Sánchez-Hernández, I. et al. (2012) 'Dual inhibition of V600EBRAF and the PI3K/AKT/mTOR pathway cooperates to induce apoptosis in melanoma cells through a MEK-independent mechanism', Cancer letters, 314(2), pp. 244-255.

Available at https://doi.org/10.1016/j.canlet.2011.09.037

Dual inhibition of ^{V600E}BRAF and the PI3K/AKT/mTOR pathway cooperates to induce apoptosis in melanoma cells through a MEK-independent mechanism

Irene Sánchez-Hernández[1], Pablo Baquero[1], Laura Calleros[2], Antonio Chiloeches[1]#

[1]Department of Biochemistry and Molecular Biology, [2]Department of Physiology; Faculty of Medicine, University of Alcalá, Madrid, Spain

Key words: BRAF, MEK, ERK, melanoma, apoptosis, PI3K/AKT/mTOR

#To whom correspondence should be addressed: Antonio Chiloeches, Dpto. Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Alcalá, Ctra. Madrid-Barcelona Km.33,6; E-28871 Alcalá de Henares, Madrid, Spain.
Phone: 0034-918854583
Fax: 0034-918854585
E-mail: antonio.chiloeches@uah.es

Abstract

BRAF is a main oncogene in human melanomas. Here, we show that BRAF depletion by siRNA or inhibition of its activity by treatment with RAF inhibitor Sorafenib induces apoptosis in NPA melanoma cells expressing oncogenic ^{V600E}BRAF. This effect is mediated through a MEK/ERK-independent mechanism, since treatment with the MEK inhibitor U0126 does not exert any effect. Moreover, we demonstrate that inhibition of the PI3K/AKT/mTOR cascade alone does not increase apoptosis in these cells. However, the blockage of this pathway in cells lacking either BRAF expression or activity cooperates to induce higher levels of apoptosis than those achieved by inhibition of BRAF alone. Consistently, we demonstrate that abrogation of BRAF expression increases AKT and mTOR phosphorylation, suggesting the existence of a compensatory pro-survival mechanism after BRAF depletion. Together, our data provide a rationale for dual targeting of BRAF and PI3K/AKT/mTOR signalling to effectively control melanoma disease.

1. Introduction

Melanoma is the most aggressive form of skin cancer. A significant advance in the biology of melanoma was the identification of an activating BRAF mutation in over 60% of these tumors [1]. The most common BRAF mutation is the substitution of a valine residue at position 600 for glutamic acid (V600E). This ^{V600E}BRAF mutant has constitutive protein kinase activity promoting sustained activation of its direct downstream target, MEK, and of the ERK1/2 kinases in turn [2-4]. The constitutive activation of the MEK/ERK pathway induced by BRAF mutations is required for the proliferation, resistance to apoptosis and invasion of melanoma cells [4-6]. It is well established that blockade of BRAF and the MEK/ERK pathway inhibits melanoma cell growth and increases apoptosis in ^{V600E}BRAF melanoma cells [4,7-9]. These findings have focused efforts to develop inhibitors to target the BRAF/MEK/ERK pathway. Thus, it has been observed regression of metastatic mutant BRAF melanomas as consequence of administration of BRAF inhibitors [10,11], suggesting that induction of apoptosis may be a major biological consequence of inhibition of mutant BRAF. Several mechanisms have been reported to contribute to apoptosis induced by inhibition of ^{V600E}BRAF. These include dephosphorylation of Bad, translocation of Bmf, upregulation of Bim_{EL}, and downregulation of Mcl-1 [12-14]. Despite this, the molecular mechanisms underlying the prosurvival role of BRAF in melanoma cells is not well understood. Several BRAF inhibitors tested showed increased apoptosis *in vitro* and strong antitumor effects in patients with ^{V600E}BRAF melanomas [10,11,15]. However, chronic administration to cell lines with the BRAF inhibitors PLX4032 or PLX4720 and most of patients treated with them develop drug resistance associated with BRAF-independent survival [16,17].

Another important signalling pathway in melanoma is the phosphoinositide-3-OH kinase PI3K/AKT/ mammalian target of Rapamycin (mTOR) pathway. The activation of this cascade leads to increased growth and apoptosis resistance via different targets, including downregulation of many proapoptotic proteins and upregulation of antiapoptotic proteins [18-21].

There is accumulating evidence that the Ras/RAF/MEK/ERK and the PI3K/AKT/mTOR pathways closely cooperate in the transduction of survival signals [22,23]. Thus, it has been shown that both BRAF and PI3K are required for protection of apoptosis of melanoma cells after loss of contact with an extracellular matrix environment [24] and that knockdown of AKT3, the main AKT isoform activated in melanoma, cooperates with BRAF targeting to promote apoptosis [25,26]. Moreover, constitutive activation of the PI3K/AKT pathway seems to mediate resistance of ^{V600E}BRAF melanoma cells to inhibition of BRAF [27,28]. The overexpression of constitutive active AKT3 renders melanoma cells resistant to apoptosis by BRAF knockdown or PLX4720 treatment [27]. Finally, the treatment of pancreatic neuroendocrine tumor cells with the RAF inhibitor RAF265 increases AKT phosphorylation, indicating a possible compensatory mechanism in response to RAF inhibition [29].

Here, we examined the role of BRAF/MEK/ERK and PI3K/AKT/mTOR pathways and the relationship between them on apoptosis of NPA melanoma cells. We show, unexpectedly, that BRAF can promote survival by an MEK/ERK-independent mechanism. Our results also show increased activation of the PI3K/AKT/mTOR pathway after BRAF targeting, suggesting the existence of a novel compensatory mechanism for acquired resistance to BRAF inhibition and a rationale for dual targeting of both pathways in melanoma.

2. Materials and Methods

2.1.Reagents

Antibodies used in this work were as follows: anti-BRAF, anti-ERK2, anti-AKT, anti-Bcl-XL, and anti-Bcl-2 and anti-p70S6K (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-tubulin (Sigma); anti-CRAF and anti-caspase-3 (BD Biosciences), anti phospho-AKT (Ser 473), anti-phospho-AKT (T308) and anti-phospho-p70S6K (T389) (Cell Signalling Technology); anti-Bim (Stressgen) and peroxidase (HRP)-conjugated antirabbit IgG and antimouse IgG (DAKO). The MEK inhibitor U0126 was from Promega; Sorafenib (BAY 43-9006) was kindly provided by Dr. R. Marais (Institute of Cancer Research, London, UK); the PI3K inhibitors LY294002 and Wortmannin were from Stressgen and Sigma, respectively; Rapamycin was from Calbiochem, the AKT inhibitor ALX (1L-6-Hydroxymethyl-chiro-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate) was from Alexis; caspases inhibitor Z-VAD-FMK was from BD Biosciences. Etoposide, cicloheximide and tetramethylrhodamine methyl ester (TMRM) were from Sigma.

2.2. Cell culture

Human melanoma NPA87 tumor cells were kindly provided by A. Fusco (Institute of Endocrinology and Experimental Oncology, Naples, Italy). All cells were cultured in DMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin at 37ºC in a 5% CO_2 atmosphere. These cells were previously described as papillary thyroid carcinoma cells and later identified as melanoma cells. Identity was confirmed *vs.* published data [30], using sequencing standar techniques.

