G1 phase arrest by the phosphatidylinositol 3-kinase inhibitor LY 294002 is correlated to up-regulation of $p27^{Kip1}$ and inhibition of G1 CDKs in choroidal melanoma cells

Fabrice Casagrande[a], Daniel Bacqueville[b], Marie-Jeanne Pillaire[a], François Malecaze[a], Stéphane Manenti[a], Monique Breton-Douillon[b], Jean-Marie Darbon[a,*]

[a] *INSERM CJF 95-10, Pavillon Rayer, CHU Purpan, 31059 Toulouse Cedex, France*
[b] *INSERM U326, Institut Fédératif de Recherche 30, CHU Purpan, 31059 Toulouse, France*

Received 28 November 1997; revised version received 7 January 1998

Abstract We have investigated the effect of the flavonoid derivative LY 294002, a potent and selective phosphatidylinositol 3-kinase inhibitor, on cell cycle progression in human choroidal melanoma cells. We demonstrate that LY 294002 induces a specific G1 block in asynchronously growing cells leading to an almost complete inhibition of cell proliferation after three days of treatment. When melanoma cells are released from a nocodazole-induced G2/M block, LY 294002 is shown to delay and greatly restrain the G1/S transition. The inhibitor is able to exert its action as long as it is added during the G1 progression and before the cells enter in S phase. We report that the LY 294002-induced G1 arrest is closely correlated to inhibition of CDK4 and CDK2 activities leading to the impairment of pRb phosphorylation which normally occurs during G1 progression. While the inhibition of CDK4 may be attributed at least in part to the decline in CDK4 protein level, CDK2 activity reduction is rather due to the up-regulation of the CDK inhibitor $p27^{Kip1}$ and to its increased association to CDK2.

Key words: Flavonoid; Phosphatidylinositol 3-kinase; Cyclin-dependent kinase; CDK inhibitor; Choroidal melanoma cell

1. Introduction

Flavonoids and their derivatives have been shown to inhibit the proliferation of several human cancer cell lines including breast, leukemic, ovarian and gastro-intestinal tumor cells by arresting cell cycle progression at specific stages [1]. While the flavone quercetin has been reported to induce a G1 phase block [2,3], the isoflavone genistein has been shown to arrest cells at the G2/M boundary [4]. The synthetic quercetin derivative L86-8275 appears to cause both G1/S and G2/M blocks [5]. The molecular mechanisms underlying such cell cycle arrests have not been elucidated until now, although flavonoids have been described to inhibit several serine/threonine or tyrosine protein kinases as well as lipid kinases involved in signal transduction [6]. Recently, a quercetin synthetic derivative called LY 294002 has been demonstrated as a potent and selective inhibitor of PI 3-kinases [7], a family of enzymes very likely involved in growth factor receptor signalling and cell transformation [8,9]. The agonist-dependent PI 3-kinase activation leads to rapid and transient production of phosphoinositides PI 3,4 P2 and PI 3,4,5 P3 which have been proposed to act as second messengers [10]. However, the subsequent events responsible for the induced mitogenic response are still poorly understood. Finally, the PI 3-kinase p110 subunit has been recently proposed to have also serine/threonine protein kinase activity [11,12]. The function of such activity remains to be documented [13,14].

Progression of eukaryotic cells through the cell cycle is orchestrated by sequential activation and inactivation of a series of serine/threonine kinases, the cyclin-dependent kinases (CDKs) associated with their respective cyclin subunits [15–17]. G1 phase progression and G1/S transition are believed to be regulated by CDK4 (and CDK6) which assemble with cyclins D in mid-G1 and CDK2 which combines later with cyclin E. CDK activity is counterbalanced by association with low molecular weight CDK inhibitors (CKIs) [18,19]. $p21^{Cip1}$ and $p27^{Kip1}$ proteins belong to the first family of CKIs and have a broad specificity, acting on the different G1 CDK cyclins as well as on the S phase CDK2-cyclin A complexes. The second and unrelated family of CKIs includes $p16^{INK4A}$ and $p15^{INK4B}$ proteins which specifically inhibit CDK4 and CDK6. Finally, CDK activity is regulated by a complex set of phosphorylation/dephosphorylation reactions [15–17].

