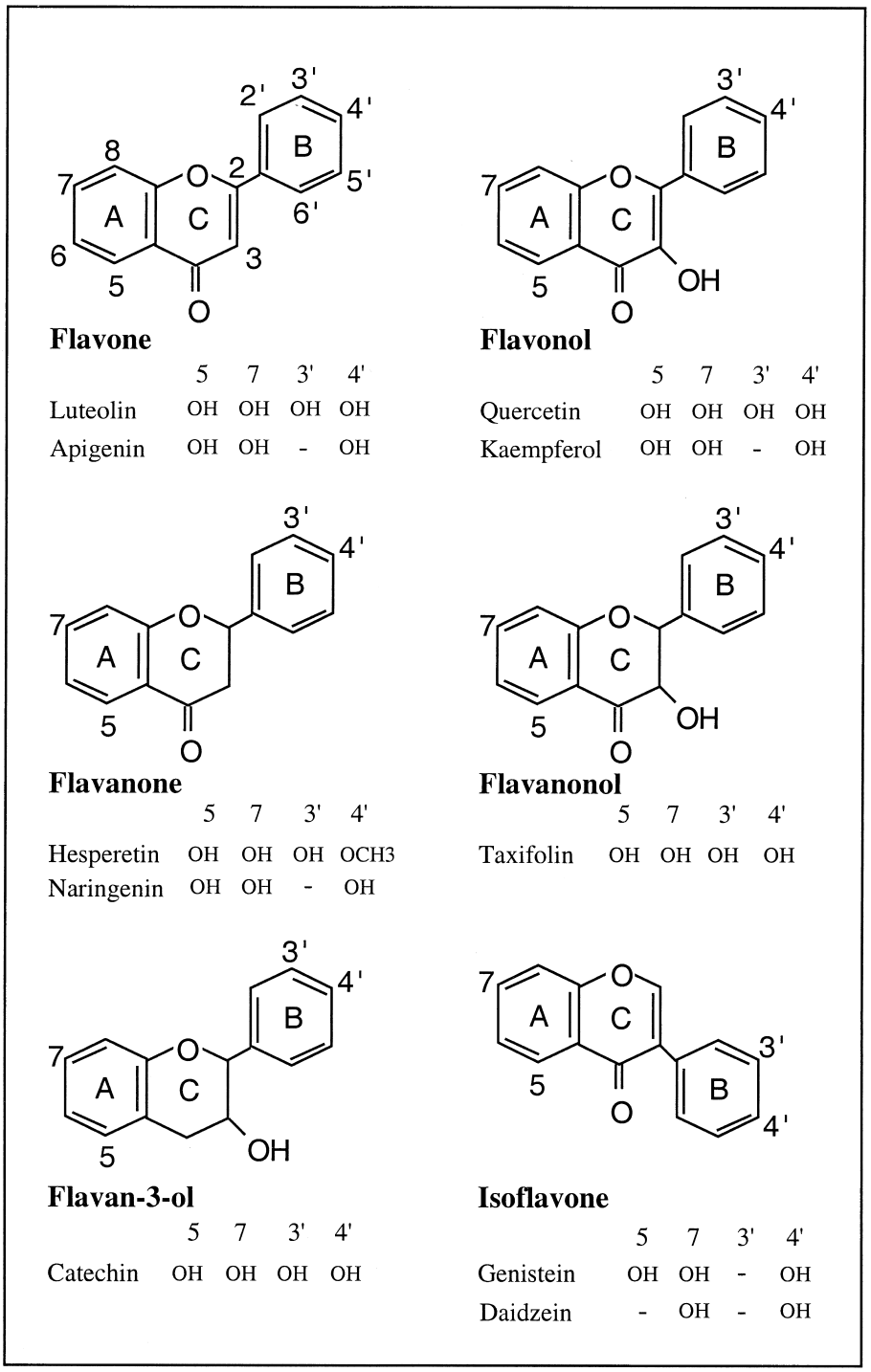


Fig. 1. Structure of the different chemical classes of flavonoids used in this study.

Genistein has been reported to arrest cells in G2 in several cell models [21,28–31] while it has been shown to induce both G1 and G2 blocks in BALB/c 3T3 fibroblasts or mouse melanoma cells [32]. Apigenin has been shown to induce a G2/M arrest in neuronal or keratinocyte cell lines [33,34] while it has been reported to block human diploid fibroblasts in both G1 and G2 [35].

Cell cycle progression is orchestrated by cyclin-dependent kinases (CDKs) whose activity is firstly dependent on association with cyclin subunits [36]. G1 progression and G1/S transition are regulated by CDK4 (and CDK6) which assembles with cyclins D in mid-G1 and CDK2 which combines later with cyclin E. While CDK2 controls S-phase when associated with cyclin A, G2/M transition is regulated

by CDK1 in combination with cyclins A and B. Activation of CDKs also needs a complex set of phosphorylations and dephosphorylations on specific residues [36]. In particular, dephosphorylation of CDK1 on Thr14 and Tyr15 residues by the dual-specificity phosphatase CDC25C has been demonstrated to be an absolute requirement for the onset of mitosis [37]. Finally, CDK activity is counterbalanced by a variety of low molecular weight CDK inhibitors (CKIs). Two gene families of mammalian CKIs have been identified, one including $p21^{CIP1}$ and $p27^{KIP1}$ and the other one $p16^{INK4A}$ and $p15^{INK4B}$ [38]. The INK4 family specifically binds CDK4 (and CDK6) and inhibits complex formation with cyclins D. The CIP/KIP family seems to function on a broader spectrum of CDKs and inactivates CDK-cyclin complexes by stoechiometric binding.