2.3. Cell transfection

For siRNA silencing, cells were seeded at $2x10^5$ cells/35-mm well the day before transfection. Cells were transfected using LipofectAMINE (Invitrogen) in 1 ml of OPTIMEM with 100nM BRAF-specific (5´-AACAGUCUACAAGGGAAAGUG-3´), CRAF-specific (5´-AAUAGUUCAGCAGUUUGGCUA-3´) or Silencer^{TM} negative control#1 siRNA (Ambion, Inc.). After 6 h incubation with the RNA-complex, medium was replaced and 2 ml of fresh medium containing 10% FBS was added. Cells were treated and harvested at the indicated times after the transfection as stated in figure legends.

2.4. Cell lysis and Western blot analysis

After incubation, cells were harvested into 200 μl lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 10% glycerol, 100 mM KCl, 1% Triton X-100, 0.3% 2-mercaptoethanol, 5 mM NaF, 0.2 mM Na_3VO_4, 5 mM $MgCl_2$) supplemented with protease inhibitors. Cell extracts were normalized for protein

content. Proteins were separated by SDS-PAGE and Western blot analysis was performed following standard protocols using the indicated antibodies.

2.5. Quantification of sub-G1 DNA content by flow cytometry

Quantification of apoptotic cells was carried out by measurement of the fraction of cells with sub-G1 DNA content by flow cytometry analysis of propidium iodide (PI)-stained cell. Adherent and floating cells were collected after treatment, washed with ice-cold PBS, and fixed with 70% ice-cold ethanol (30 min, 4ºC). Fixed cells were washed twice with PBS and treated with RNase (1 mg/ml; 30 min, 37ºC). Cellular DNA was stained with 5 ng/ml PI in PBS and cells were analyzed on a FACScan flow cytometer (Becton Dickinson). Percentages of cells in different cell cycle phases were calculated from DNA histograms. Cells with sub-G_1 DNA content were considered apoptotic.

2.6. Assessment of apoptosis by Annexin V and PI staining

Apoptosis was also detected by Annexin V-FITC Apoptosis Detection Kit (Calbiochem). Briefly, cells were double stained with annexin V-FITC and propidium iodide (PI) following manufacturer's instruction. Staining was measured by flow cytometry on the FACSCalibur (Becton-Dickinson). The distribution of cells was analyzed using CellQuest™ software (Becton-Dickinson) in the flow cytometer. Data from 10,000 cells was collected for each data file. Early apoptosis was defined as Annexin V-FITC-positive and PI-negative cells, and late apoptosis was defined as double positive Annexin V-$FITC^+$/PI^+ staining.

2.7. Measurement of mitochondrial membrane potential (Δψm)

After treatments, adherent and floating cells were collected, washed with ice-cold PBS, and stained with TMRM (50 nM) in PBS at 37°C for 15 min. After staining, cells were immediately subjected to flow cytometry using a FACScan flow cytometer (Becton Dickinson) with a 488 nm laser. The fluorescence emission of TMRM was collected through a 585/42 nm filter. Experiments were carried out in triplicate and 10000 cells were acquired for each test sample. The Data were analyzed using CellQuest™ software (Becton-Dickinson).

3. Results

3.1. Inhibition of ^{V600E}BRAF induces apoptosis in NPA cells through a MEK/ERK-independent mechanism

To investigate the role of oncogenic BRAF signalling on apoptosis in melanoma cells, we used the NPA melanoma cell line that harbours the activating V600E mutation in the BRAF gene. This NPA cell line was previously misidentified as papillary thyroid carcinoma cell line, however it has been recently demonstrated that it has a melanoma origin [30]. We first examined MEK-ERK signalling in

NPA cells by using RNA interference to deplete individual RAF isoforms. Under the experimental conditions used here, each small interfering RNA (siRNA) oligonucleotide was specific for its target protein. The CRAF siRNA only depleted CRAF and the BRAF siRNA only targeted BRAF (Figure 1A). We also determined ERK1/2 activity and observed that BRAF silencing completely suppressed ERK phosphorylation in these cells, whereas abrogation of CRAF expression did not show any effect (Figure 1A). These data are consistent with findings showing that cells harbouring BRAF mutations are significantly more dependent on BRAF signalling than cells where BRAF is not mutated [3,4,6].

We next examined the involvement of BRAF in cell survival. For this purpose, we analyzed the sub-G1 hypodiploid cell population by flow cytometry in NPA cells that did not express either CRAF, BRAF or both proteins by transfection with specific siRNA. We observed that BRAF depletion induced a significant increase on the sub-G1 population after 96 h (Figure 1B). We also examined the percentage of cells undergoing a decrease in mitochondrial membrane potential, another characteristic parameter of the process of apoptosis, by using the TMRM probe. As shown in Figure 1C, the abrogation of BRAF expression increased the percentage of cells with low mitochondrial membrane potential. By contrast, CRAF depletion failed to induce either of these markers of apoptosis. Abrogation of both CRAF and BRAF expression exerted similar effect to that of depletion of BRAF alone (Figure 1B, 1C). To verify the role of BRAF in apoptosis, we analyzed caspase 3 activation. To this end, we determined the levels of complete protein after silencing the expression of RAF proteins with siRNA. As can be seen in figure 2A, depletion of BRAF in these cells reduced the levels of inactive caspase 3, while removal of CRAF had no effect. Furthermore, we examined whether abrogation of BRAF expression produced apoptosis through activation of caspases in NPA cells. Thus, we transfected these cells with BRAF siRNA and treated them with the caspase inhibitor Z-VAD-FMK (Figure 2B). These results showed that treatment of NPA cells with Z-VAD-FMK in BRAF knock-down cells reversed the cell death induced by the lack of BRAF (Figure 2B), indicating that ^{V600E}BRAF provides a survival signal to NPA cells. Moreover, and that the cell death observed by abrogation of BRAF expression was apoptosis induced through activation of caspases.

Other authors have shown that overexpression of ^{V600E}BRAF decreases both the level and activity of some proteins of the proapoptotic Bcl-2 family, such as BIM and BAD [14]. Since our data showed that abrogation of ^{V600E}BRAF expression increased apoptosis in NPA cells, we wondered whether any protein of this family would be a target of ^{V600E}BRAF in these cells. We studied the effect of silencing BRAF on the expression levels of proapoptotic protein BIM and the antiapoptotic proteins Bcl-2 and Bcl-XL by western blot (Figure 2C). We observed that the ^{V600E}BRAF depletion decreased the expression levels of Bcl-2 and Bcl-XL, while it increased the levels of BIM protein (Figure 2C), indicating that ^{V600E}BRAF induces cell survival by regulating the expression of these proteins in the apoptotic machinery of cells.