Several inhibitors of G1 phase progression including TGFβ [20,21], cyclic AMP [22], rapamycine [23,24] or γ radiations [25] have been shown to interact with the CDK regulatory network, exerting effects at both the levels and activities of CDK-cyclin complexes. CKIs have been shown to play an essential role in the G1 phase arrest. For example, the immunosuppressant rapamycine has been shown to maintain a high level of p27 in T-cell [26] or macrophages [22] which is mainly responsible of the induced G1 phase block. Interestingly, rapamycine has been shown to act by interfering with the FRAP/RAFT-1/mTOR protein [27,28] which exhibits distant homology with PI 3-kinases [13,14].

In this paper, we investigated the effect of the flavonoid derivative LY 294002 on cell cycle and proliferation in human choroidal melanoma cells. We report that the PI 3-kinase inhibitor induces a specific G1 phase arrest correlated to up-regulation of the CDK inhibitor p27 and inhibition of CDK4 and CDK2 activities.

*Corresponding author. Fax: +33 5 61 77 25 68.
E-mail: darbon@purpan.inserm.fr

Abbreviations: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; PI 3-kinase, phosphatidylinositol 3-kinase; FCS, fetal calf serum; PBS, phosphate-buffered saline; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LY 294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one

PII S0014-5793(98)00043-X

2. Materials and methods

2.1. Cell culture

Human choroidal melanoma cells (spindle-shape OCM-1 cell line) were maintained at 37°C in RPMI-1640 medium, pH 7.3, supplemented with 100 UI/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine and 5% FCS in humidified 5% CO_2, 95% air. All culture reagents 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 studies

For cell growth measurement, melanoma cells were seeded at an initial density of 3×10^4 cells per 35-mm dish. Indicated concentrations of LY 294002 (purchased from Biomol Research Laboratories) were added at day 0 and 2. Control dishes received the same volume of the solvent DMSO. Cells were harvested with trypsin-EDTA and cell number was determined by using a Coulter Counter (Coultronics).

2.3. Cell cycle progression analysis

Melanoma cells were plated at a density of 2×10^5 cells per 100-mm dish in the absence or presence of 20 μM LY 294002. After one or two days, cells were harvested by brief trypsinisation and centrifuged at $500\times g$ for 5 min. The cell pellet was washed twice in PBS, labelled with propidium iodide and the cell cycle distribution was determined by flow cytometry analysis using a Coulter Elite.

In order to synchronize cells in G2/M phase, 100 ng/ml nocodazole was added to the culture media for 14–16 h. The detached cells were centrifuged at $500\times g$ for 5 min, washed twice with PBS and then replated in FCS medium without or with 20 μM LY 294002. Cells were collected at the indicated times and the cell cycle distribution was analyzed by flow cytometry. Concurrently, total cell extracts were prepared by direct lysis of cells either in Laemmli sample buffer or in modified RIPA buffer (see below).

2.4. Immunoblotting and immunoprecipitation

Proteins were separated by electrophoresis on 7.5% or 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 h 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. Anti-CDK4 (C-22), anti-CDK2 (M2), anti-cyclin A (BF 683) and anti-p27 (C-19) antibodies were from Santa Cruz Biotechnology. Anti-pRb (G3-245) was from Pharmingen.

For immunoprecipitation experiments, cells were lysed in modified 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% β-glycerophosphate, 0.1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM PMSF, 0.1% Tween 20 and 10% glycerol. The lysates were clarified by centrifugation at $12\,000\times g$ for 5 min at