In this paper, we attempt to establish the possible relationships between the structure of flavonoids and their effect on cell cycle progression in human melanoma cells OCM-1. To our knowledge, such an extensive structure/activity study on a given cell model has never been reported with regard to the cell cycle. In order to define more precisely the mechanism of action of flavonoids, we further investigated their effect on CDK2 and CDK1 activities, shown to control respectively the G1/S and G2/M transitions. We also looked at their ability to up-regulate the CKIs $p21^{CIP1}$ and $p27^{KIP1}$ and to alter the phosphorylation state of CDK1.

## 2. Material and methods

### 2.1. Cell culture

Human choroidal melanoma cells (spindle-shape OCM-1 cell line) were cultured in RPMI-1640 medium, pH 7.3, supplemented with 100 IU/mL penicillin, 100 $\mu$g/mL streptomycin, 2.5 $\mu$g/mL amphotericin B, 2 mM L-glutamine, and 5% fetal calf serum (FCS). Cells were grown at 37° under 5% $CO_2$ atmosphere. All culture reagents and media were from Gibco. Culture media were changed every 2–3 days. When they reached confluence, cells were dissociated by 0.05% trypsin-0.02% EDTA and replated at 1:30 dilution.

### 2.2. Cell proliferation measurement

For cell growth measurement, melanoma cells were seeded at an initial density of $3 \times 10^4$ cells per 35-mm dish. Indicated concentrations of flavonoids (Alexis) dissolved in DMSO were added at day 0. Control dishes received the same volume of the solvent DMSO (final concentration of 0.1%). After 48 hr, cells were harvested with trypsin-EDTA and cell number was determined by using a Coulter Counter (Coultronics).

### 2.3. Cell cycle progression analysis

Cells were plated at a density of $2–4 \times 10^5$ cells per 100-mm petri dish in the absence or presence of the indicated concentrations of flavonoids. Control cells were incubated with the same final concentration of solvent. After 1 to 3 days, approximately $10^6$ cells were harvested by brief trypsinization and centrifuged at 500 g for 5 min. The cell pellet was washed twice in PBS and fixed by the gradual addition of ice-cold 70% ethanol. Cells were then labelled with propidium iodide and the cell cycle distribution was determined by flow cytometry analysis using a Coulter Elite.

### 2.4. Immunoblotting and immunoprecipitation

Whole cell lysates were prepared by directly lysing cells in sample buffer. Proteins (approximately 100 $\mu$g) were separated by electrophoresis on 10% SDS-PAGE. Gels were either stained with Coomassie blue to control for balanced loading or electroblotted to nitrocellulose membranes (BA85 from Cera-Labo) for 1 hr at 20 V using a semi-dry transfer apparatus. Immunoblots were developed by using the ECL detection system (Amersham) according to the manufacturer's instructions.

For immunoprecipitation experiments, cells ($1–2 \times 10^6$) were lysed in RIPA buffer containing 50 mM HEPES, pH 7.3, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 1 mM sodium fluoride, 10 mM $\beta$-glycerophosphate, 0.1 mM sodium orthovanadate, 10 $\mu$g/mL aprotinin, 10 $\mu$g/mL leupeptin, 0.1 mM PMSF, 0.1% Tween 20 and 10% glycerol. The lysates were clarified by centrifugation at 12,000 *g* for 10 min at 4° and the protein contents were estimated by electrophoresis on SDS-PAGE and Coomassie blue staining. Immunoprecipitations were performed using the same amount of proteins (1 mg) for the different samples. Antibodies previously bound to protein A-Sepharose CL-4B beads (Pharmacia) were added to the cell lysates for 12 hr at 4° with gentle agitation. Immune complexes were collected by centrifugation and washed three times in RIPA buffer and twice in PBS. The immune complexes were fractionated on SDS-PAGE. After electroblotting, analysis of CDKs or associated CKIs was carried out by immunoblotting the membranes with specific antibodies.

### 2.5. Antibodies

Monoclonal anti-CDK1 (SC54) and anti-cyclin B1 (SC245) as well as polyclonal anti-CDK2 (SC163), anti-$p21^{CIP1}$ (SC397) and anti-$p27^{KIP1}$ (SC528) antibodies were from Santa Cruz Biotechnology. Polyclonal anti-phospho-CDK1 (Tyr15) antibody was from BioLabs.

### 2.6. Protein kinase assay

For CDK1 and CDK2 activity assays, immunoprecipitations (0.25 to $0.75 \times 10^6$ cells) were performed as described

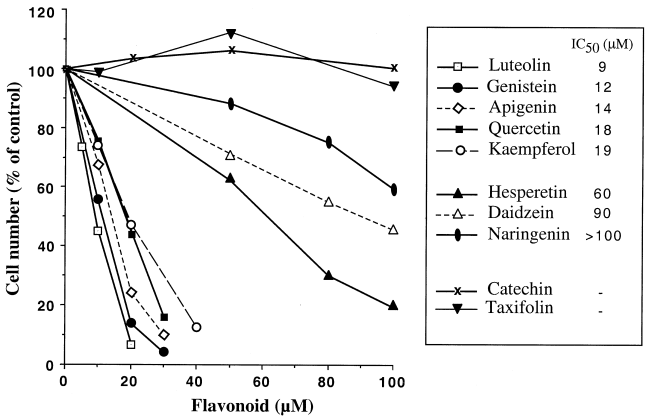


Fig. 2. Effect of flavonoids on the proliferation of OCM-1 human melanoma cells. OCM-1 melanoma cells were seeded in 35-mm dish at 3,500 cells per $cm^2$ and cultured in the absence (control) or in the presence of increasing concentrations of the indicated flavonoids. Cells were counted after 2 days of treatment. Data are the mean of two to three different experiments and are expressed as percent of control. The $IC_{50}$ values are indicated in the right panel.

above. Immunoprecipitates were washed twice with kinase buffer (25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). Beads were resuspended in 20 $\mu$L of 25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2 $\mu$ci [$\gamma$-$^{32}$P] ATP (Amersham), 200 $\mu$M cold ATP and 2 $\mu$g of histone H1 (Boehringer-Mannheim). Kinase reactions were carried out for 10 min at 30° and stopped by adding 12 $\mu$L of 4X Laemmli sample buffer. The samples were heated for 45 min at 30°. Phosphorylation of histone was analyzed by SDS-PAGE and autoradiography of the electroblotted gel. The amounts of CDK1 or CDK2 were determined by immunoblotting and quantified by computer densitometry (Gel Doc 1000 system, Bio-Rad).