Next, we examined whether BRAF protected from apoptosis through activation of the MEK/ERK pathway. We performed similar experiments using the RAF multikinase inhibitor Sorafenib and the MEK inhibitor U0126. As expected, the treatment with both inhibitors completely abolished ERK phosphorylation in NPA cells (Figure 3A). We then determined apoptosis levels in NPA cells, untreated or pretreated with these inhibitors for 24, 48 and 72 hours (Figure 3). First, we observed that treatment with the MEK inhibitor U0126 did not increase the sub-G1 hypodiploid cell population at any

time assayed; however, the RAF inhibitor Sorafenib increased the sub-G1 hypodiploid cell population in a time-dependent manner (Figure 3B). To confirm these results, we determined the level of phosphatidyl serine exposed outside the cell membrane, by labelling with Annexin V-FITC and subsequent analysis by flow cytometry, after treatment of NPA cells with the inhibitors U0126 and Sorafenib for 24, 48 and 72 hours (Figure 3C, 3D). Accordingly, the results revealed that the U0126 treatment did not produce an increase in basal levels of Annexin V^+-marked cells, whereas incubation with Sorafenib increased the percentage of cells labelled only with Annexin V after 48 hours (Figure 3C, 3D). Moreover, the increase of apoptosis induced by Sorafenib treatment was exerted through caspase activation, since the pretreatment of NPA cells with the caspase inhibitor Z-VAD-FMK decreased by about 60% the sub-G1 population induced by the RAF inhibitor (Figure 3E). Since ERK activation is totally dependent of BRAF, these results demonstrate that oncogenic BRAF protein produces a survival signal for melanoma cells through a MEK/ERK-independent mechanism.

3.2. PI3K/AKT/mTOR pathway cooperates with ^{V600E}BRAF to protect NPA cells from apoptosis

The PI3K/AKT pathway also plays an important role in cell transformation and it has been widely reported that it is involved in cell survival and protection from apoptosis. Therefore, to determine whether the increased apoptosis induced by the absence of BRAF was also taking place through the PI3K/AKT pathway, we studied whether there was any interaction between them.

We analyzed the effect of PI3K inhibition with the specific inhibitors LY294002 and Wortmannin on the apoptosis induced by the lack of BRAF expression in NPA cells. As shown in figure 4A, neither of these inhibitors exerted any effect on the sub-G1 population in basal conditions in BRAF-expressing cells. However, both LY294002 and Wortmannin treatments in NPA cells without BRAF expression significantly increased their apoptosis compared to untreated cells without BRAF (Figure 4A). To confirm these results, we determined the percentage of cells with low mitochondrial membrane potential under the same conditions (Figure 4B). The results were similar to those observed in the determination of the sub-G1 hypodiploid population, the incubation with the LY294002 and Wortmannin inhibitors did not increase the proportion of cells with low membrane potential in the control groups, whereas treatment of cells that did not express BRAF with these inhibitors increased it significantly, with respect to untreated cells lacking ^{V600E}BRAF (Figure 4B) On the other hand, we determined apoptosis in NPA cells treated with LY294002 inhibitor alone or in combination with either Sorafenib or U0126 inhibitors (Figure 4C). In this case, besides the lack of effect of individual treatment with LY294002 seen previously, we observed that the combination of LY294002 with U0126 did not increase apoptosis, but that the simultaneous treatment with LY294002 and Sorafenib increased apoptosis of NPA cells compared to that observed after treatment with Sorafenib alone (Figure 4C). These data demonstrate that cell death induced by inhibiting ^{V600E}BRAF together with PI3K is also independent of the MEK/ERK pathway.

Once observed that inhibition of PI3K resulted in increased apoptosis when ^{V600E}BRAF protein was not expressed, we studied whether this effect was produced through its effector protein AKT, as we observed that its phosphorylation depends on PI3K (Figure 6A, 6B). We analyzed the levels of apoptosis in NPA cells expressing or lacking the BRAF protein and treated with the AKT inhibitor ALX.

As shown in figure 4D, incubation with ALX alone induced a slight increase of apoptosis, but this increase was much higher in cells in which BRAF expression had been inhibited by siRNA (Figure 4D).

It has been shown that mTOR is a point of convergence in growth control and cell survival by receiving signals from Ras and PI3K/AKT pathways [31]. This protein is involved in the suppression of tumor cell apoptosis and its role has also been linked with regulation of BIM. Since we have previously shown that abrogation of ^{V600E}BRAF expression increased the levels of this proapoptotic factor, we examined whether mTOR inhibition had any effect on apoptosis in NPA cells that either expressed or not ^{V600E}BRAF. NPA cells were treated with the mTOR inhibitor Rapamycin after silencing BRAF expression and the results obtained show that Rapamycin did not cause apoptosis in basal conditions. However, as was the case of PI3K or AKT inhibition, Rapamycin potentiated the effect induced by the lack of this oncogene, producing a significant increase in apoptosis (Figure 5A). We confirmed that inhibition of mTOR only exerted an effect on apoptosis after BRAF inhibition in cells treated with a combination of Sorafenib and Rapamycin (Figure 5B). This combined treatment significantly increased the percentage of NPA cells in the sub-G1 fraction compared with Sorafenib alone (Figure 5B). On the other hand, we checked that mTOR is a convergence point from the BRAF and PI3K pathways by inhibiting simultaneously BRAF, PI3K and mTOR. Thus, either in experiments in which BRAF expression was abolished by siRNA (Figure 5A) or inhibited with Sorafenib (Figure 5B), the combinations of the PI3K inhibitors LY294002 or Wortmannin with Rapamycin did not significantly increase apoptosis under basal conditions. Moreover, the combined treatment of these inhibitors did not induce an additional apoptosis either in BRAF-non expressing cells (Figure 5A) or in Sorafenib-treated cells as compared to cells treated with the inhibitors independently (Figure 5B).

Of the two existing mTOR complexes, mTORC1 and mTORC2, only the first one is inhibited by Rapamycin, and this complex is involved in the synthesis of proteins through the activation of the kinase p70S6K. We finally checked whether the inhibition of protein synthesis with cycloheximide also enhanced the effect of the absence of BRAF in NPA cells on apoptosis. The data in figure 5C show that in BRAF-expressing cells treated with cycloheximide there was not effect on apoptosis, whereas in cells lacking BRAF an increase in apoptosis was observed.

3.3. BRAF abrogation results in activation of the PI3K/AKT/mTOR pathway

Finally, since our data have demonstrated that the PI3K/AKT/mTOR pathway cooperates with ^{V600E}BRAF in the prevention of apoptosis and that high levels of cell death are obtained when both pathways are inhibited, we studied the relationship between them. For this purpose, we determined the activation status of AKT in NPA cells expressing BRAF and in those in which we inhibited its expression (Figure 6A, B). We observed that cells expressing ^{V600E}BRAF do not show basal phosphorylation of AKT on S473, but when we blocked BRAF expression, we observed a significant increase of its phosphorylation, without changes in its expression levels (Figure 6A). Since S473 can also be phosphorylated by mTORC2, we examined the phosphorylation of T308 of AKT, which is depedent on PDK1 activated by PI3K. We also observed that although NPA cells show basal phosphorylation on this residue, the abrogation of BRAF expression increased it (Figure 6B).

