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Highlights

- Vitamin K2 blocks IFN-α-2b-induced reduction in size and volume of the altered hepatic foci in rats
- Vitamin K2 inhibits IFN-α-2b-induced apoptosis in rat preneoplastic livers
- Vitamin K2 inhibition of IFN-α-2b-induced apoptosis is mediated by increased levels of total hepatic Bcl-2 in rat preneoplastic livers

Vitamin K2 supplementation blocks the beneficial effects of IFN-α-2b administered on the early stages of liver cancer development in rats

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Abstract

Objective: Vitamin K2 is present in dairy products, and it has been recommended its supplemteation as a micronutrient in humans. Vitamin K2 has special interest as it holds anticancerous properties. On the other hand, IFN-α-2b administered during development of hepatic preneoplasia decreased both number and volume percentage of altered hepatic foci (AHF) by increasing apoptosis in the foci. The aim of this study was to evaluate the effects of IFN-α-2b treatment supplemented with vitamin K2 in an early stage of liver cancer development in rats.

Methods: Adult male Wistar rats were subjected to a two-phase model of hepatocarcinogenesis (initiated-promoted, IP group). Animals were divided into 4 groups: untreated (IP), IP treated with IFN-α-2b ($6{,}5x10^5$ U/Kg), IP treated with vitamin K2 (10 mg/Kg), and IP treated with both compounds.

Results: We found that vitamin K2 blocks IFN-α-2b-induced reduction in size and volume of the altered hepatic foci. Also, vitamin K2 inhibits IFN-α-2b-induced apoptosis, and finally, vitamin K2 inhibition of IFN-α-2b-induced apoptosis is mediated by increased levels of total hepatic Bcl-2 in rat preneoplastic livers.

Conclusion: These findings highlight that supportive vitamin supplements/therapies are not always safe, as they could put at risk the life of patients treated with IFN-α-2b.

Keywords Apoptosis, cytokine, hepatocellular carcinoma, proliferation, vitamin supplementation

Abbreviations

HCC, hepatocellular carcinoma; AHF, altered hepatic foci; ROS, reactive oxygen species; MK, menaquinone; DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; rGST-P, anti-pi class of rat glutathione *S*-transferase antibody; PCNA, proliferating cell nuclear antigen; IP, initiated-promoted or initiation-promotion; PI, proliferative index; H&E, hematoxylin & eosin.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with an estimated incidence of more than one million new cases per year [1]. Many cancer patients combine some forms of complementary and alternative medicine therapies with their conventional therapies. The most common choice of these treatments is the nutritional administration of certain essential compounds such as vitamins [2][3]. In these connection, vitamin K is a fat-soluble essential vitamin; its only known physiological role is as a cofactor for γ-glutamyl-carboxylase, which acts on several blood-clotting proteins [4]. Vitamin K is similarly involved in the regulation of bone metabolism by γ-carboxylation of bone matrix proteins [5].

There are three types of vitamin K: naturally occurring vitamin K1 (phylloquinone) and vitamin K2 (menaquinone, MK) and chemically synthesized vitamin K3 (menadione). Vitamin K1 and vitamin K2 are non-toxic even at high doses. Vitamin K3 is toxic causing hemolytic anemia, liver toxicity and allergic reactions [6].

Vitamin K2 is also known as MK-n (n =1 to 14), where n stands for the number of repeating isoprenyl units in its side chain. The most common form of vitamin K2 in animals is MK-4, which is produced by intestinal bacteria or is metabolically converted from other vitamins K [7], i.e. MK-7 shows a pharmacological effect since it is converted to MK-4 in the liver [8].

Vitamin K2 is present in dairy products, such as meat and eggs [9]. Epidemiological studies have indicated that populations taking these foods rich in micronutrients have lower incidence of cancer or cancer mortality [10]. In this connection, it was suggested that micronutrients may have some anticancer properties [11]. Therefore, there is a significant interest in determining whether vitamin K2 offers protection against HCC [10][11].

There has been continuing interest for the use of vitamin K2 in the chemoprevention of HCC in Asian countries due to its long-term safety [12]. It has been shown that vitamin K suppresses growth and induces apoptosis and differentiation in various cancer cells, including

HCC cells *in vitro* and *in vivo* with little, if any, toxicity in adult HCC patients [13]. However, the precise mechanism of its antitumoral action has not been clarified, yet. A few studies have demonstrated reduction of HCC recurrence upon vitamin K2 supplementation; however, to confirm the beneficial effect or lack of it upon vitamin K2 administration, larger, higer quality trials are still required [14][15].

Recent studies have shown that vitamin K2 can be combined with antitumor drugs to enhance their antiproliferative effects. It has been reported that the combined use of perindopril, an angiotensin-converting enzyme inhibitor drug, with vitamin K2 attenuates preneoplastic hepatic lesions in rats [16]. Even more, a synergistic action that suppresses cell migration of human hepatoma cells has also been demonstrated with sorafenib, the only drug approved by the FDA for the specific treatment of liver cancer [17].

IFN is known to be a multifunctional cytokine exhibiting various biological functionsand it has been used for antiviral treatment in patients with chronic hepatitis C [18]. IFN-α has been shown to induce apoptosis in tumor cells *in vivo* using a similar dose to that used clinically [19]. It has been stated that IFN-α applied in the early stages of tumor development could have an important clinical effect [20]. In this regard, we have demonstrated that IFN-α-2b administered during the development of hepatic preneoplasia significantly decreased both number and volume percentage of altered hepatic foci (AHF) by an increased programmed cell death in the foci. The apoptotic effect of IFN-α-2b on preneoplastic livers was mediated by an increase in reactive oxygen species (ROS) and depends, to a large extent, on the deregulation of the Bcl-2 family, with a substantial increase in Bax levels in mitochondria and overexpression of p53 [21].

In the present study we evaluated the effects of adding vitamin K2 to the traditional IFN-α-2b treatment in an early stage of liver cancer development in rats. The effects of this combination on the development of liver cancer have not been tested experimentally *in vivo*.

Materials and Methods

Chemicals

Diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), and vitamin K2 (MK-4) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). IFN-α-2b was obtained from BioSidus Laboratory (Buenos Aires, Argentina) and anti-P class of rat glutathione *S*-transferase (rGST-P) antibody from Stressgen Bioreagents (Ann Arbor, MI, USA). Antibodies against Bax, Bcl-2, Bcl-$x_{L,}$ cytochrome c, proliferating cell nuclear antigen (PCNA), cyclin D1, p27, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Pierce-enhanced chemiluminescence (ECL) Western Blotting Substrate was from Thermo Fisher Scientific (Rockford, IL, USA). All other chemicals were of the highest grade commercially available.