## 3. Results

### *3.1. Inhibition of OCM-1 cell proliferation by flavonoids*

We have investigated the effect of a series of flavonoids on OCM-1 melanoma cell proliferation. The list of the flavonoids was selected among the different chemical classes of this family (Fig. 1), to allow the discovery of potential structure/activity relationships. As shown on Fig. 2, three groups of compounds may be clearly distinguished: a first group of very active compounds ($IC_{50}$ between 9 and 19 $\mu$M) which comprises flavones (luteolin and apigenin), flavonols (quercetin and kaempferol) and the isoflavone genistein; a second group of poorly active compounds ($IC_{50}$ between 60 and 100 $\mu$M) which is constituted by the isoflavone daidzein and the flavanones naringenin and hesperetin; and a third group of compounds which are completely inactive up to 100 $\mu$M such as the flavanonol taxifolin and the flavan-3-ol catechin. In other words, with the exception of daidzein, the flavones, flavonols and isoflavones were found to be potent antiproliferative compounds whatever the number of hydroxyl groups but unless the benzopyrone ring is conserved. By contrast, the flavonoids which lack the double bond C2-C3 like the flavanones naringenin and hesperetin as well as the flavanonol taxifolin or those which lack both this double bond and the oxy function at position 4 of the ring C (catechin) were poorly or completely inactive.

### *3.2. Effects of flavonoids on OCM-1 cell cycle distribution*

As conflicting results have been reported with regard to the specific stage at which flavonoids arrest cell cycle progression, we analyzed by flow cytometry the OCM-1 cell cycle distribution after 1 to 3 days of treatment with concentration of flavonoid 2 to 4 times the $IC_{50}$ value that was determined for cell proliferation inhibition. As shown on Fig. 3, the flavonol quercetin and the flavone luteolin induced a clear accumulation of cells in G1, with more than 25% increase. By contrast, the flavonol kaempferol and the flavone apigenin which differ respectively from quercetin and apigenin by the lack of a hydroxyl group at the 3′-position of the ring B, caused the accumulation of cells in G2/M (with an increment of approximately 30%). Interestingly, prior to the G2/M block induced by kaempferol and apigenin at day-2, a partial accumulation of cells in S was also detected (not shown). Furthermore, the isoflavone genistein led to a clear-cut arrest of cells in G2/M, while daidzein which differs from genistein by the lack of the hydroxyl at the 5-position of the ring A, induced on the contrary, an accumulation of cells in G1 (with an increase of approximately 15%). These data are summarized on Table 1. As expected, neither catechin nor taxifolin (100 $\mu$M), which were shown to be inactive for inhibiting OCM-1 cell proliferation, caused an alteration of cell cycle distribution. Surprisingly, naringenin and hesperetin which significantly affected cell proliferation at 100 $\mu$M did not exert any effect on cell cycle distribution (not shown). These later results might be explained by an unspecific lengthening of all the cell cycle phases in cells treated with these compounds. Alternately, the apparent inhibition of cell growth exerted by naringenin and hesperetin might be mostly due to the cytotoxic effect of high concentrations of these flavonoids. Indeed, while the cytotoxicity of the different compounds under study was found to be low up to 30–40 $\mu$M concentrations (giving less than 30% cell lysis as determined using the lactate deshydrogenase assay), it markedly increased for concentrations higher than 80–100 $\mu$M, except in the case of daidzein which caused only 20% and 25% cell lysis at 100 and 150 $\mu$M, respectively (not shown). In other words, the concentrations of flavonoids used in this study (including quercetin which caused less than 35% cell lysis at 70 $\mu$M) may be considered as poorly cytotoxic on melanoma cell cultures.