Furthermore, this increase in AKT phosphorylation in the absence of BRAF seems to be due to PI3K activation, since inhibition of PI3K activity with LY294002 abolished this increase (Figure 6A, B). To verify that this effect was transmitted through the PI3K/AKT/mTOR pathway, we studied the effect of abrogating ^{V600E}BRAF expression on the phosphorylation status of the mTOR substrate p70S6K (Figure 6C). The analysis of p70S6K phosphorylation levels on T389 showed parallel results to those observed with AKT; p70S6K was not phosphorylated under basal conditions, but there was an increase in its phosphorylation after inhibition of ^{V600E}BRAF expression (Figure 6C). Furthermore, this higher p70S6K phosphorylation was also dependent on PI3K and mTOR, since treatment with the inhibitors of these proteins, LY294002 and Rapamycin, respectively, blocked this phosphorylation (Figure 6C). These data indicate that ^{V600E}BRAF could act as an inhibitor of the PI3K/AKT/mTOR/ pathway and that its absence increases the activity of this pathway.

4. Discussion

The Ras/RAF/MEK/ERK cascade and the PI3K/AKT/mTOR cascade are prototypic survival pathways that have been implicated in tumorogenesis of many cancer cells including melanoma and thyroid cancer. Thus, down-regulation of these pathways contributes to the inhibition of tumor development and progression, and improves responses to common therapies [32,33,34]. The role of oncogenic BRAF on apoptosis has been mainly linked to the activation of the MEK/ERK cascade. Here, however, we show that either BRAF depletion with small interference RNA or treatment with the RAF inhibitor Sorafenib promotes apoptosis in NPA melanoma cells in a MEK/ERK-independent manner, since the inhibition with the MEK inhibitor U0126 did not exert any effect in these cells. We also show that BRAF targeting causes deregulation of the cellular apoptotic machinery; in particular, decreasing the expression of antiapoptotic proteins Bcl-2 and Bcl-XL, promoting the expression or stabilization of the proapoptotic protein BIM and activation of caspases. Thus, although abrogation of BRAF expression and treatment with Sorafenib is lethal to melanoma cell lines, the mechanisms of action of BRAF may be considerably more complex than those proposed for MEK inhibition. These data suggest the existence of at least two distinctive response patterns to inhibition of BRAF. In some cancer cells, its inhibition could modulate apoptosis through a MAPK-independent process, whereas other cells could respond to this inhibition as they might to a MEK inhibitor. The MEK/ERK-independent functions of oncogenic BRAF can also be observed in other cancers such as thyroid cancer, for instance. Thus, it is not known why the BRAF mutation induces a different gene expression pattern and is associated with a higher aggressiveness and recurrence of these tumors, compared to other genetic alterations related to the MEK/ERK pathway, such as transversion of the receptor tyrosine kinase RET/PTC or oncogenic mutations of Ras protein [35]. Regarding apoptosis, it has been observed that inhibition of MEK does not decrease the higher levels of the antiapoptotic proteins c-IAP1 y c-IAP3 induced by oncogenic BRAF or that the deregulation of Bcl-2 and Bcl-XL caused by Sorefenib is independent of the MEK-ERK activity [9,28,36,37].

At present, there is no known substrate for BRAF other than MEK through which it exerts its protection against cell death. One possibility could be through the demonstrated dimerization of BRAF

with CRAF, which has been shown to promote survival by modulating the activity of several effectors, such as ASK1, MST2 or NF-κB, regardless of MEK kinase activity [38]. Therefore, in certain circumstances, BRAF may signal through CRAF and this could be a different mechanism of action from the Ras and MEK/ERK pathway. However, our results rule out CRAF in mediating ^{V600E}BRAF-induced survival, as we have shown that inhibition of CRAF expression does not change the levels of cell death in our model. In this sense, it is widely reported that, whereas in cells with wild-type BRAF and CRAF, many functions are dependent on these two proteins, cells bearing ^{V600E}BRAF mutation become "addicted to this oncogene" and the role of CRAF is not as relevant.

A particularly interesting finding from our studies is the role of the PI3K/AKT/mTOR signalling in BRAF-dependent survival. We observed that inhibition of the PI3K signalling pathway at different levels with specific inhibitors has no effect on apoptosis. However, in cells lacking ^{V600E}BRAF activity, due to treatment with Sorafenib or elimination of its expression with siRNA, the inhibition of this pathway with either PI3K, AKT or mTOR inhibitors, synergizes to increase apoptosis levels to a greater extend than that achieved by the lack of expression or activity of ^{V600E}BRAF alone. This indicates that inhibition of individual components of the PI3K/AKT/mTOR pathway only enhances apoptosis in the absence of this oncogene. Moreover, the fact that the simultaneus inhibition of PI3K and mTOR induces the same levels of apoptosis that the inhibition of mTOR alone in cell with abrogated BRAF expression or inhibited by treatment with Sorafenib show that mTOR is a convergence point of BRAF and PI3K signals in these cells.

Several studies have proposed the collaboration of the Ras/RAF/MEK/ERK and PI3K/AKT/mTOR pathways in the survival process [22,39,40]. Thus, the dual inhibition of the Ras/RAF/MEK/ERK and PI3K/AKT/mTOR cascades has a strong rationale in different types of cancer, showing evidences that simultaneous inhibition of both signalling pathways is effective *in vitro* and in animal models [22,32,39,40]. It has been demonstrated in melanoma cells that BRAF and PI3K are necessary for the protection of cell death induced by loss or inappropriate contact with the extracellular matrix [24,27]. However, it has also been observed that the dual inhibition of both the PI3K/AKT/mTOR pathway and the RAF/ERK pathway lowered the incidence of tumors and induced a cytostatic response *in vitro* and in xenograft tumors, exceeding responses to the MEK inhibitor or mTORC1 inhibitor alone, but not affecting apoptosis [41].

The cross talk between the two pathways can result in activation of one pathway if the other is inhibited singly. Thus, we observed that BRAF silencing in NPA cells increases AKT phosphorylation on S473 and T308 and p70S6K on T389 and that both phosphorylations of AKT are reversed by inhibition of PI3K with LY294002, as well as by inhibiting mTORC1 with Rapamycin, in the case of p70S6K. Although AKT could also be phosphorylated by mTORC2 and this kinase can be inhibited by the LY294002 inhibitor, these effects seem to be dependent on PI3K activation because T308 is phosphorylated by PDK1 after activation by PI3K and not by mTORC2. Moreover, we observed similar effects on phosphorylation of T389 of p70S6K, which is phosphorylated by mTORC1 but not by mTORC2, after treatment with Rapamycin, which inhibits mTORC1 but not mTORC2. These data show a compensatory mechanism of the PI3K/AKT/mTOR pathway on the apoptosis induced by BRAF targeting. These results would explain why the levels of apoptosis observed with the abrogation of ^{V600E}BRAF are lower than those observed after dual inhibition of both pathways, due to up-

regulation of the phosphorylation of AKT/mTOR which counteracts this effect; therefore, it is necessary to inhibit both pathways to produce a greater decrease in cell survival. In this sense, it has been observed that activation of the PI3K/AKT pathway mediates acquired resistance to apoptosis induced by inhibition of BRAF with the inhibitors Sorafenib, PLX4720 or SB-590885 [28,42,43] and that overexpression of AKT3 in melanoma cells mediate resistance to apoptosis induced by BRAF inhibition [27]. However, in these studies an increase in AKT activation after BRAF inhibition has not been shown. The only compensatory activation of AKT in response to RAF inhibition has been demonstrated by Zitzman et al., who showed that of the three neuroendocrine tumor cells lines, the inhibition of RAF with the RAF625 inhibitor only increased AKT phosphorylation in BON-1 cells, but without a parallel increase in p70S6K phosphorylation, as we observed [29].