Animals and Treatment

Adult male Wistar rats (from ICIVET Litoral, UNL-CONICET) (250–280 g) were maintained in suspended stainless steel wire-bottom cages to prevent coprophagy (2 per cage) in a room at constant temperature with a 12 h light–dark cycle, with food and water supplied *ad libitum*. Experimental protocols were performed according to the NIH "Guide for the Care and Use of Laboratory Animals" (Publication no. 25-28, revised 1996), and approved by the local animal care and use committee.

All animals were subjected to a 2-phase model of rat hepatocarcinogenesis (initiation-promotion, IP). An overview of the experimental setup is provided in Fig. 1A. The initiation stage was performed by the administration of 2 intraperitoneal necrogenic doses of DEN (150 mg/kg body weight) 2 weeks apart. Administration of 2-AAF was performed 1 week after the last injection of DEN. The 2-AAF was dissolved in dimethyl sulfoxide and then suspended in corn oil to a final concentration of 8 mg/mL. The rats received 20 mg/kg body weight of 2-AAF/corn oil suspension by gavage for 3 consecutive days per week for 3 weeks. After the IP treatment, animals were divided into 4 groups of 5 rats each: IP, IP-IFN (from now on group IFN), IP-VK2 (from now on group VK2) and IP-IFN+VK2 (from now on group IFN+VK2). Group IFN received IFN-α-2b (6.5 $x10^5$ U/kg body weight) intraperitoneally 3 times per week

for 3 weeks. The dose of IFN-α-2b used was comparable to that used for therapeutic purpose [22]. Group VK2 received 10 mg/kg vitamin K2 [23] by gavage, daily 3 times per day for 3 weeks (vitamin K2 was previously dissolved in corn oil). Group IFN+VK2 received both drugs at the doses administered individually.

At the end of the ninth-week of treatment rats were bled through a cardiac puncture after administration of ketamine-xylazine (70 mg/kg body weight and 2.1 mg/kg body weight, respectively), and livers were removed and processed.

Immunohistochemical studies

Quantitation of AHF

rGST-P has been described as the most effective single marker of hepatic preneoplasia in the rat. Thus, immunohistochemical detection of rGST-P is the most widely used method for identification, quantitation and assessment of rat altered hepatic foci (AHF) [24]. Liver slices from different lobes were fixed in 10% v/v formalin solution and embedded in low melting paraffin. Immunohistochemical staining was performed using the antibody raised against rGST-P. The slices were incubated with biotinylated goat anti-rabbit secondary antibody and then with horseradish-peroxidase-conjugated streptavidin (HRP CytoScan Detection Kit, Cell Marque). Signals were detected with DAB Sustrate Kit (Cell Marque) followed by hematoxylin counterstaining. A representative number of field sections (usually between 1 to 1.5 cm^2 of tissue) was evaluated per animal. Images were collected using a CCD color video camera (Sony SSC-c370) attached to a Zeiss Axiolab microscope. Images were processed using a NIH imaging analysis system (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). The number of AHF per liver and AHF as percentage of liver were calculated according to the modified Saltykov's method [25] using the digitized images.

Determination of proliferative index

To investigate differences in proliferation activity in rGST-P-positive foci between the experimental groups, serially sectioned slides were examined by immunohistochemical staining

with anti-rGST-P and anti-PCNA antibodies. PCNA was visualized by the method of Greenwell et al. [22] using as primary antibody anti-PCNA from Santa Cruz Biotechnology (Santa Cruz, CA). A representative number of random fields of liver sections (400X magnification) were evaluated and scored. All PCNA-positive cells in G1, S, M, and G2 phases were combined as total proliferating cells. The number of proliferating cells within rGST-P-positive lesions was determined by examining at least 1,000 hepatocytes. Proliferative index (PI) was expressed as proliferating cells scored per 1,000 hepatocytes.

For Bcl-2 immunohistochemistry, serially sectioned slides were examined by immunohistochemical staining with anti-rGST-P and anti-Bcl-2 antibodies. Bcl-2 was visualized by the method of Greenwell et al. [22] using as primary antibody anti-Bcl-2 from Santa Cruz Biotechnology (Santa Cruz, CA).

Hepatic collagen evaluation

Liver fibrosis was evaluated by digital image analysis on sections stained with 1% Direct Red 80/picric acid. This semi-quantitative technique allows the estimation of total amount of hepatic collagen using several microphotographs slices obtained from each liver slice, as previously described[27]. Results were expressed as area occupied by collagen/area of the picture (in cm^2).

Immunoblotting

Tissue samples were homogenized in 150 mM KCl with protease inhibitors. Cytosolic, mitochondrial, and nuclear extracts were prepared as described previously [28][29]. The homogenates were centrifuged at 1,000*g* to remove unbroken cells, nuclei and heavy membranes. Nuclei were sedimented by centrifugation at 1,000*g* (First centrifugation). Pellets from first centrifugation were washed and resuspended in RIPA buffer containing 20 mmol phosphate-buffered solution (pH 8), 1% Triton, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mmol EDTA, 200 mmol NaCl, and protease inhibitors. Pellets were incubated on ice for 1 hour, and centrifuged (8,000*g*, 15 minutes, 4°C) to obtain nuclear fraction. Supernatant from first centrifugation was then used to obtain mitochondria fractions by centrifugation at

9,000*g* at 4°C for 15 minutes. Supernatant from this centrifugation was then washed in $CaCl_2$ 0.1M and centrifuged at 27,000*g* and 4°C for 15 minutes. Pellet was discarded, and supernatant was kept as the cytosolic fraction.

Protein concentration was determined by the Lowry method [30]. Equal amounts of protein were subjected to electrophoresis on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (LD400 microplate reader, Beckman Coulter Inc, Fullerton, CA, USA). Membranes were blocked with T-PBS-10% nonfat milk, washed and incubated overnight at 4ºC with primary antibodies. Finally, membranes were incubated with peroxidase-conjugated secondary antibodies and bands were detected by the ECL™ detection system and quantified by densitometry using the Gel-Pro Analyzer software. Both, equal loading and protein transfer for each membrane were checked by incubations with the proper antibodies and by Ponceau S staining.