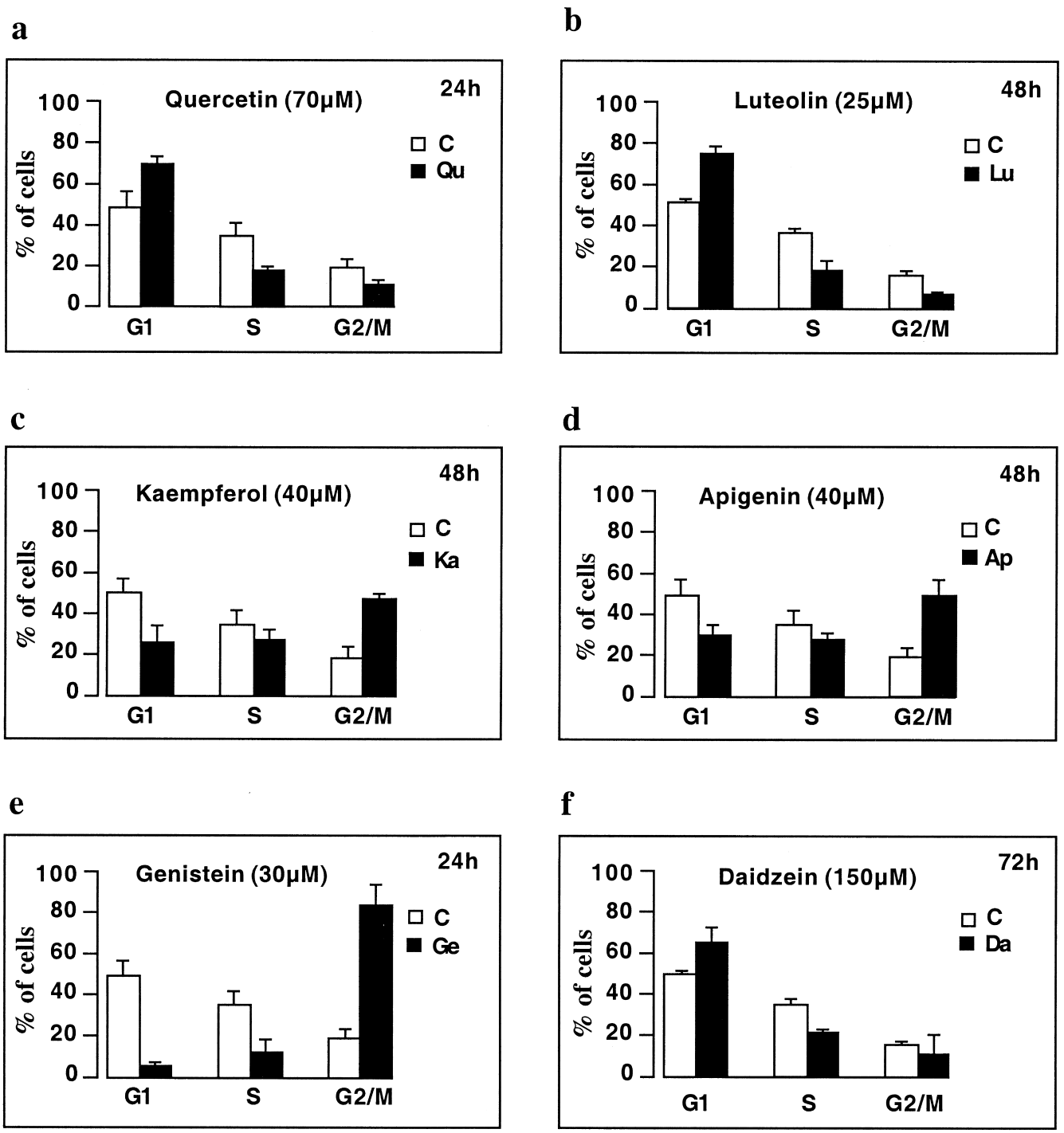


Fig. 3. Effect of flavonoids on the cell cycle distribution of OCM-1 melanoma cells. OCM-1 melanoma cells were cultured for 24–72 h in the absence (Control) or in the presence of 70 $\mu$M quercetin (Qu, panel a), 25 $\mu$M luteolin (Lu, panel b), 40 $\mu$M kaempferol (Ka, panel c), 40 $\mu$M apigenin (Ap, panel d), 30 $\mu$M genistein (Ge, panel e) or 150 $\mu$M daidzein (Da, panel f). Cell cycle distribution was analyzed by flow cytometry. Data (% of cells in the indicated phases) are the means ± SD of three to five independent measurements.

### *3.3. Effects of flavonoids on CDK activities*

In an attempt to define the molecular mechanisms of the different active flavonoids at the level of the cell cycle, we investigated their effect on the activities of CDK2 and CDK1, which have been shown to control respectively the G1/S and G2/M transitions. We measured the activities of the specifically immunoprecipitated kinases from cells treated with the same concentrations of flavonoids than those used in cell cycle distribution analyses. As shown on Fig. 4, flavonoids like quercetin, daidzein or luteolin which induced a cell cycle block in G1, inhibited the activity of

Table 1
Comparison of the effects of flavonoids on cell cycle proteins and cell signaling kinases

| | Hydroxyl position | | | | | a | | | | | b | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 5 | 7 | 3′ | 4′ | Block | CDK2 | CDK1 | p27 | p21 | PKC | PI 3-K | RTK |
| Flavonol | | | | | | | | | | | | | |
| Quercetin | OH | OH | OH | OH | OH | G1 | ▼ | □ | ▲ | △ | ▼ | ▼ | □ |
| Kaempferol | OH | OH | OH | — | OH | G2/M | □ | ▼ | △ | □ | ▽ | ▽ | ▼ |
| Flavone | | | | | | | | | | | | | |
| Luteolin | — | OH | OH | OH | OH | G1 | ▼ | □ | ▲ | △ | ▼ | ▼ | □ |
| Apigenin | — | OH | OH | — | OH | G2/M | □ | ▼ | △ | □ | ▽ | ▽ | □ |
| Isoflavone | | | | | | | | | | | | | |
| Genistein | | OH | OH | — | OH | G2/M | □ | ▼ | □ | ▲ | □ | □ | ▼ |
| Daidzein | | — | OH | — | OH | G1 | ▼ | □ | △ | △ | ? | ? | □ |
| Flavanone | | | | | | | | | | | | | |
| Naringenin | — | OH | — | — | OH | □ | □ | □ | △ | □ | ? | ? | ? |
| Hesperetin | — | OH | OH | OH | OCH3 | □ | □ | □ | △ | □ | □ | □ | □ |
| Flavanonol | | | | | | | | | | | | | |
| Taxifolin | OH | OH | OH | OH | OH | □ | □ | □ | □ | □ | ▽ | □ | □ |
| Flavan-3-ol | | | | | | | | | | | | | |
| Catechin | OH | OH | OH | OH | OH | □ | □ | □ | □ | □ | □ | □ | □ |

(a) The data of the present study are summarized. Inhibitory or stimulatory effects are indicated by upward or downward arrows respectively, the relative darkness of the arrow corresponding to the intensity of the effect. Empty bars indicate a lack of variation and question marks are for "not determined."
(b) Inhibitory effects on protein kinase C (PKC), PI 3-kinase (PI 3-K) and EGF receptor tyrosine kinase (RTK) are summarized from [14,15,18].