We still do not know the mechanism by which the absence of BRAF increases the PI3K/AKT/mTOR pathway; we tested whether it could be that BRAF inactivates the pathway by direct inhibition of any of its components, but we have not observed a direct interaction of BRAF with PI3K or AKT (data not shown). Then, a possible explanation would be that the lack of BRAF induces an autocrine loop to activate a pathway to support apoptosis resistance. In this sense, it has been observed enhanced AKT phosphorylation levels associated with increased IGF-1R signalling in melanoma BRAF-inhibitor resistant cells [43]. Moreover, a negative feedback loop activating AKT in response to MEK in a EGFR-dependent fashion has been observed in breast cancer cells [44]. However, no effects on apoptosis were shown.

Our results, along with others, demonstrate that targeting solely the BRAF/MEK/ERK pathway is not sufficient to induce high levels of apoptosis in melanoma cells and that the PI3K/AKT/mTOR pathway must also be inhibited to further decrease the cell survival [22,45,46]. We show that: (1) ^{V600E}BRAF melanomas are addicted to this oncogene. Thus, targeting this protein renders cells susceptible to apoptosis; (2) the conventional MEK/ERK pathway is no essential for survival of the ^{V600E}BRAF melanoma cells; (3) activation of the PI3K/AKT/mTOR pathway resulting from BRAF inhibition acts as a protective cellular mechanism to promote survival of ^{V600E}BRAF melanoma cells; and (4) concomitant BRAF and PI3K inhibition leads to higher levels of apoptosis in ^{V600E}BRAF melanoma cells. Results from clinical studies with small molecule inhibitors of BRAF mutants have been very encouraging in the treatment of melanoma, but frequent anti-cancer drug resistance remains a major obstacle for more successful treatment of the disease. Our study not only establishes a mechanism of resistance to BRAF inhibition but also proposes a strategy to overcome it. We show that combining BRAF and PI3K/AKT/mTOR inhibitors constitutes a promising approach to decrease cell survival and resistance to therapy. Thus, our findings warrant further investigation to address the mechanisms underlying PI3K activation after BRAF inhibition and suggest that combination strategies targeting BRAF and PI3K should be considered as a potential approach to treat melanomas refractory to BRAF inhibitors.

Conflict of Interest

No potential conflicts of interest are disclosed by the authors.

Acknowledgments

We are grateful to Dr. A. Jiménez–Ruiz and Dr. D. Moreno-Mateos for technical assistance and Dr. L. Puebla for her linguistic assistance. The work was supported by Grants from FIS of the Instituto de Salud Carlos III (ref PI060109) and from UAH (ref GC2009-002) I. S-H. is a recipient of a predoctoral fellowship from the FPI-UAH program.

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Figure legends

Fig. 1. Inhibition of BRAF expression induces apoptosis in NPA melanoma cells. (A) Representative western blot for BRAF, CRAF and ERK2 expressions, and phospho ERK, 72 h after transfection of NPA cells with siRNA oligonucleotides specific for BRAF (siBRAF), CRAF (siCRAF) or both (siB/CRAF) and a scrambled oligo control (siControl). The results show a representative experiment repeated three times with similar results. (B) Cells were transfected as in (A) for 96 h; after this time medium and NPA cells were collected and apoptosis was identified by quantification of the sub-G1 fractions of PI-stained cells by flow cytometry. Upper panel, results shown are the mean ± SEM of three independent experiments performed in duplicate; bottom panel, histograms of one representative experiment. (C) Percentage of cells with low mitochondrial membrane potential (low $\Delta\Psi m$) measured by flow cytometry after TMRM staining in cells treated as in (B). Upper panel, data shown are the mean ± SEM of three independent experiments performed in duplicate; bottom panel, dot plots of one representative experiment, arrows indicate cell populations with low mitochondrial membrane potential. **0.001<p<0.01, ***p<0.001, significant differences compared to the corresponding control.

Fig. 2. Inhibition of BRAF expression deregulates the cellular apoptotic machinery. (A) Cells were transfected with siRNA oligonucleotides specific for BRAF (siBRAF), CRAF (siCRAF) or both (siB/CRAF) and a scrambled oligo control (siControl) and western blot was performed for caspase-3. Blots were reprobed with anti-tubulin antibody as loading control. Results shown are the means ± SEM from three experiments performed with similar results and blots are from one typical experiment. (B) The sub-G1 fraction measurements in NPA cells untreated or treated with Z-VAD-FMK (40 μM) for 72 h, in which BRAF was depleted (siBRAF) compared with cells transfected with a scrambled control (siControl) and untransfected cells. % Sub-G1 population was normalized and expressed as fold decrease from control (siBRAF). Data are the mean ± SEM of three independent experiments performed in duplicate. (C) Bcl-2, Bcl-XL and BIM expression in NPA cells transfected with siRNA oligonucleotide specific for BRAF (siBRAF) or a scrambled oligo control (siControl) for 72 h. Data are the mean ± SEM of three independent experiments. Western blot showing BRAF, Bcl-2, Bcl-XL and BIM expressions and tubulin, as loading control, are from a representative experiment. **0.001<p<0.01, ***p<0.001, significant differences compared to the corresponding control.

Fig. 3. MEK/ERK activation is not required for BRAF-induced apoptosis. (A) Representative western blot for phospho-ERK and ERK2, as control, in NPA cells treated with DMSO (-) as vehicle control, Sorafenib (10 μM) or U0126 (10 μM) for 48 h. (B) NPA cells were treated with DMSO (-), Sorafenib (10 μM) or U0126 (10 μM) for 48 h and 72 h. Proportion of sub-G1 cells was quantified by flow cytometry. Data showing the mean ± SEM are complied from three independent experiments performed in duplicate. (C and D) NPA cells were treated as indicated in (B) and then stained with Annexin V-FITC and PI followed by flow cytometry analysis. Early apoptosis is defined by Annexin V^+/PI^- staining (Q4)

and late apoptosis is defined by Annexin V^+/PI^+ staining (Q2); (C) Data are the mean ± SEM of three independent experiments performed in duplicate, (D) Dot plots of a representative experiment. (E) NPA cells were pretreated with Z-VAD-FMK (40 μM) for 30 min or remained untreated and then treated with Sorafenib for another 48 h. Cell death was measured as described in (B). % Sub-G1 population was normalized and expressed as fold decrease from control (siBRAF). Data are the mean ± SEM of three independent experiments performed in duplicate. **0.001<p<0.01, ***p<0.001, significant differences compared to the corresponding control.