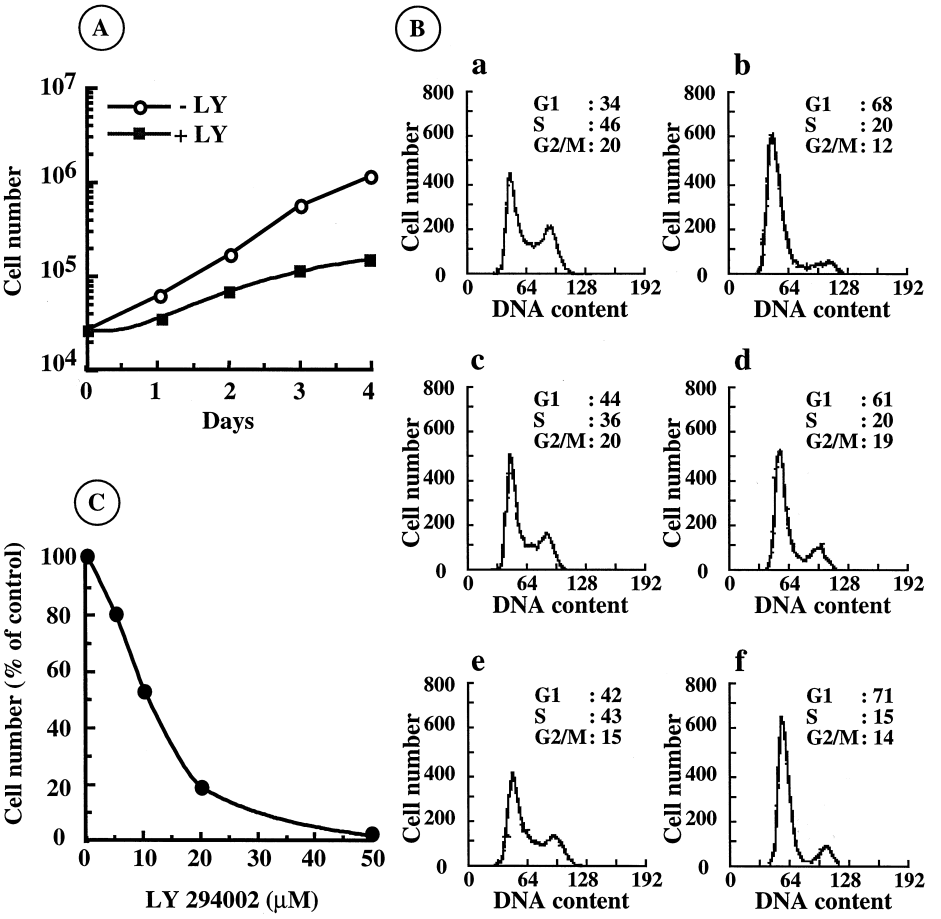

Fig. 1. Effect of LY 294002 on cell cycle progression and cell proliferation of asynchronous melanoma cells. Asynchronous melanoma cells were cultured for the indicated times in the absence (−LY) or presence (+LY) of 20 μM LY 294002 (A, B), or for 3 days in the absence (control) or presence of increasing concentrations of the drug (C). Cell number was evaluated by counting (A, C) and results are the means of triplicate measurements performed on separate dishes (cell number variation below 10%). In C, data are expressed as percent of cell number in the absence of LY 294002 (control). Alternatively, cell cycle distribution was analyzed by flow cytometry (B) after 24 h (a, b) or 48 h (c–f) of culture without (a, c) or with (b, d, f) the drug. In e, LY 294002 was washed out from the culture after 24 h and cells were cultured for the next 24 h in the absence of the drug. In f, LY 294002 was added twice, at time 0 and after 24 h of culture. Results are, as in A and C, from one experiment representative of three different experiments.