Caspase-3 activity assay

Caspase-3 activity was determined using EnzChek1 Caspase-3 Assay Kit #1 (Molecular Probes Inc., Eugene, OR, USA), according to the manufacturer's suggestions. Briefly, hepatic total homogenates from each sample were mixed with substrate solution. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm in a DTX 880 multimode detector (Beckman Coulter Inc, Fullerton, CA, USA).

Statistical Analysis

Data are presented as means ± SEM. Statistical significance was evaluated by unpaired two-tailed Student's test. Time course studies were evaluated by two-way ANOVA followed by Bonferoni post-test (GraphPad PRISM® 4 software), p value <0.05 was interpreted as a significant difference.

Results

General findings

During the experiment, food consumption was similar in all groups and no signs of dehydration were observed along the treatment (data not shown). The ratio liver/body weight was similar in all groups. H&E staining and further examination of the liver showed basically no differences between livers from all different groups (Fig. 1B) with no signs of acute or chronic inflammation in spite of the AHF development (arrows). We found non-significant differences in the activity of the hepatic enzymes AST and ALT in plasma (Fig 1C).

Vitamin K2 blocks IFN-α-2b-induced reduction in size and volume of the AHF

Changes in number and volume percentage of the liver rGST-P-positive foci are shown in Fig 2. The number and volume percentages of AHF per liver in IFN group were significantly decreased compared to IP group (-39% and -65%, respectively). Surprisingly, VK2 did not show any differences on the number and volume percentages of AHF per liver respect to IP group, alone (VK2 group) or combined with IFN (IFN+VK2 group). Furthermore, the animals that received both drugs showed a trend toward an increment in number and volume percentages of AHF per liver, although this trend was not statistically significant.

Vitamin K2 does not improve total collagen deposition

Collagen deposition upon the different treatments is shown in Fig. 3. As expected, IFN-α-2b treatment markedly reduced hepatic collagen deposition, therefore the degree of fibrosis compared to IP rats (-32 %). In line with results for the number and volume percentage of liver occupied by AHF, VK2 alone did not show any effects on collagen deposition. However, a reduction was observed for the combined IFN+VK2 group (-31%), similar to IFN group, indicating that the vitamin treatment did not have an additive action on the beneficial effects of IFN-α-2b.

Vitamin K2 partially blocks the apoptotic effect of IFN-α-2b

As previously shown, immunoblot analysis of proapoptotic protein Bax, showed an increase in mitochondrial protein expression in IFN as well as in IFN+VK2 groups (+219% and +97% respectively) respect to IP. No changes were observed in VK2 group (Fig. 4A). Levels of cytosolic cytochrome c were evaluated by immunoblotting. Cytosolic cytochrome c increased in IFN group compared to IP animals (+135%); the other groups did not show any differences respect to IP (Fig 4B).

It was previously demonstrated that p53 induces apoptosis in HCC [31]. To determine whether p53 is a major mediator of apoptosis in preneoplastic foci of rats treated with IFN-α-2b, we performed immunoblot analysis of p53 using nuclear fractions. Administration of IFN-α-2b increased the expression of p53 (+75, 2%) protein compared to IP group, whereas vitamin K2 treatment reduced the levels (-54, 3%) of the protein (Fig 4C).

Also, the caspase cascade is now believed to be the main phatway by which the apoptosis is orchestrated. The most prevalent caspase in the cell is caspase 3 [32]. We determined caspase 3 activity in all studied groups. Fig. 4D shows that caspase-3 activity was significantly increased in IFN group respect to IP animals. Vitamin K2 had no effects on caspase-3 activity in any of the other groups.

Vitamin K2 blocks the apoptotic effect of IFN-α-2b through an induction of the antiapoptotic protein Bcl-2

Regarding the antiapoptotic protein Bcl-x_L, no significant differences were observed for any groups; however, a non-statistically significant trend to a decrease was observed in the IFN group respect to the IP group (Fig 5A). The analysis of the antiapoptotic protein Bcl-2 showed an increase in mitochondrial protein expression in VK2 (+94%) and IFN+VK2 (+43%) groups (Fig. 5B). Interestingly, when Bcl-2 was evaluated by immunohistochemistry, we found a very interesting distribution pattern. An astonishing staining outside the AHF was observed the IP group. IFN group, however, presented with less stained cells, but Bcl-2-possitive cells were distributed both inside and outside the AHF. VK group presented with a pattern similar to IP

group; but in contrast, many Bcl-2-positive cells were found within the AHF. A similar pattern in the combined group was observed (Fig. 5C).

Finally, Bax/Bcl-2 ratios were calculated for each group. Setting arbitrarily IP ratio as 1, we found a significant increase only in IFN group ratio (3.2±0.2), reinforcing our previous results where IFN-α-2b induced apoptosis in preneoplastic livers in rats (Fig 5D).

Vitamin K2 does not affect cell proliferation and the cell cycle

Fig. 6A and table 1 show the analysis of PCNA protein by both immunoblotting and immunohistochemistry, respectively. Immunoblotting showed no differences in the expression of PCNA protein between all the studied groups. In line, no changes were observed in the PI within the foci.

One proposed antitumoral mechanism of vitamin K2 is its ability to arrest the cellular cycle [6], thus, we measured the expression of two key proteins involved in the cycle: cyclin D1 and p27. Analysis of protein expression in nuclear extracts showed no differences in the expression of cyclin D1 protein in any of the studied groups (Fig. 6B). There was an increase in p27 protein in IFN group respect to IP group (+113%) (Fig. 6C). However, its expression level is not enough to show any effects on cell cycle arrest.

Discussion

Today, chemotherapy remains one of the main approaches for the treatment of HCC [33]. Despite the significant advances in HCC management lately, there are no effective chemoprevention policies, and only sorafenib treatment has been approved for patients with advanced tumors [34]. However, sorafenib efficacy is now on debate since despite the survival benefit (3 months, vs. placebo patients) [35], resistance and intolerance to sorafenib are very common. Thus, new and old systemic therapies need to be (re)considered for HCC patients with resistance or intolerance to sorafenib.