CDK2 by 40–60% (Fig. 4a and 4b). By contrast, the flavonoids which caused an accumulation of cells in G2/M like kaempferol, apigenin and genistein were without effect on CDK2. On the other hand, while quercetin, daidzein and luteolin did not alter the activity of CDK1, kaempferol, apigenin and genistein inhibited this kinase by 50–70% (Fig. 4c and 4d; see Table 1 for summarized results).

As several flavonoids have been demonstrated as inhibitors of protein kinases like PKCs or PI 3-kinases [14,15,18] and because flavopiridol, a flavonoid derivative, has been reported to inhibit directly CDK2 and CDK1 [39,40], we measured the effects of adding serial concentrations of flavonoids on specific CDK2 and CDK1 immunoprecipitates from control cells. We found that almost all the flavonoids under study were without effect on both CDK2 and CDK1 activities when their concentrations were below 100 $\mu$M. Even at this very high concentration, they inhibited kinase activities by less than 20–30%. The only exception was quercetin which inhibited CDK2 by 45% at 70 $\mu$M and by 70% at 100 $\mu$M (not shown).

### 3.4. Effects of flavonoids on CKI levels

As CDK activity is highly regulated by association with CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$, we examined the possible up-regulation of these proteins in cells treated with the different flavonoids. The prediction was that the compounds which led to CDK2 inhibition and G1 cell cycle arrest would be able to increase the content of cellular $p21^{CIP1}$ or $p27^{KIP1}$. As expected and shown in Fig. 5 (a and b), the flavonol quercetin and the flavone luteolin markedly up-regulated the level of $p27^{KIP1}$ within 24 hr (and until 48–72 hr) as did the quercetin derivative LY294002 (panel a; [41]). The two compounds also increased the content of $p21^{CIP1}$ but lately (after 48–72 hr) and to a lower extent (a and b). The flavonol kaempferol and the flavone apigenin which arrest cells in G2/M but were without effect on CDK2 activity, affected only marginally the level of $p27^{KIP1}$ and did not induce $p21^{CIP1}$ (panel c and d). Surprisingly, the isoflavone genistein which also caused a G2/M block and did not affect CDK2 activity, led to a clear increase in the content of $p21^{CIP1}$. By contrast, it was without effect on the level of $p27^{KIP1}$ (panel e). Daidzein, which inhibited CDK2 and induced a G1 block, up-regulated $p21^{CIP1}$ and, to a lower extent, $p27^{KIP1}$ (panel f). As expected, flavonoids which did not affect cell proliferation such as catechin and taxifolin, had no effect on CDK inhibitor levels. Those which exerted a weak effect on cell growth such as hesperetin and naringenin, caused only a marginal increase of $p27^{KIP1}$ (not shown). These data are summarized on Table 1.

### 3.5. Effects of flavonoids on CKI association to CDK

We next wonder whether the up-regulation of CDK inhibitors induced by some of the flavonoids could be responsible for the inhibition of CDK2 or CDK1 and for the subsequent cell cycle arrest. We therefore investigated the ability of flavonoids to induce the association of $p27^{KIP1}$ and $p21^{CIP1}$ to CDK2 or CDK1 by blotting the respective kinase immunoprecipitates with specific antibodies. As shown on Fig. 6a, the inhibition of CDK2 by quercetin was correlated to a striking increase of the binding of $p27^{KIP1}$ to the kinase. Association of $p21^{CIP1}$ was also observed but to a lower extent. Similar results, although more discrete, were obtained with luteolin (not shown). Daidzein caused a marked