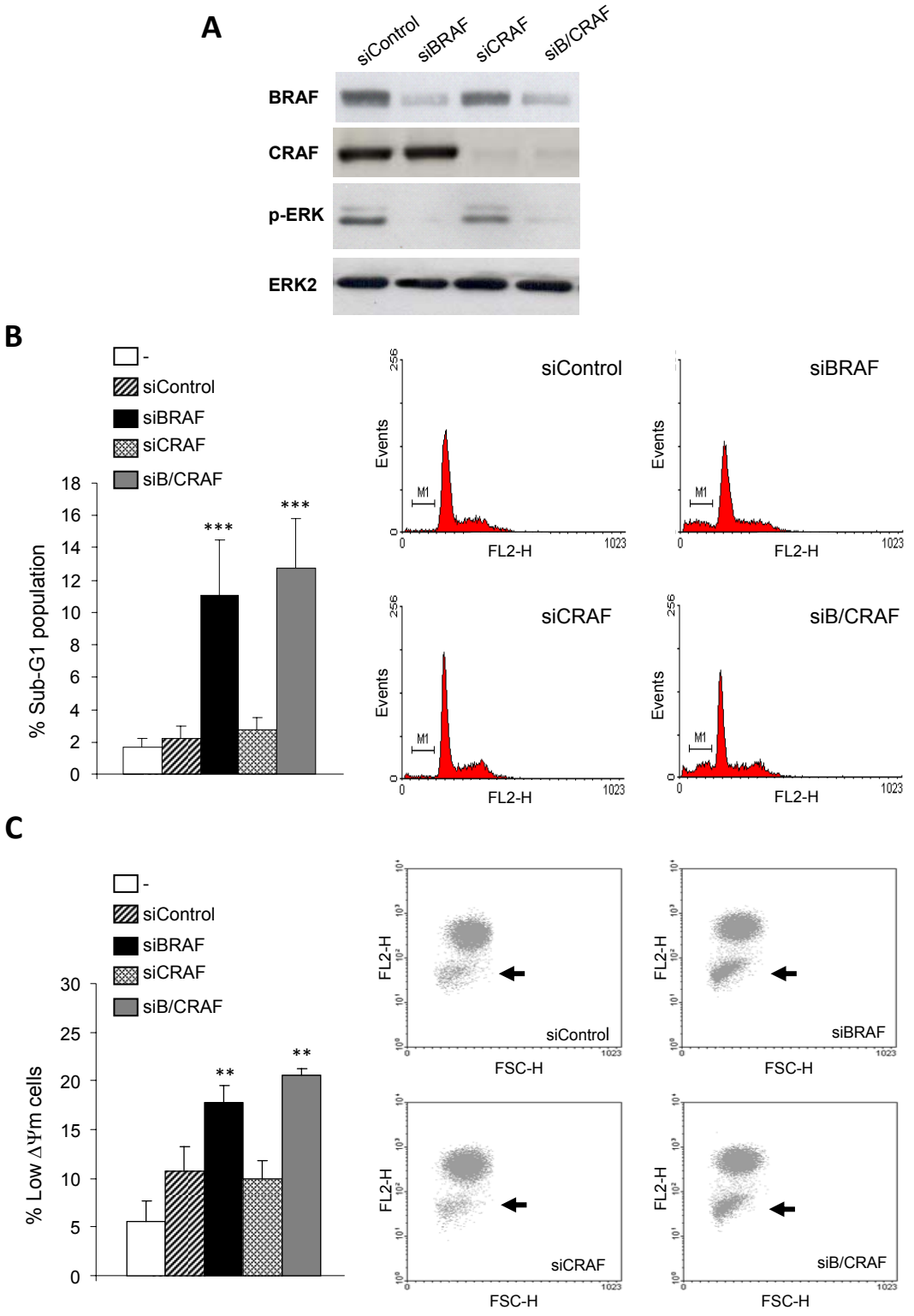
Fig. 4. PI3K and AKT cooperate with BRAF to protect NPA cells from apoptosis. (A) Cells were transfected with the indicated siRNA oligos and then incubated for 48 h with 20 μM LY294002 or 100 nM Wortmannin; medium and cells were then collected and apoptosis was identified and quantified by FACS with PI staining. (B) Mitochondrial membrane potential (ΔΨm) was measured in TMRM-stained cells treated as indicated in (A). (C) Cells were treated with Sorafenib (10 μM) or U0126 (10 μM) for 48 h or 72 h, in the absence or presence of 20 μM LY294002, and sub-G1 fractions were identified and quantified by flow cytometry with PI staining. (D) Cells were transfected as in (A) and incubated in the absence or presence of 30 μM ALX for the last 48 h. At the end of each experiment, sub-G1 fractions were identified and quantified by flow cytometry in PI-stained cells. Results shown are the mean ± SEM of three independent experiments performed in duplicate. *0.01<p<0.05, **0.001<p<0.01, ***p<0.001, significant differences compared to the corresponding control.

Fig. 5. Inhibition of mTOR increases BRAF-induced apoptosis. (A) NPA cells were transfected with siRNA control (siControl) or with specific siRNA for BRAF (siBRAF) and incubated in the absence or presence of 20 μM LY294002, 100 nM Wortmannin or 50 nM Rapamycin alone or in combination for the last 48 h. (B) Cells were treated with Sorafenib (10 μM), Rapamycin, Rapamycin plus Sorafenib, and combinations of Rapamycin plus LY294002 (LY + Rapa) or Wortmannin (Wort + Rapa) in the absence or presence of Sorafenib for 72 h. (C) Cells were transfected as in (A) and treated with 5 μg/ml of cicloheximide (CHX) for the last 48 h. At the end of each experiment, sub-G1 fractions were identified and quantified by flow cytometry in PI-stained cells. Data showing the mean ± SEM are complied from three independent experiments performed in duplicate. **0.001<p<0.01, ***p<0.001, significant differences compared to the corresponding control.

Fig. 6. BRAF abrogation increases phosphorylation of AKT and mTOR. Representative western blots for phospho-AKT (S473) (A) and phospho-AKT (T308) (B) in NPA cells treated with or without 20 μM LY294002 for the last 48 h, in which BRAF expression was depleted with specific siRNA oligonucleotide (siBRAF), compared with cells transfected with a scrambled control (siC) or no transfected, for 96 h. (C) Representative western blot showing phospho-p70S6K (T389) in NPA cells untreated or treated with 20 μM LY294002 or 50 nM Rapamycin, in which BRAF was depleted

(siBRAF) as in (A). For each pair of rows, an image of the phospho-specific blot is shown with the reprobed membrane with anti-AKT or anti-p70S6K antibody, respectively, as controls. Representative blots from experiments performed three times for p-AKT (S473) and p-p70S6K (T389), and two times for p-AKT (308), with similar results are shown.