The use of antivirals and vaccination has successfully diminished the incidence of hepatitis B-related HCC [34]. In this sense, IFN-α has been clinically used for delaying the progression of liver function impairment or for the prevention of HCC development in patients with chronic hepatitis B or C [36][37]. The clinical outcome of IFN-α monotherapy has not been satisfactory [38]; however, several studies have reported a strong antitumor activity and survival benefit of IFN-α-based combination therapy in HCC [39][40]. Also, patients undergoing IFN-α-2b treatment are susceptible to several secondary effects [37]. Thus, combining certain alternative medicinal therapies, such as vitamins, with conventional therapies is a common choice for many cancer patients. It is believed that these complementary therapies strengthen the general weakness from such patients.

In line, several clinical trials have attempted to elucidate the role of vitamin K2 in preventing the development of HCC in women with viral cirrhosis, and also the suppression of recurrence of HCC, leading to a greater survival [41][42]; however, results are still controversial [42]–[44].

Our group has been devoted to the study of the early stages of liver carcinogenesis for many years. In this sense, we found that administration of IFN-α-2b during the development of hepatic preneoplasia prevents the increment in the number and size of AHF [21][45]. In this study, we sought to evaluate the effect of IFN-α-2b administered after the end of the carcinogenic treatment, in order to compare the reversion effect of IFN-α-2b rather than the prevention effect. To our knowledge, this is the first study showing the revertive effects of IFN-α-2b in a rat

model of early hepatocarcinogenesis. In addition, we also evaluated the revertive effect of vitamin K2 using the same model, and studied whether the combination of both compounds had a beneficial (additive or synergic) effect on liver cancer development compared to the individual treatments. In these sense, we found interesting but unexpected results, since combination of IFN-α-2b and vitamin K2, at previously individually tested doses [21][23], did not show any beneficial effects on the development of liver cancer. Furthermore, vitamin K2 seems to have a blocking effect on here presented and also all the previously reported IFN-α-2b actions [21][28][45].

Here, we reported that vitamin K2 blocks IFN-α-2b-induced reduction in size and volume of the AHF. A very thorough study of the vitamin K2 dose to be administered was made before the start of the treatments. Doses ranging between 3 mg/Kg to 20 mg/Kg were found in literature. Based on the AHF reducing effect of vitamin K2, we used the dose of 10 mg/Kg body weight administered 3 days a week for 3 weeks. Yoshiji et al. reported an AHF reducing effect using 3 mg/Kg after a partial hepatectomy during 8 weeks [16]. Despite we used 10 mg/Kg; vitamin K2 was not able to reduce the number or the volume of the AHF. However, it is interesting to note that vitamin K2 combined with IFN-α-2b had absolutely any effect on the development of the AHF; this is, AHF sizes and numbers were not different from the foci from IP livers, showing that vitamin K2 would signal though a pathway interfering with the IFN-α-2b signaling pathway.

IFN-α is an interesting agent against liver fibrosis. Intrinsic antifibrogenic properties of this compound has been established by *in vitro* studies [46][47]. Indeed, IFN-α exerts its antifibrogenic effects in different rat models of hepatic fibrosis [48][49] and also in patients with hepatitis C virus [50].We previously found that IP rats presented exacerbated liver fibrosis compared to control rats [51]. In this study, we showed that IFN-α-2b treatment improved the deposition of total collagen in IP rats. Interestingly, vitamin K2 had no effect on collagen deposition, neither alone or with IFN-α-2b. In this regard, to our knowledge, it was never reported a beneficial effect of vitamin K2 on collagen deposition. Even more, it was claimed that vitamin K2 may contribute to collagen assembly and deposition in osteoblastic cells [52][53].

Vitamin K2 induces apoptosis in a wide range of tumoral cells [7][54]–[58]. Unexpectedly, we did not find any signs of apoptosis induced by vitamin K2 in our model. As previously shown in a model of prevention of hepatocarcinogenesis [21] , here we found that IFN-α-2b induces Bax translocation to the mitochondria in livers from IP rats, inducing apoptosis; however, in this model the combination with vitamin K2 seemed to block IFN-α-2b apoptotic effect. When other apoptotic proteins and activities were studied, we found a similar pattern, were IFN-α-2b induced-apoptosis is blocked by vitamin K2, with no apoptotic effect of the vitamin alone. In this sense, it was previously reported that vitamin K2 did not show apoptosis on Huh7 cells, where the effect appears only when vitamin K2 is combined with another compound [59]. When p53 protein expression was evaluated, vitamin K2 alone induced a marked decrease in protein expression.

Finally, we found that the levels of anti-apoptotic protein Bcl-2 were increased when IP rats were treated with vitamin K2 alone. This increment impairs the mithocondrial apoptotic pore formation leading to a decreased or null apoptosis activation [60]. Furthermore, when the hepatic apoptotic index was calculated, no global effects of vitamin K2 on apoptosis were found, showing once again that vitamin K2 has a blocking effect on the IFN-α-2b-induced apoptotic effect.

It is interesting to note that Bcl-2 acts inhibiting vitamin K2 apoptotic action. Furthermore, it was demonstrated that the antitumor effect of vitamin K2 might be improved by silencing BCL-2 expression in HCC [61].

Besides, programmed cell death is facilitated in part through the production of free radicals via oxidative pathways. In this connection, it has been proposed that Bcl-2 acts inhibiting cell death by interfering with the production of oxygen-derived free radicals induced by a wide variety of stimuli such as IFN-α-2b [45][62].

Although it was shown that vitamin K2 induced growth inhibition via cell cycle arrest and apoptosis in a dose dependent manner for glioma cells in both rat and human cell types [63], in this study we found no changes in cell proliferation in livers from IP rats treated or not with IFN-α-2b, vitamin K2 or both. It was previously shown that vitamin K2 has no effect on cell

proliferation inside liver tumors. Authors claimed that in order to pursue its antiproliferative effects, doses of vitamin K2 should be extremely elevated compared to those used clinically [16]. In line, in a previous report from our group, we found that proliferative index was unchanged upon IFN-α-2b administration [21].

It is interesting to note that naturally occurring vitamins K had poor effect on cell growth and none on the cyclins [64]. Furthermore, vitamin K2 administered together with IFN-α in cell lines presented with little effect on cell cycle [65]. In this study; however, we showed that vitamin K2 administration in the early stages of liver cancer development, has no effect on cell proliferation or apoptosis. Even more, combination with IFN-α-2b seemed to block the antiapoptotic effects of IFN-α-2b, through a mechanism linked to an increase in the antiapoptotic protein Bcl-2. Since both agents are widely used in clinical practice, this combination regimen may be evaluated against HCC in the future.