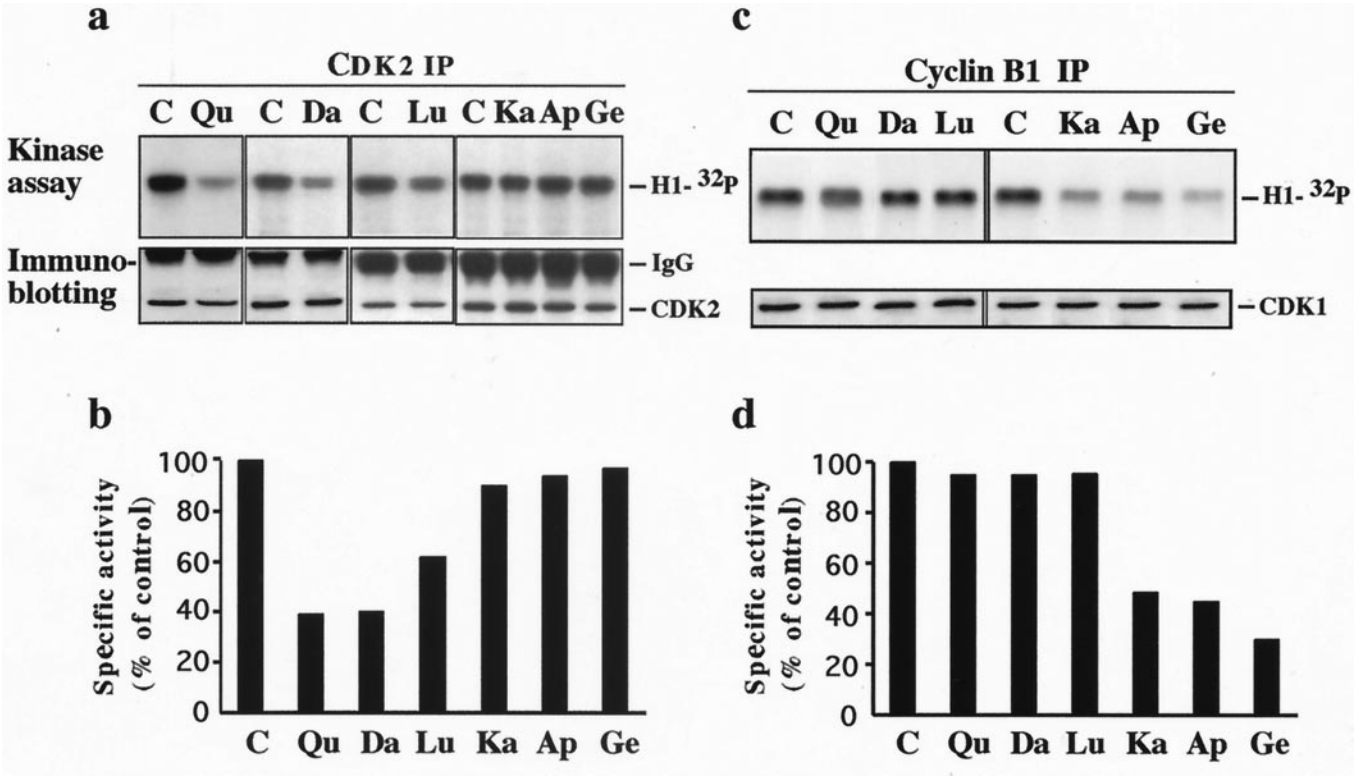


Fig. 4. Effect of flavonoids on cellular CDK2 and CDK1 activities. OCM-1 melanoma cells were cultured for 48 h in the absence (Control, C) or in the presence of 70 $\mu$M quercetin (Qu), 100 $\mu$M daidzein (Da), 25 $\mu$M luteolin (Lu), 40 $\mu$M kaempferol (Ka), 40 $\mu$M apigenin (Ap) or 30 $\mu$M genistein (Ge). CDK2 (a, b) and CDK1 (c, d) were immunoprecipitated from the respective RIPA lysates using specific CDK1 and cyclin B1 antibodies. Kinase activities were measured on the immune complexes using histone H1 as a substrate. Phosphorylation of histone was analyzed by SDS-PAGE and autoradiography of the electroblotted gels. The level of CDK2 and CDK1 was evaluated by blotting the fractionated immune complexes. Specific activities (b and d) were evaluated by counting the radioactive histone H1 bands and by quantifying the levels of CDK2 and CDK1 by computer densitometry with a known amount of recombinant proteins as a reference. They were calculated as pmol $^{32}$P per $\mu$g of CDK2 (b) or CDK1 (d) and expressed as percent of control. Data are representative of at least two different experiments.

association of $p21^{CIP1}$ but only a weak rise of $p27^{KIP1}$ (Fig. 6a), in agreement with the modest up-regulation of this latter in daidzein-treated cells (see Fig. 5f). Similarly, we were unable to show any increase in the binding of $p27^{KIP1}$ to CDK2 from cells treated with apigenin or kaempferol. Obviously, these CDK2 immunoprecipitates were devoid of $p21^{CIP1}$ as the cellular content of this latter is not induced by apigenin and kaempferol (see Fig. 5c and 5d). By contrast, we have previously shown that genistein was able to cause the association of $p21^{CIP1}$ to CDK2 but we demonstrated that the amount of the inhibitor was not sufficient to cause the inhibition of the kinase activity [30].

### *3.6. Effects of flavonoids on CDK1 phosphorylation state*

Finally, we were not able to demonstrate an association of $p27^{KIP1}$ or $p21^{CIP1}$ to CDK1 immunoprecipitated from cells treated with any of the flavonoids under study (not shown). As these data suggested that the inhibition of CDK1 by the flavonoids leading to a G2/M cell cycle arrest was very likely not due to CDK inhibitors, we wonder whether the phosphorylation state of CDK1 could be affected in cells treated with these compounds. As shown on Fig. 6b, genistein, apigenin and kaempferol led to an increase of the slower electrophoretic mobility form of CDK1 which is believed to be the Tyr15-phosphorylated (inactive) form of the kinase. By using a specific anti-phospho-CDK1 (Tyr15) antibody, we confirmed that the three flavonoids increased the content of Tyr15-phosphorylated CDK1 (panel c). By contrast, the flavonoids which affected CDK2 but not CDK1 did not change the Tyr15 phosphorylation level of CDK1.

## 4. Discussion

In the present study, we have established that several structurally related flavonoids were able to strongly inhibit the proliferation of human OCM-1 melanoma cells. We have demonstrated that a ring C with oxo function at position 4 and a C2-C3 double bond was required for maximal anti-proliferative activity. Taxifolin which lacks the C2-C3 double bond and catechin which lacks both the double bond and the C4 oxo group are unable to inhibit cell proliferation. Naringenin and hesperetin which also lack the C2-C3 double bond are found to be very poorly efficient. These results