Figure 1. Sánchez-Hernández I. *et al.*



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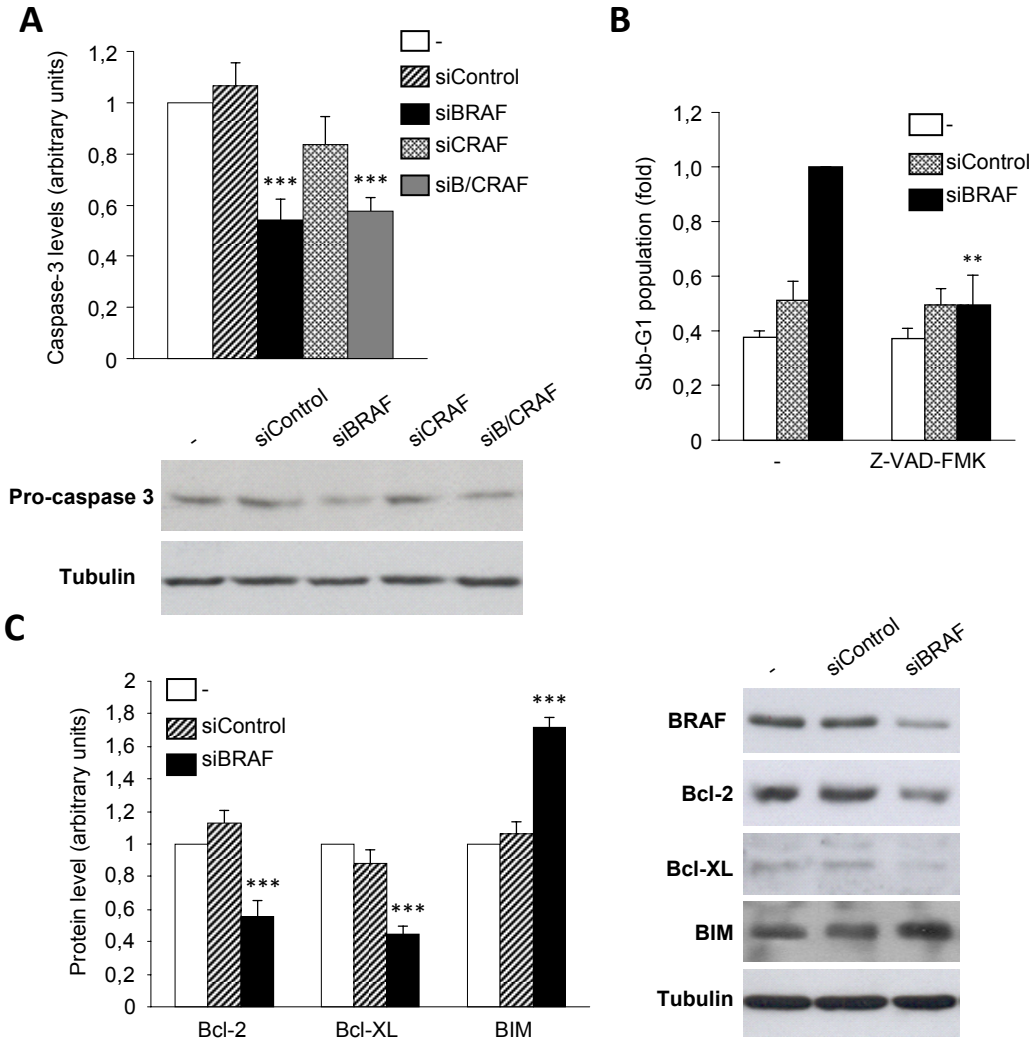

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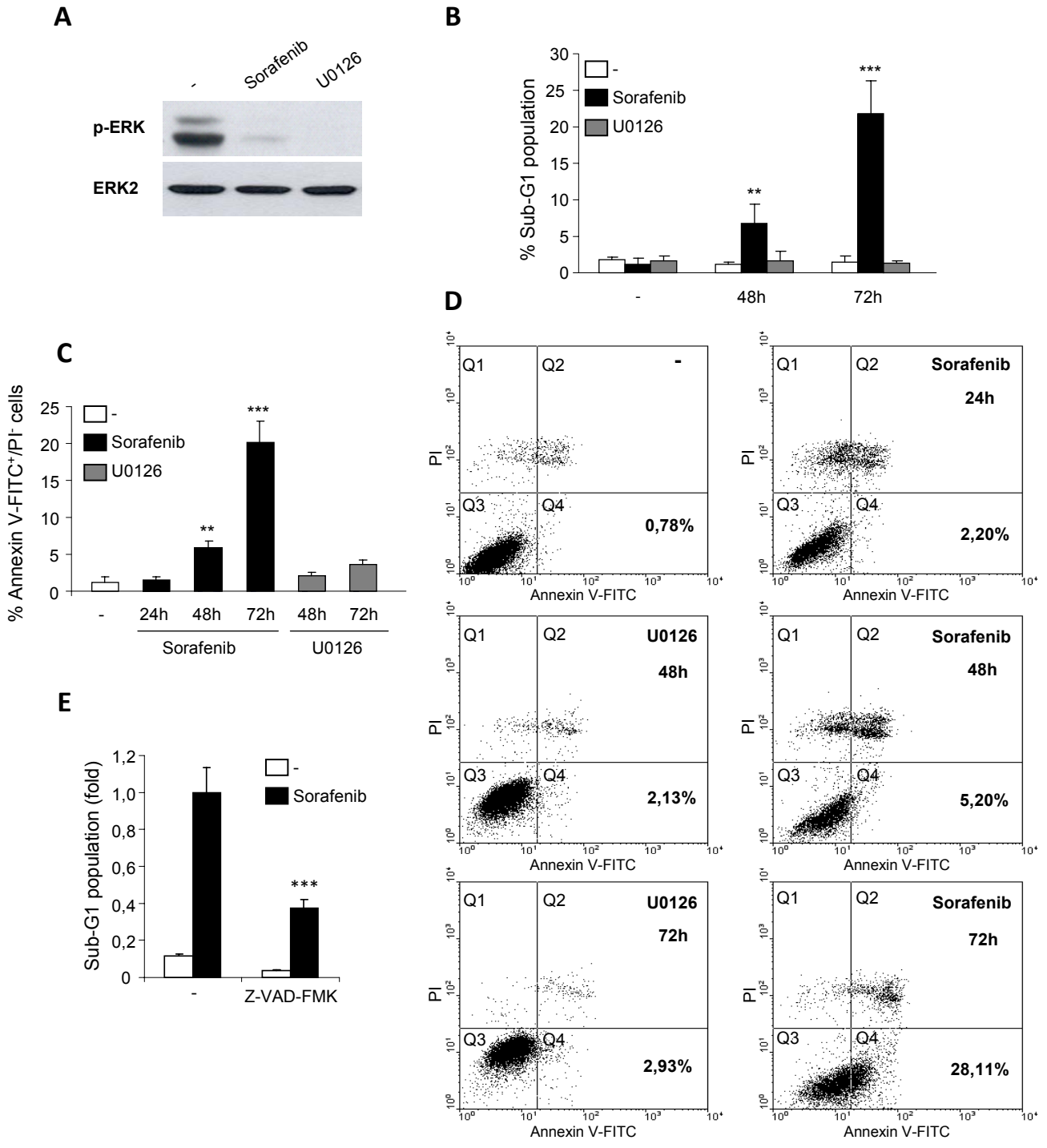