In summary, we did not see a beneficial effect as a result of treating IP rats with vitamin K2, alone or in combination with IFN-α-2b, since there is no decrease in the number and size of foci, decrease in proliferation, or increased apoptosis. Furthermore, it seems clear that vitamin K2 interferes in IFN-α-2b cellular actions. In this sense, we believe there is an unknown interaction between the signaling pathways of IFN-α-2b and vitamin K2 that leads to the modulation of other signaling pathways with undesired final effects. This interaction might be mediated by the antiapoptotic protein Bcl-2.

Our findings are interesting in many ways. First, this is the only study performed in rats evaluating the effects of IFN-α-2b and vitamin K2 combined therapy on the early stages of liver carcinogenesis. Second, this study highlights that supportive vitamin supplements/therapies are not always safe, as they could put at risk the life of patients treated with IFN-α-2b. Finally, administered doses should be studied careful and individually in order to decrease at a maximum level the risk of secondary effects in patients treated for liver cancer.

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Compliance with ethical standards

Confict of interest All authors have no fnancial disclosures and no confict of interest

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Table 1. PI in AHF

Group	PI
IP	331.0±12.95
IFN	328.1±5.20
VK2	279.3±43.98
IFN+VK2	355.5±74.05

NOTE. PI was expressed as proliferating cells per 1000 hepatocytes. All values represented mean ± SEM.

Figure legends

Figure 1. **No signs of liver damage in animals undergoing hepatic preneoplasia**. A) Animal treatment protocol. Male Wistar rats were subjected to a two-stage model of hepatocarcinogenesis. IP group received two intraperitoneal doses of DEN (150 mg/Kg body weight) 2 weeks apart. One week after the last injection, the animals received 20 mg/Kg body weight of 2-AAF by *gavage* during 3 consecutive days per week for 3 weeks. Group IFN: IP rats received IFN-α-2b $6.5x10^5$ U/kg intraperitoneally 3 times per week for 3 weeks (weeks 6 to 9); Group VK2: IP rats received 10 mg/kg vitamin K2 orally 3 times per day for 3 weeks (weeks 6 to 9); and Group IFN+VK2: IP rats received both compounds at equal doses as per separate. The animals were sacrificed at the end of week 9. B) Liver H&E staining for evaluation of hepatic architecture (100X). No signs of acute or chronic inflammation despite the AHF development (arrows). C) Plasma transaminase levels, as a hepatic damage marker.

Figure 2. **Liver immunohistochemical study of rGST-p expression**. A) Representative images showing the size of rGST-p-positive foci in IP, IFN, VK2, and IFN+VK2 groups, magnification 100X. Changes in (B) number of AHF per liver and (C) percentage of liver as AHF. Each bar indicates mean ± SEM. *p<0.05 vs IP group.

Figure 3. Study of collagen deposition in the liver. A) Representative images of liver collagen deposition. Collagen fibers are showed in red color (arrows), magnification 200X. B) Total liver collagen estimation. Each bar represents the mean ± SEM. *p<0.05 vs IP group.

Figure 4. Analysis of hepatic proapototic markers. A) Study of Bax mitochondrial levels, (B) cytochrome c cytosolic levels and (C) p53 nuclear levels by immunoblotting. The bars below each immunoblot panel represent the densitometric analysis of the bands. Each bar represents the mean ± SEM. *p<0.05 vs IP group. D) Caspase-3-like activity in hepatic total homogenates. Data are expressed in percent values as mean ± SEM; caspase-3 activity in IP group is considered 100%. *p<0.05 vs IP group.

Figure 5. Analysis of hepatic antiapoptotic markers. A) Expression levels of Bcl-X_L, and (B) Bcl-2 in liver mitochondrial fractions by immunoblotting. The bars below each immunoblot panel represent the densitometric analysis of the bands. (C) Immunostaining for Bcl-2. Immunohistochemistry was performed using paraffin-embedded liver sections. The arrows show the presence of the AHF (100X). D) Bar graph showing the ratio between optical density for Bax bands over Bcl-2 bands for individual immunoblottings (apoptotic index): Bax/Bcl-2. Results are expressed relative to IP group, to be considered 1. Bars represent mean ± SEM. *$p<0.05$ vs IP group.

Figure 6. Analysis of cellular proliferation and cell cycle proteins. A) Immunoblotting for PCNA, (B) cyclin D1, and (C) p27 proteins in liver nuclear fractions. Bars represent mean ± SEM. *$p<0.05$ vs IP group.