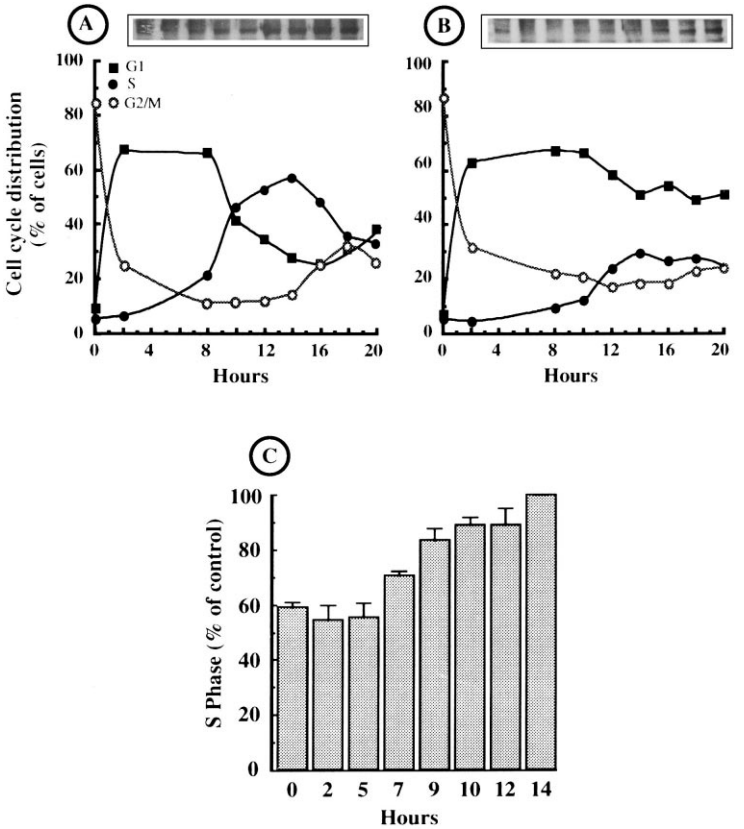

Fig. 2. Effect of LY 294002 on cell cycle distribution of melanoma cells released from a nocodazole-induced G2/M block. Melanoma cells were synchronized in G2/M phase by previous treatment for 14–16 h with 100 ng/ml nocodazole. Cells were then released from this block by serum stimulation in the absence (A) or presence (B) of 20 μM LY 294002. Flow cytometry analysis was performed at the indicated times. S phase entry was further evaluated by blotting, in parallel, cell lysates with an anti-cyclin A antibody (inserts: each lane corresponds to each time point). Alternatively, 20 μM LY 294002 was added at various times after the nocodazole block release (C). Cell cycle distribution was analyzed 14 h after the release and the number of cells in S phase following LY 294002 treatment expressed as percent of cells in S phase after 14 h of serum stimulation in the absence of the drug.

4°C and the protein contents were estimated by electrophoresis on SDS-PAGE and Coomassie blue staining. Immunoprecipitations were performed using the same amount of proteins (1.5 mg) for the different samples. Antibodies previously bound to protein A-Sepharose CL-4B beads (Pharmacia) were added to the cell lysates for 12 h at 4°C with gentle agitation. Immune complexes were collected by centrifugation and washed three times in RIPA buffer, twice in PBS and twice in kinase assay buffer (25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). After kinase assays (see below), the samples were fractionated on SDS-PAGE and gels were electroblotted. Analysis of CDKs or associated CKIs was carried out by immunoblotting using specific antibodies.

2.5. Protein kinase assays

Kinase reactions (total volume 20 μl) were performed for 10 min at 30°C in 25 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 5 μci [γ-^{32}P]ATP (Amersham) with 50 μM ATP (CDK4) or 200 μM ATP (CDK2). Two μg of histone H1 (Boehringer-Mannheim) or 2 μg of pRb (QED Bioscience Inc.) were added into the respective CDK2 and CDK4 assays. The reactions were stopped by adding 12 μl of 4×Laemmli sample buffer and heated for 45 min at 30°C. Phosphorylation of the substrates was analyzed by SDS-PAGE and autoradiography of the electroblotted gel.

3. Results

3.1. Inhibition of asynchronous melanoma cell proliferation by LY 294002

We investigated the effect of the PI 3-kinase inhibitor LY 294002 on cell proliferation of choroidal melanoma OCM-1 cells. As shown on Fig. 1A, the flavonoid derivative markedly decreased the proliferation rate of asynchronously growing OCM-1 cells leading to an almost complete inhibition of cell proliferation after three days of treatment. Flow cytometric analysis demonstrated that this growth inhibitory action was the consequence of a specific G1 phase arrest (Fig. 1B). However, a significant proportion of melanoma cells escape the block at day 1, agreeing with the fact that complete inhibition of cell proliferation requires several days of treatment. The effect of LY 294002 was reversible since after removal of the drug, arrested cells reentered the cycle as assessed by flow cytometric analysis (Fig. 1B, panel e). Fig. 1B also evidences that a stronger effect of LY 294002 was obtained when the drug was added twice during a 2-day treatment (panel f), very likely reflecting the instability of the flavonoid derivative in the cell. As shown on Fig. 1C, the IC50 observed for cell proliferation inhibition was about 10 μM, a concentration higher than, but compatible with, the one (1.4 μM) reported for in vitro inhibition of PI 3-kinase activity [7].