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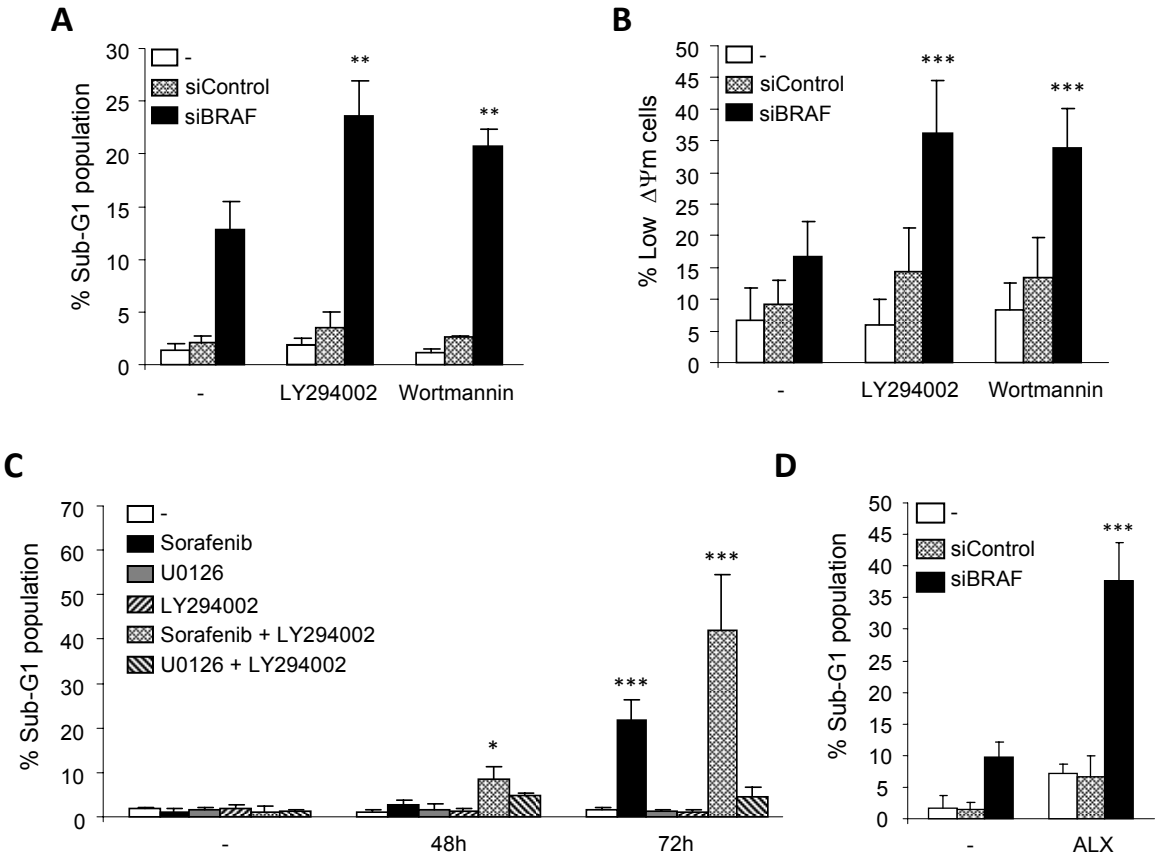

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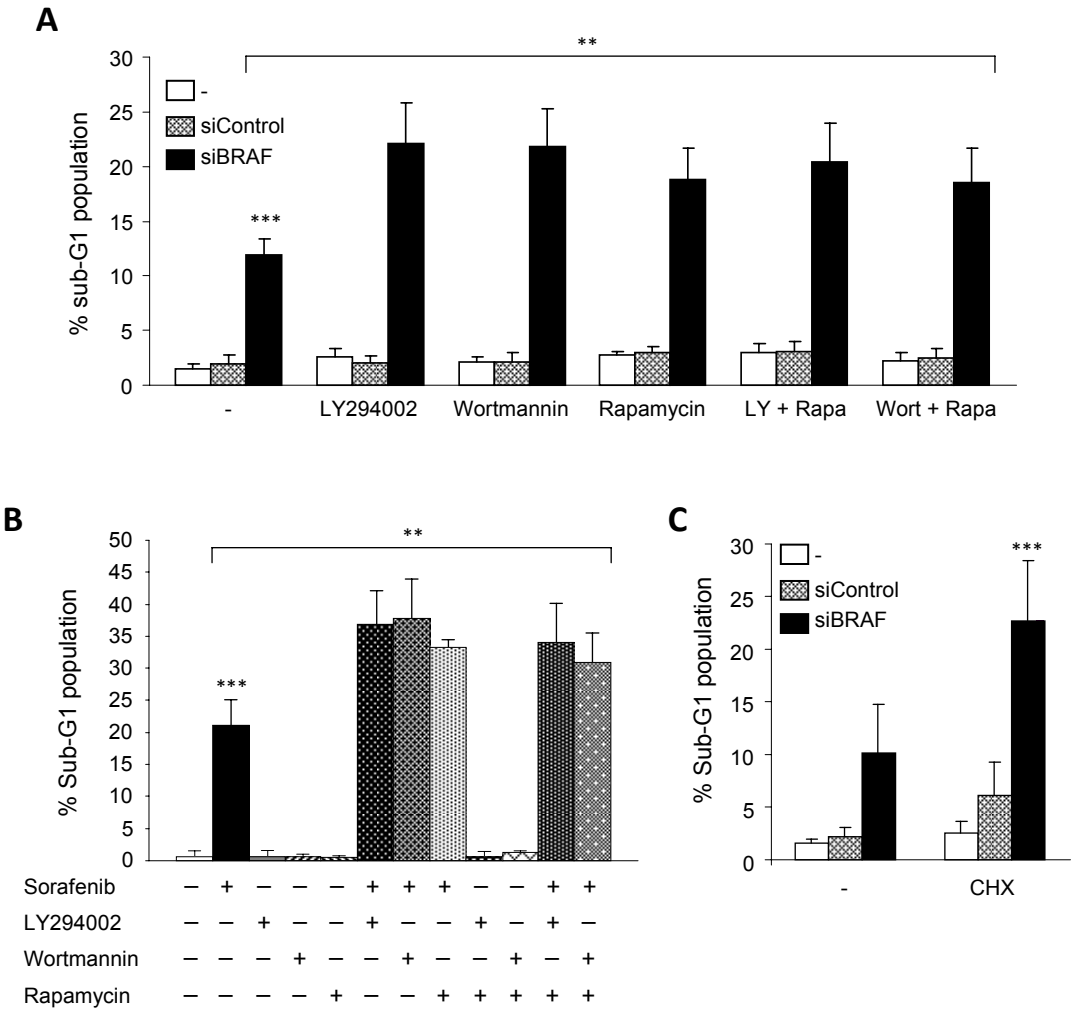

Figure 6. Sánchez-Hernández I. *et al.*