Figure 1

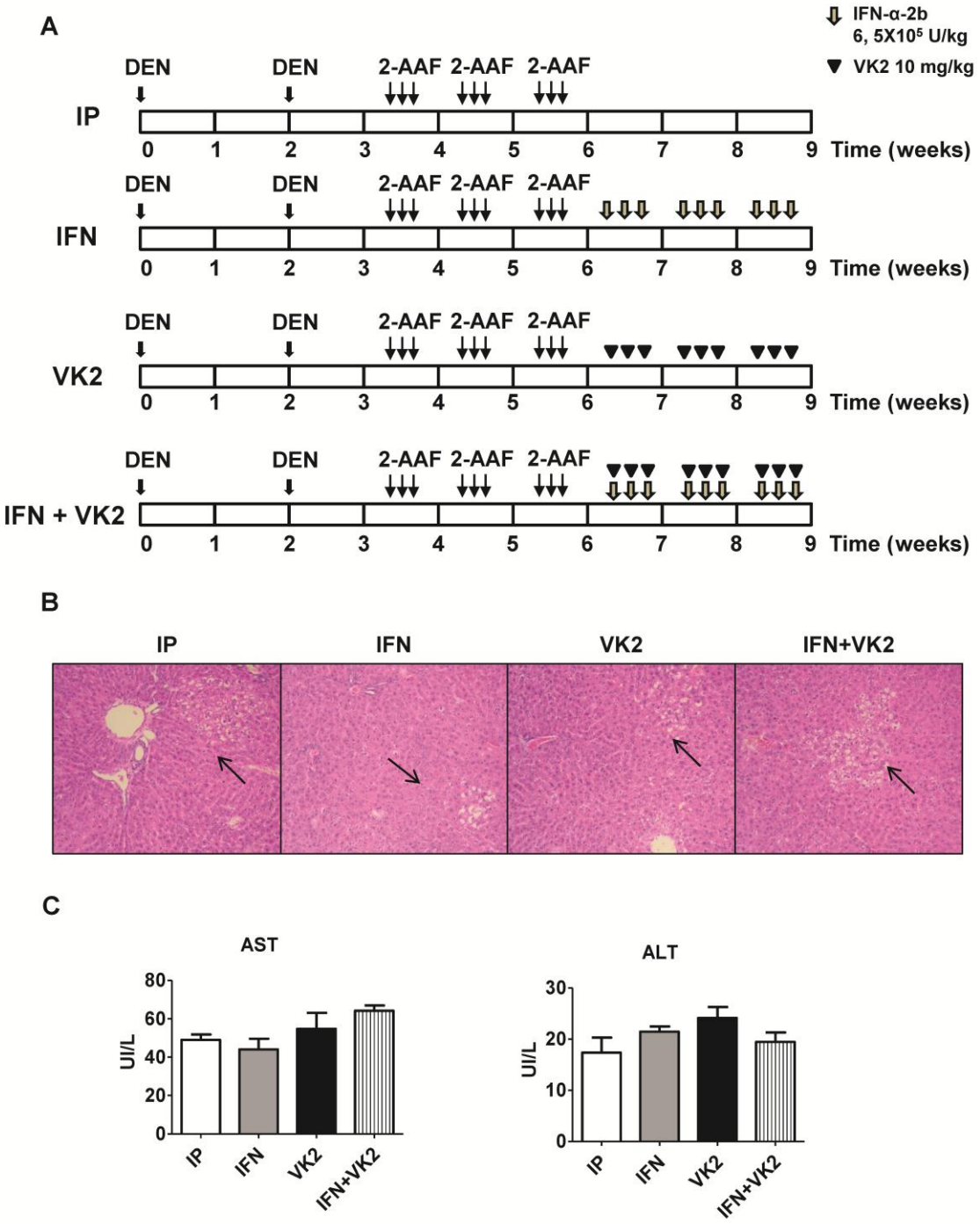

A
IFN-α-2b
6, 5X10⁵ U/kg
VK2 10 mg/kg
DEN
DEN
2-AAF 2-AAF 2-AAF
IP
IFN
VK2
IFN + VK2
0 1 2 3 4 5 6 7 8 9 Time (weeks)
B
IP
IFN
VK2
IFN+VK2
C
AST
UI/L
80
60
40
20
0
IP
IFN
VK2
IFN+VK2
ALT
UI/L
30
20
10
0
IP
IFN
VK2
IFN+VK2