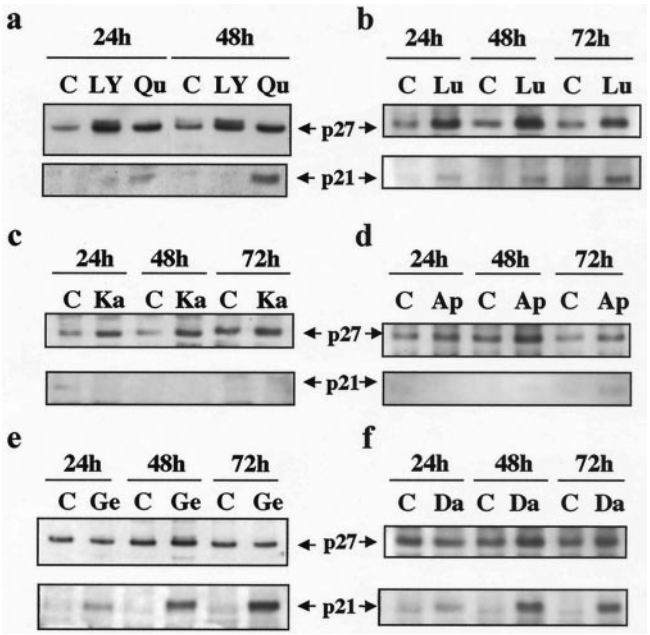


Fig. 5. Effect of flavonoids on the cellular content of CDK inhibitors. OCM-1 melanoma cells were cultured for 24 h–72 h in the absence (C) or in the presence of 70 $\mu$M quercetin (Qu, panel a), 25 $\mu$M luteolin (Lu, panel b), 40 $\mu$M kaempferol (Ka, panel c), 40 $\mu$M apigenin (Ap, panel d), 30 $\mu$M genistein (Ge, panel e) or 100 $\mu$M daidzein (Da, panel f). Alternatively, cells were exposed to 20 $\mu$M LY294002 (LY, panel a). Whole cell extracts were fractionated on SDS-polyacrylamide gels and electroblotted proteins were probed with anti-$p21^{CIP1}$ and anti-$p27^{KIP1}$ antibodies.

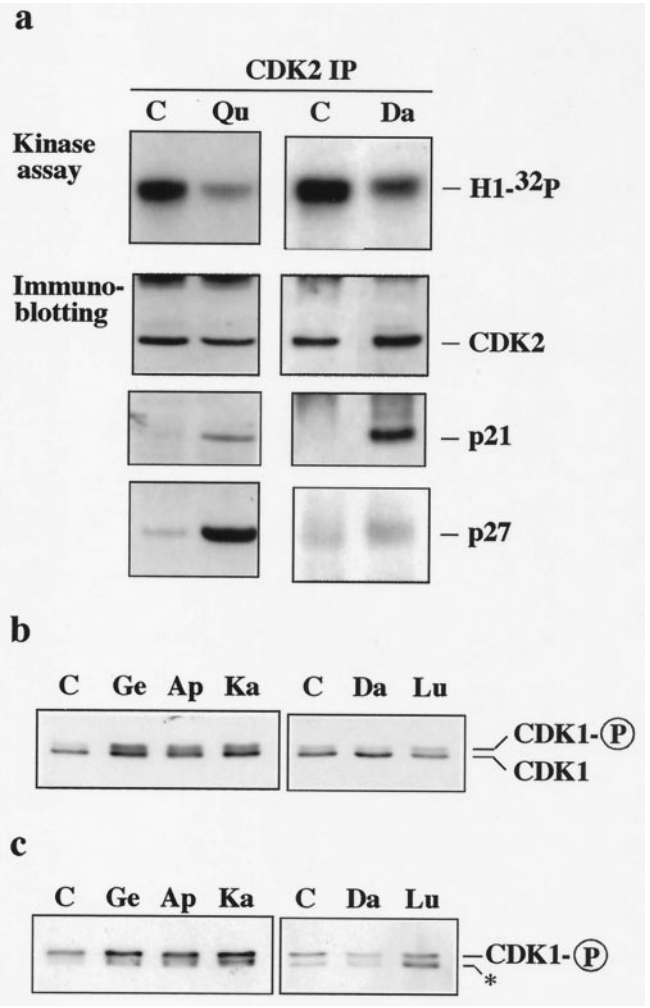


Fig. 6. Effect of flavonoids on the association of CKIs to CDK2 and on the phosphorylation state of CDK1. OCM-1 cells were cultured for 48 h in the absence (C) or in the presence of flavonoids as indicated in the legend to Fig. 5. (a) CDK2 was immunoprecipitated and the kinase activity was measured on the immune complexes. After fractionation on SDS-polyacrylamide gel, the electroblotted gel was analyzed by autoradiography (kinase assay) and the levels of CDK2 and associated $p21^{CIP1}$ and $p27^{KIP1}$ were evaluated by specific immunoblotting of the membrane. (b) and (c) Whole cell extracts were directly fractionated by SDS-PAGE and electroblotted proteins were probed with anti-CDK1 (b) or anti-phospho-CDK1 (Tyr15) antibody (c). In addition to the main protein recognized by this latter, which corresponds to the upper band in b (the phosphorylated form of CDK1), a minor and unspecific band (marked with a star in c) was occasionally detected.

are in agreement with those previously reported on endothelial cells [11].

Interestingly, a similar structure requirement was demonstrated with regard to inhibition of PKC [14,15] and PI 3-kinase [15,18]. However, these studies revealed that the presence of hydroxyl groups at the 3′- and 4′-positions of ring B confered the maximal inhibitory effect. A model of the minimal structural features needed for PKC inhibition postulated a planar benzopyrone ring with a 7-hydroxyl and a coplanar 3′, 4′ dihydroxy-phenyl ring at the 2-position [14]. Our data summarized on Table 1, differ from these requirements as kaempferol and apigenin which lack the 3′ OH are still potent inhibitors of cell proliferation. The same is true for the isoflavone genistein whose the phenyl ring is at the 3-position of ring B and which has been shown to affect neither PKC nor PI 3-kinase activities [15].

Our results suggest that the 3′ OH of the ring B might be important for the level at which the cell cycle arrests. Indeed, the presence of this hydroxyl group in quercetin and luteolin correlates to a G1 block while its absence in kaempferol and apigenin correlates to a G2 block. That the hydroxyl at 5-position of the benzopyrone could play a similar pivotal role with regard to the isoflavone class of compounds may be hypothetized as genistein which contains this hydroxyl group arrested cells in G2 while daidzein which lacks it induced an accumulation of cells in G1.