3.2. Induction by LY 294002 of a mid-late G1 phase arrest in synchronized melanoma cells

To further study the LY 294002-induced G1 phase arrest, melanoma cells were previously synchronized in G2/M by

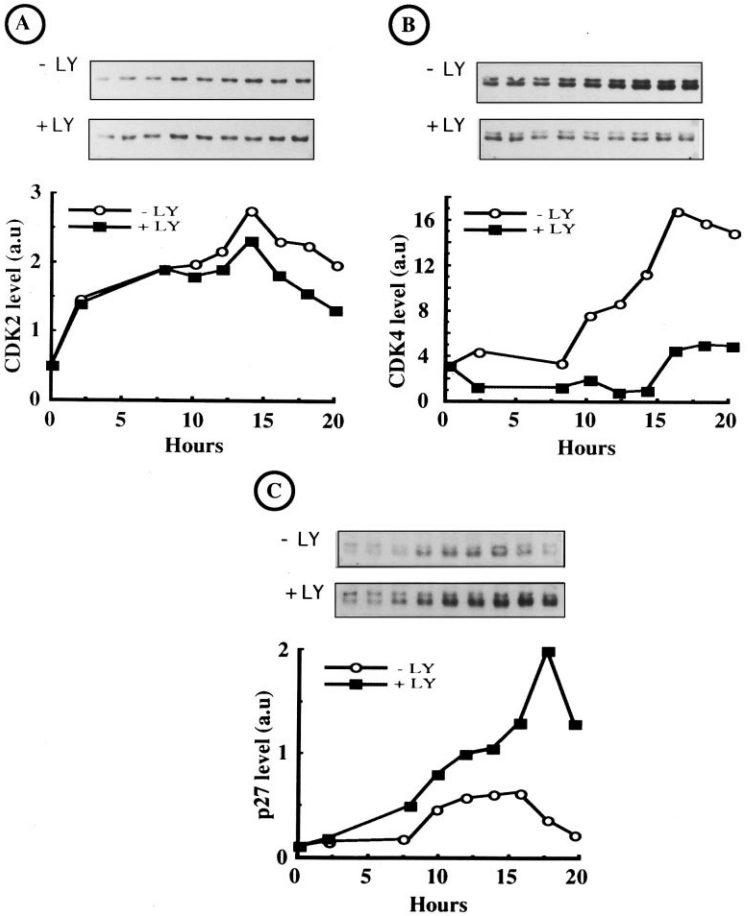

Fig. 3. Effect of LY 294002 on CDK2, CDK4 and p27 levels. G2/M synchronized melanoma cells were stimulated by serum for the indicated times in the absence (−LY) or presence (+LY) of 20 μM LY 294002. Respective cell extracts were fractionated by SDS-PAGE and the levels of CDK2 (A), CDK4 (B) and p27 (C) were evaluated by blotting with specific antibodies. Quantitative evaluations of immunoblots were performed using the Biorad Gel Doc video system.

a nocodazole treatment and then released from this block by serum stimulation in the absence (Fig. 2A) or in the presence (Fig. 2B) of LY 294002. Flow cytometric analysis shows that the PI 3-kinase inhibitor delayed (by 2–4 h) and markedly restrained (by 40–50%) the G1/S transition. This result was confirmed when cyclin A expression was evaluated as a marker of S phase entry (Fig. 2A and B, inserts).

We then attempted to determine how long an exposure to LY 294002 was required to induce the G1 phase arrest. This was done by adding the flavonoid derivative at various times after the nocodazole block release. Fig. 2C shows that the PI 3-kinase inhibitor was effective to prevent cells from entering S phase when added until 5 h after the release. Drug addition at a later time (7 h) maintained fewer cells in G1 phase and, once cells neared the G1/S transition (9–10 h), LY 294002 was ineffective in preventing S phase entry.

3.3. *Up-regulation by LY 294002 of the CDK inhibitor p27 and inhibition of CDK4 and CDK2 activities*

To understand the molecular events involved in the flavonoid derivative action on cell cycle progression, we investigated the effect of the compound on G1 CDK levels and activities along the course of G1 progression in nocodazole synchronized melanoma cells.