Figure 2

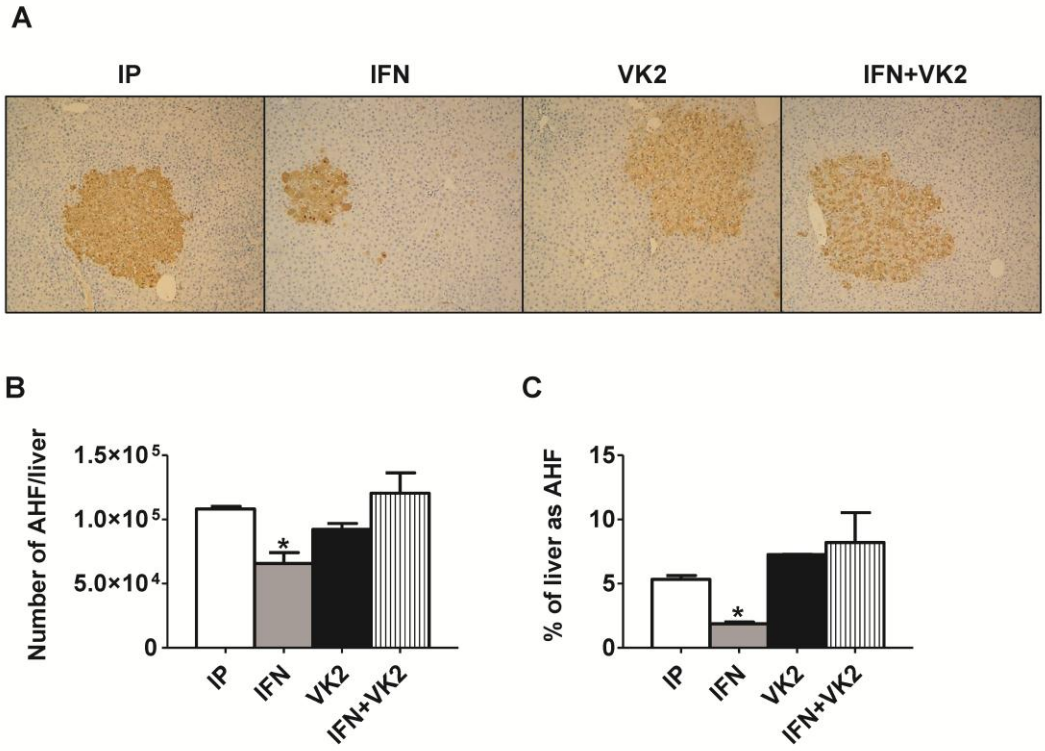


Figure 3

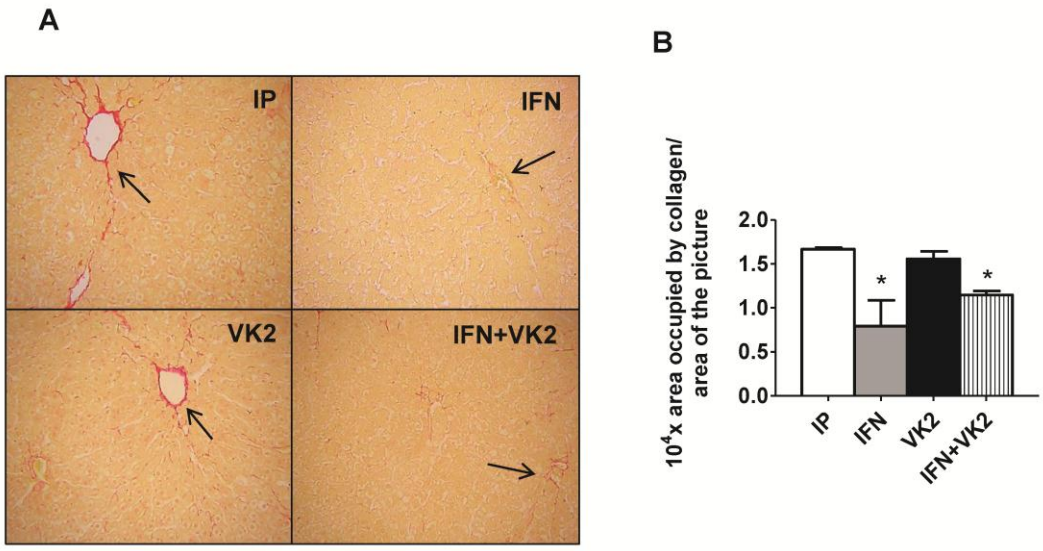


Figure 4

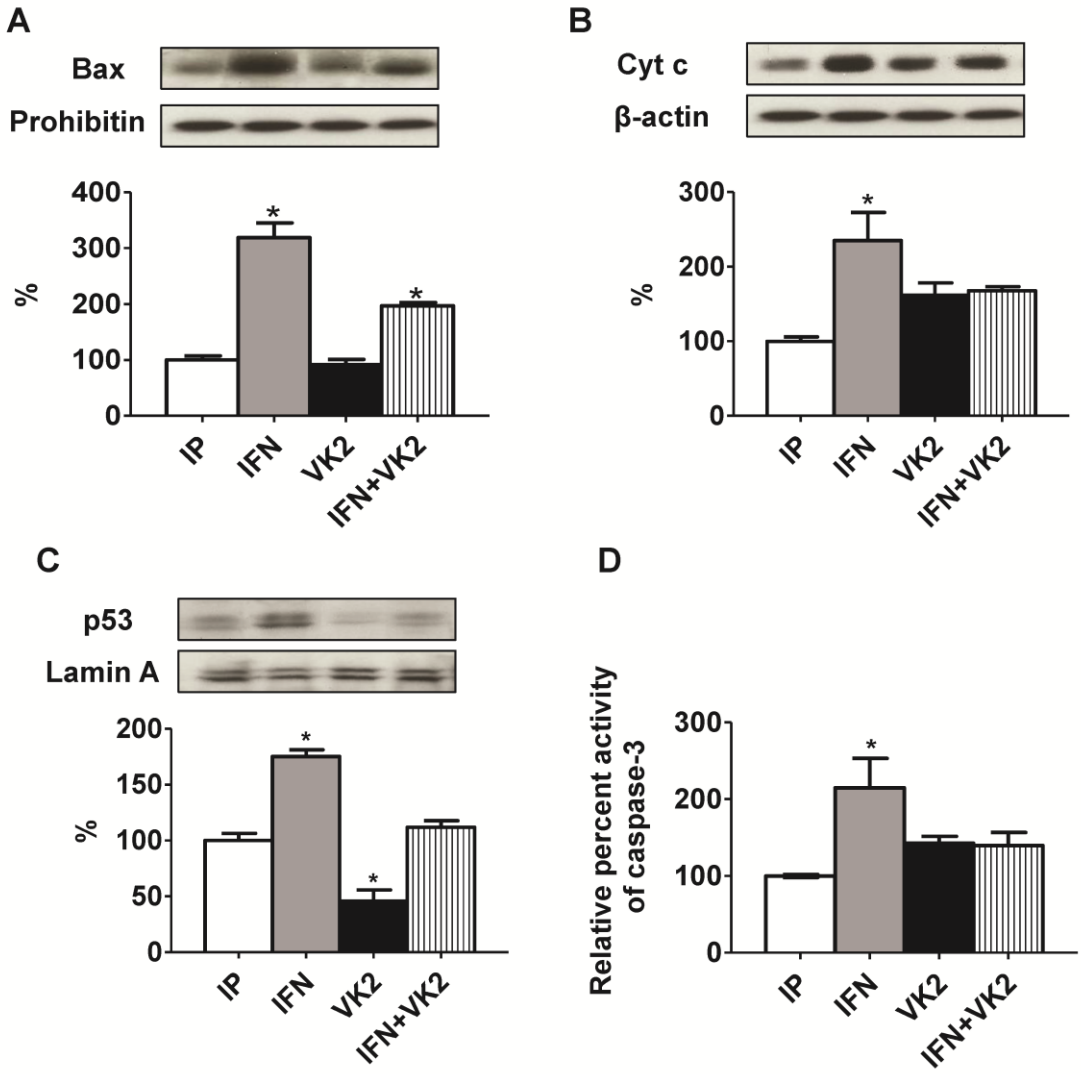

A
Bax
Prohibitin
400
300
200
100
0
%
*
*
IP
IFN
VK2
IFN+VK2
B
Cyt c
β-actin
300
200
100
0
%
*
IP
IFN
VK2
IFN+VK2
C
p53
Lamin A
200
150
100
50
0
%
*
*
IP
IFN
VK2
IFN+VK2
D
Relative percent activity of caspase-3
300
200
100
0
*
IP
IFN
VK2
IFN+VK2