Although our study does not reveal a clear relationship between the structure of flavonoids and their ability to up-regulate one CKI or another, it demonstrates that the G1 cell cycle arrest depends on the degree of the CKI increase. Indeed, flavonoids which up-regulated both $p21^{CIP1}$ and $p27^{KIP1}$ (quercetin, luteolin and daidzein) led to G1 arrest, and this is particularly true for the compounds which caused a marked increase of $p27^{KIP1}$ like quercetin and luteolin. By contrast, the flavonoids which up-regulated poorly $p27^{KIP1}$ and not $p21^{CIP1}$ (kaempferol and apigenin) or which induced only $p21^{CIP1}$ (genistein) were unable to arrest cells in G1 (Table 1).

We have previously reported that the PI 3-kinase inhib-

itor LY294002 increased the $p27^{KIP1}$ content in OCM-1 melanoma cells and induced a G1 phase block [41]. Here, we confirm that PI 3-kinase is likely implicated in the regulation of $p27^{KIP1}$ as all flavonoids which have been shown to inhibit PI 3-kinase *in vitro* [15,18] up-regulated the CKI. However, all did not block cells in G1 as they increased $p27^{KIP1}$ at different degrees. Indeed, apigenin and kaempferol which still inhibit PI 3-kinase (although to a lower extent than quercetin and luteolin), led to a cell cycle arrest in G2 as they induced only a slight increase in the level of $p27^{KIP1}$. In other words, our results emphasize the lack of a close correlation between the level at which flavonoids arrest the cell cycle and their ability to inhibit PI 3-kinase (or PKC). Similarly, cell cycle arrest in G2/M was not dependent on the ability of flavonoids to inhibit tyrosine kinase as apigenin which does not inhibit this type of enzyme [15], induced a G2/M block as kaempferol and genistein did.

It has been recently reported that the induction of $p21^{CIP1}$ by flavone or quercetin was p53-independent [42,43]. An inverse relationship between $p21^{CIP1}$ and p53 has also been shown in mammary cancer cells exposed to genistein [44]. However, several flavonoids have been found to increase the steady-state level of p53 [26,45]. Whether the up-regulation of $p21^{CIP1}$ we observed in OCM-1 melanoma cells treated with certain flavonoids is p53-dependent or not remains to be established. However, we have demonstrated that genistein increased the steady state level of p53 in these cells [46].

Our present study clearly establishes that flavonoids which arrest cells in G1 inhibit CDK2 while those which block cells in G2 inhibit CDK1. This finding was reminiscent of similar effects induced by the flavone derivative flavopiridol on CDK2 and CDK1 activities with subsequent cell cycle arrest at either G1 or G2 [39,40]. In these studies, flavopiridol was shown to inhibit directly the kinases by interacting with the ATP binding domain and competing with the nucleotide. By contrast, the inhibitions we describe here were not direct as we demonstrate that flavonoid concentrations below 100 $\mu$M were without significant effect when the compounds were added directly to the CDK2 or CDK1 immunoprecipitates. However, we cannot exclude that, *in cellulo*, high concentrations of flavonoids could inhibit directly CDK2 or CDK1.

Finally, we demonstrate that the up-regulation of CKIs (high increase of $p27^{KIP1}$ or moderate increase of both $p21^{CIP1}$ and $p27^{KIP1}$) is likely responsible for inhibition of CDK2 (and subsequent G1 cell cycle arrest) while inhibition of CDK1 (and following G2/M block) was rather due to the phosphorylation of the kinase on Tyr15.

While up-regulation of CKIs by certain flavonoids has been already reported [42,43,47–50], it has been correlated to an inhibition of CDK2 only in few cases, mainly in human diploid fibroblasts treated with apigenin [35] and in human prostate carcinoma cells exposed to silymarin [51] or silibinin, the main constituent of this flavonoid mixture [52]. In these studies, the increases of $p21^{CIP1}$ [35] or both $p21^{CIP1}$ and $p27^{KIP1}$ levels [51,52] were correlated with inhibitions of CDK4 or CDK2 activities and with subsequent G1 cell cycle arrest. The up-regulation of $p21^{CIP1}$ has been reported to correlate with a G2 block in breast [47,48], prostate [49] and lung [50] cancer cells exposed to genistein. In most of these studies, the increase of $p21^{CIP1}$ was correlated to cell apoptosis [47,49,50]. Our data do not support these findings. Indeed, in OCM-1 melanoma cells, most of the flavonoids (except quercetin) did not induce apoptosis even at high concentration (Casagrande F. and Darbon J.M., unpublished data). Moreover, we demonstrate that the G2/M cell cycle arrest induced by certain flavonoids including genistein, was correlated to an inhibition of CDK1 without any association of $p21^{CIP1}$ (or $p27^{KIP1}$) to the kinase. Finally, we have recently reported that genistein could induce a G2 arrest in cells devoid of $p21^{CIP1}$ [31].

The G2/M arrest induced by apigenin in keratinocytes has also been correlated to inhibition of CDK1 activity [34]. However, this effect was not attributed to an alteration of the phosphorylation state of CDK1 but rather to a decline in the cyclin B1 content. Similarly, the genistein-induced G2/M arrests of breast or prostate cancer cells were correlated to a decrease in cyclin B1 [48,49]. By contrast, in OCM-1 melanoma cells, we have demonstrated that genistein increased the content of cyclin B1, likely due to the accumulation of cells in G2 (data non shown). In these cells, the G2/M arrest induced by apigenin, kaempferol and genistein is clearly the consequence of an impairment of the dephosphorylation of CDK1 on Tyr15, likely due to the inactivation of the phosphatase CDC25C, as demonstrated in the case of genistein [31,46].

## Acknowledgments

The authors are very indebted to Marie Penary and Brigitte Serres for their excellent technical assistance and to Georges Cassar for the flow cytometry analyses. We acknowledge Dr Maria A Saornil, Valadolid Institute, Spain, for the gift of human choroidal melanoma cells OCM-1. This work was supported by Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Université Paul Sabatier, Région Midi-Pyrénées, Fondation de France and Ligue Nationale Contre le Cancer.

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