As shown on Fig. 3, LY 294002 did not significantly affect the CDK2 protein level. By contrast, CDK4 level decreased as a function of the drug exposure period, with a significant higher effect after 10–14 h of treatment. As a main feature, the CDK inhibitor p27 was shown rapidly up-regulated by the PI 3-kinase inhibitor.

Fig. 4 shows that the CDK4 and CDK2 kinase activities were markedly reduced in the LY 294002-treated cells as assessed by enzymatic assays of specific immunoprecipitates. These declines were not the consequence of a direct inhibition of the kinases as neither CDK4 nor CDK2 activity was affected when the drug was added directly to immunoprecipitates from untreated cells (not shown). CDK2 activity reduction was rather due to an increase of the ratio of p27 associated to the kinase (Fig. 4A). As we were unable to detect any variation of p27 or p21 associated to CDK4 and because p15 is very likely absent in melanoma OCM-1 cells (unpublished results), the CDK4 kinase activity inhibition (Fig. 4B) was probably due to the decrease of the protein level. In any case, the CDK4 and CDK2 kinase reductions were observed at early points after release from nocodazole block, suggesting their involvement in the G1 arrest caused by LY 294002. Noteworthy, the PI 3-kinase inhibitor markedly reduced the pRb phosphorylation which normally occurs during the G1 progression (Fig. 4C).

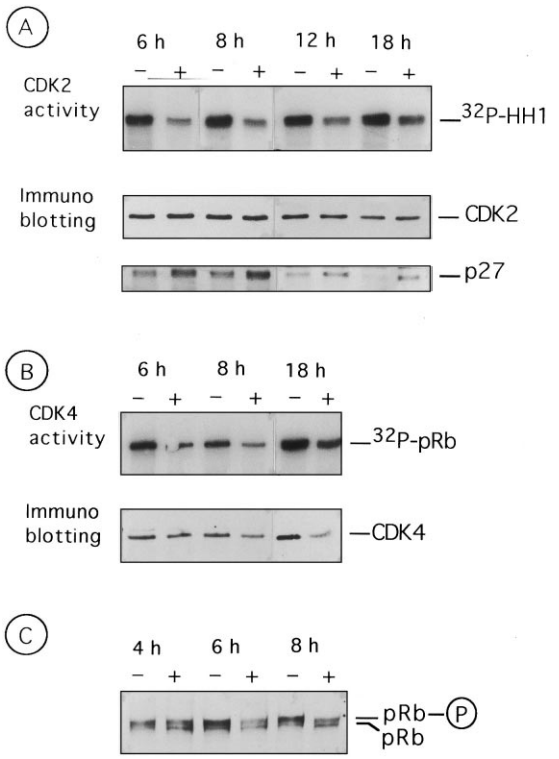

Fig. 4. Effect of LY 294002 on CDK2 and CDK4 activities, and on pRb phosphorylation. G2/M arrested cells were incubated for the indicated times in FCS medium without (−) or with (+) 20 μM LY 294002. CDK2 (A) and CDK4 (B) were immunoprecipitated from the respective RIPA lysates and kinase activities measured on the immune complexes by using respectively histone H1 (HH1) and pRb as substrates. Phosphorylation of the substrates was analyzed by SDS-PAGE and autoradiography of the electroblotted gel. The levels of CDK4 and CDK2 as well as the associated p27 were evaluated by specific blotting. Aliquots of RIPA lysates were, in parallel, analyzed for their pRb contents by SDS-PAGE and specific blotting (C).

4. Discussion

Flavonoid compounds have been reported to inhibit cell proliferation by inducing specific blocks either at the G1/S or G2/M boundaries [1–4]. In this paper, we demonstrate that the quercetin derivative LY 294002 causes a specific G1 phase arrest in human choroidal melanoma cells. This result agrees with a recent report showing that the drug inhibited the proliferation of smooth muscle cells in cultured rabbit aortic segments [7]. We report that, in human melanoma cells, the flavonoid derivative exerts its action by inhibiting the G1 CDK activities and the subsequent phosphorylation of the retinoblastoma protein pRb. These inhibitory effects are, at least in part, due to an induced up-regulation of the CDK inhibitor p27. This latter result is likely not restricted to tumor cells as a similar finding has been recently reported in NIH 3T3 fibroblasts where LY 294002 has been shown to prevent the down-regulation of p27 induced by EGF or phorbol-12,13-dibutyrate and to inhibit the DNA synthesis [29].