Figure 5

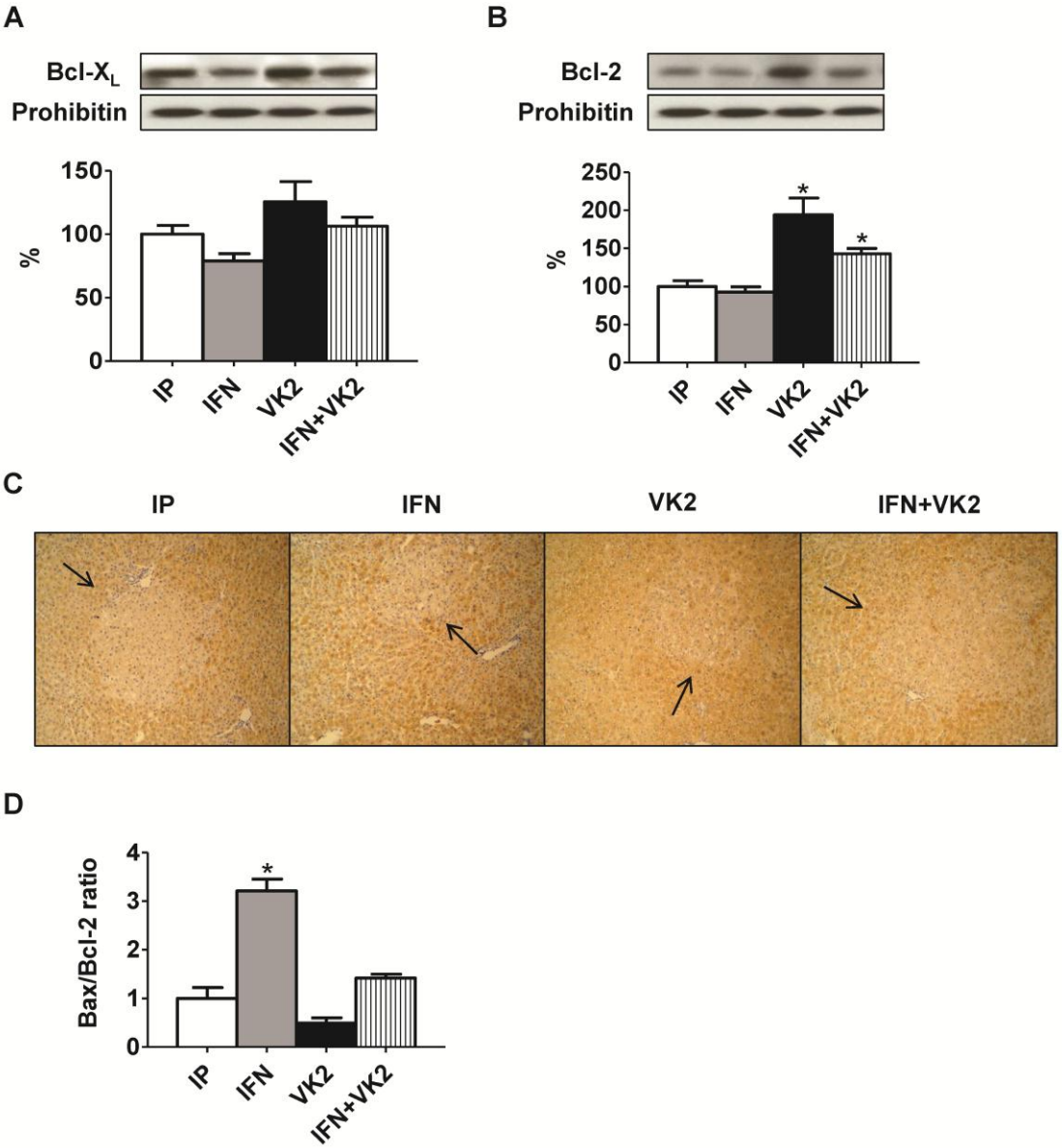
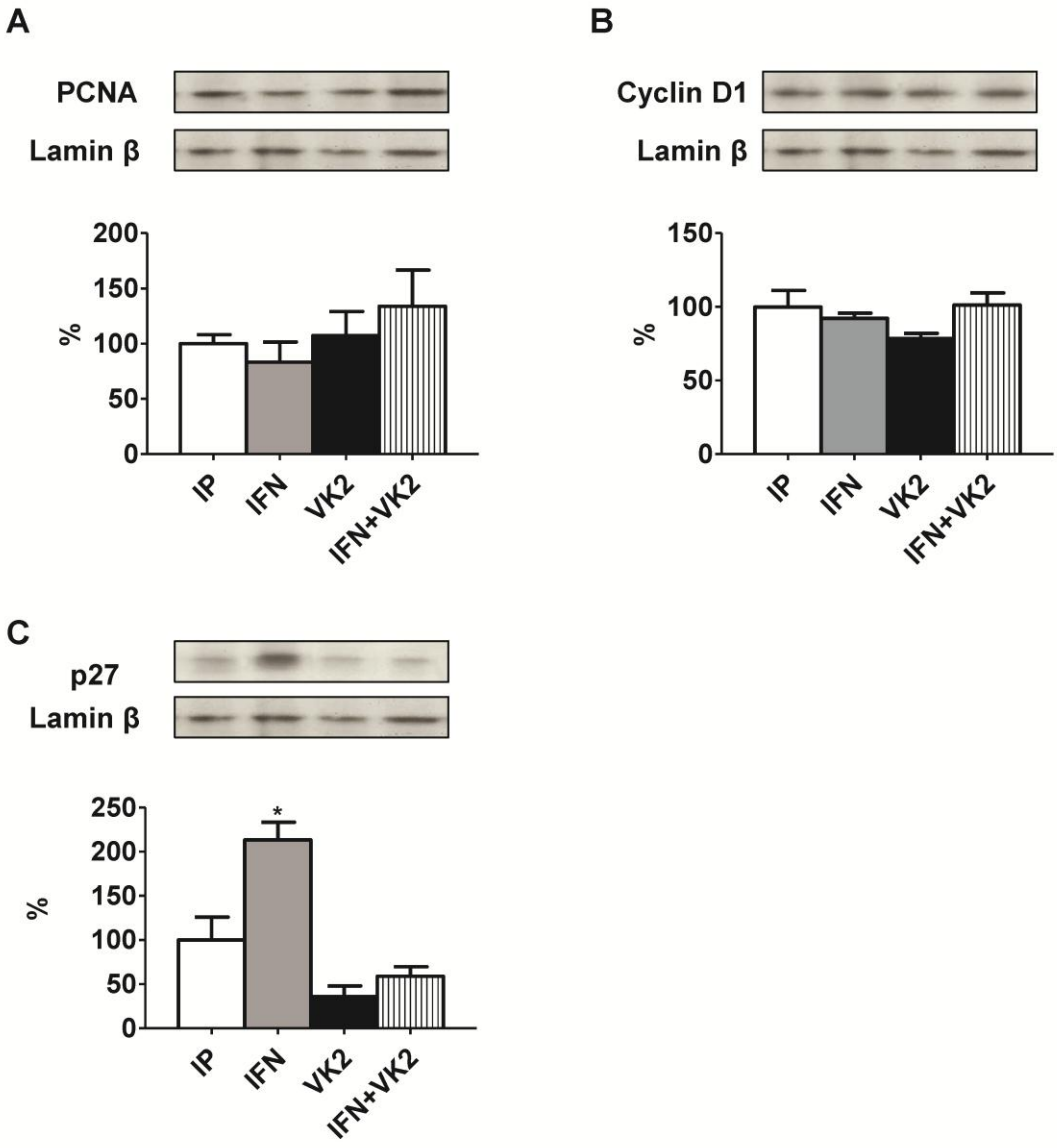


Figure 6


A
PCNA
Lamin β
200
150
100
50
0
%
IP
IFN
VK2
IFN+VK2
B
Cyclin D1
Lamin β
150
100
50
0
%
IP
IFN
VK2
IFN+VK2
C
p27
Lamin β
250
200
150
100
50
0
%
*
IP
IFN
VK2
IFN+VK2