LY 294002 has been shown to be a potent inhibitor of PI 3-kinase activity, acting as a competitive inhibitor for ATP binding site of the enzyme [7]. This effect was highly specific as other lipid kinases as well as a series of serine/threonine kinases (including PKC, MAP kinase and S6 kinase) or tyrosine kinases (including EGF receptor and c-src tyrosine kinases) were not affected by the compound. In agreement with this selectivity, we find that the flavonoid derivative does not directly inhibit the CDK activities. By contrast, other flavonoids have been shown to inhibit several classes of protein or lipid kinases [6]. For example, quercetin inhibits PKC, receptor and non-receptor tyrosine kinases as well as PI 3- and PI 4-kinases while genistein affects the activity of several tyrosine kinases. Interestingly, the flavonoid L86-8275 has been shown to directly inhibit cdc2 kinase activity [5]. All these observations suggest that LY 294002 exerts its effects in a much more specific way than the flavonoid compounds studied until now. However, several types of PI 3-kinases have been identified so far. The most characterized group are heterodimers composed of a p85 regulatory subunit and a p110 catalytic subunit [30]. In contrast, the recently cloned PI 3-kinase p110γ does not complex to p85 and is activated by Gβγ subunits [31]. A third group of PI 3-kinases includes the human homolog of Vps34 gene product from *Sacharomyces cerevisiae* [32]. Whether LY 294002 inhibits identically all these PI 3-kinase species remains to be determined.

In fact, the implication of PI 3-kinases in mitogenesis has been really demonstrated with regard to the p85-p110 dimeric enzyme only. Most convincing was the report that inhibitory antibodies to the p110 subunit blocked the DNA synthesis occurring in response to PDGF and EGF [33].

In any case, whether the LY 294002 action on cell cycle regulation that we report here is only targeted by inhibition of PI 3-kinase is questionable. Indeed, PI 3-kinases have been recently shown to be distant homologs of two others classes of proteins, the FRAP/RAFT-1/mTOR protein which is believed to be a serine/threonine kinase, and a family of proteins involved in DNA repair including the double-stranded DNA-dependent protein kinase [13,14]. One cannot exclude at the present that LY 294002 may inhibit the activity of other members of this superfamily of proteins. In agreement with such a speculation, wortmannin, another specific inhibitor of PI 3-kinase, has been shown to inhibit the DNA-dependent protein kinase, even though the IC50 for this inhibition is two orders of magnitude higher than for PI 3-kinase. By contrast, wortmannin does not inhibit FRAP which has been demonstrated involved in rapamycine-induced cell cycle regulation [13].

Altogether, our results bring important light on the effect of the flavonoid derivative LY 294002 on the cell cycle machinery. Further, they give insight on a possible link between PI 3-kinase activation pathway and cell cycle regulation.

Acknowledgements: This work was supported by the Institut National de la Santé et de la Recherche Médicale, by the Ligue Nationale contre le cancer and by the Region Midi-Pyrénées, France. We are indebted to Georges Cassar for the flow cytometry analysis and to Dr. M.A. Saornil from the Valadolid Institute, Spain for the gift of the melanoma cell line OCM-1.

References

[1] Middleton, E. and Kandaswami, C. (1993) in: The Flavonoids: Advances in Research Since 1986 (Harborne, J.H., Ed.), pp. 619–652, Liss, New York.
[2] Yoshida, M., Sakai, T., Hosokawa, N., Marui, N., Matsumoto, K., Fujioka, A., Nishino, H. and Aoike, A. (1990) FEBS Lett. 260, 10–13.
[3] Yoshida, M., Yamamoto, M. and Nikaido, T. (1992) Cancer Res. 52, 6676–6681.

[4] Matsukawa, Y., Marui, N., Sakai, T., Satomi, Y., Yoshida, M., Matsumoto, K., Nishino, H. and Aoike, A. (1993) Cancer Res. 53, 1328–1331.
[5] Losiewicz, M.D., Carlson, B.A., Kaur, G., Sausville, E.A. and Worland, P.J. (1994) Biochem. Biophys. Res. Commun. 201, 589–595.
[6] Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Rémésy, C., Chap, H. and Payrastre, B. (1997) Biochem. Pharmacol. 53, 1649–1657.
[7] Vlahos, C.J., Matter, W.F., Hui, K.Y. and Brown, R.F. (1994) J. Biol. Chem. 269, 5241–5248.
[8] Varticovski, L., Harrison-Findik, D., Keeler, M.L. and Susa, M. (1994) Biochim. Biophys. Acta 1226, 1–11.
[9] Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B.C., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Cell 64, 281–302.
[10] Kapeller, R. and Cantley, L.C. (1994) BioEssays 16, 565–576.
[11] Carpenter, C.L., Auger, K.R., Duckworth, B.C., Hou, W.M., Schaffhausen, B. and Cantley, L.C. (1993) Mol. Cell. Biol. 13, 1657–1665.
[12] Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M.J., Giut, I., Totty, N.F., Truong, O., Vicendo, P., Yonezawa, K., Kasuga, M., Courtneige, S.A. and Waterfield, M.D. (1994) EMBO J. 13, 522–533.
[13] Hunter, T. (1995) Cell 83, 1–4.
[14] Carpenter, C.L. and Cantley, L.C. (1996) Curr. Opin. Cell Biol. 8, 153–158.
[15] Morgan, D.O. (1995) Nature 374, 131–134.
[16] Nigg, E.A. (1995) BioEssays 17, 471–480.
[17] Pines, J. (1995) Biochem. J. 308, 697–711.
[18] Elledge, S.J., Winston, J. and Harper, J.W. (1996) Trends Cell Biol. 6, 388–392.
[19] Sherr, C.J. and Roberts, J.M. (1995) Genes Dev. 9, 1149–1163.
[20] Koff, A., Ohsuki, M., Polyak, K., Roberts, J.M. and Massagué, J. (1993) Science 260, 536–539.
[21] Reynisdottir, I., Polyak, K., Iavarone, A. and Massagué, J. (1995) Genes Dev. 9, 1831–1845.
[22] Kato, J.-Y., Matsuoka, M., Polyak, K., Massagué, J. and Sherr, C.J. (1994) Cell 79, 487–496.
[23] Albers, M.W., Williams, R.T., Brown, E.J., Tanaka, A., Hall, F.L. and Schreiber, S.L. (1993) J. Biol. Chem. 268, 22825–22829.
[24] Morice, W.G., Wiederrecht, G., Brunn, G.J., Siekierka, J.J. and Abraham, R.T. (1993) J. Biol. Chem. 268, 22737–22745.
[25] Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J. and Reed, S.I. (1994) Cell 76, 1013–1023.
[26] Nourse, J., Firpo, E., Flanagan, W.M., Coats, S., Polyak, K., Lee, M.-H., Massagué, J., Crabtree, G.R. and Roberts, J.M. (1994) Nature 372, 570–573.
[27] Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S. and Schreiber, S.L. (1994) Nature 369, 756–758.
[28] Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Temps, P. and Snyder, S.H. (1994) Cell 78, 35–43.
[29] Takuwa, N. and Takuwa, Y. (1997) Mol. Cell. Biol. 17, 5348–5358.
[30] Carpenter, C.L., Duckworth, B.C., Auger, K.R., Cohen, B., Schaffhausen, B. and Cantley, L.C. (1990) J. Biol. Chem. 265, 19704–19711.
[31] Hiles, I.D., Otsu, M., Volinia, S., Fry, M.J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N.F., Hsuan, J.J., Courtneige, S.A., Parker, P.J. and Waterfield, M.D. (1992) Cell 70, 419–429.
[32] Volinia, S., Dhand, R., Vanhaesebroeck, B., MacDougall, L.K., Stein, R., Svelebil, M.J., Domin, J., Panayotou, G. and Waterfield, M.D. (1995) EMBO J. 14, 3339–3348.
[33] Roche, S., Koegl, M. and Courtneige, S.A. (1994) Proc. Natl. Acad. Sci. USA 91, 9185–9189.