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Identification of the Anticancer Potential of Vitamin B17 via Targeting *Raf-1* and Autophagy-related Gene Expression

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Abstract

Background: Cancer is a disease in which molecular changes of the growth factors and relevant signaling cause uncontrolled growth and division of cells. The most common factors involved in cancer initiation and development include epidermal growth factor, mitogen-activated protein kinase, and autophagy effectors.

Method: This experimental study was conducted to investigate the potential anticancer properties of a number of agents, including interferon-gamma, rapamycin, and vitamin B17, which were compared to Sorafenib in hepatocellular carcinoma HepG2 cell line and stem cells. Cells were cultured in RPMI medium with 10% fetal bovine serum, 4 mM sodium pyruvate, 4 mM L-glutamine, and 100 U/mL penicillin/streptomycin. Cell viability and levels of lactate dehydrogenase were investigated for the cytotoxic potential of these agents in both kinds of cells. The expression profile of *Raf-1*, autophagy-related *LC3B*, *TP53*, *caspase 3 (Casp3)*, and levels of released inflammatory cytokines, including IL-4 and IL-6, were monitored in response to the chemical treatment.

Results: Our findings showed insufficient inhibition of the indicated factors by interferon-gamma (IFN-γ) and rapamycin in cancer cells when compared to Sorafenib. Interestingly, vitamin B17 revealed competitive inhibition on cell proliferation of HepG2 cells compared with Sorafenib while in stem cells, vitamin B17 led to impartial consequences. Unlike *TP53* and *Casp3*, gene expressions of *Raf-1* and *LC3B* were significantly reduced in cancer cells treated with vitamin B17 at both RNA and protein levels, while their expression was markedly upregulated in the treated stem cells. Furthermore, in both cells, vitamin B17 increased the expression of IL-4 while reducing the production of IL-6.

Conclusion: These data provide evidence for the effectiveness of vitamin B17 in cancer treatment via selective regulation of *Raf-1* and autophagy-related *LC3B* in cancer cells.

Keywords: Vitamin B17, Antineoplastic agents, Autophagy, Apoptosis, Cancer treatment

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Introduction

Cell proliferation is one of the most complicated cellular processes leading to cell division and increasing the copies of each living cell. The mechanisms through which normal cells can successfully replicate include cyclin-dependent kinases (CDKs) and the epidermal growth factor receptor (EGFR).[1] The EGFR particularly stimulates downstream targets of the pro-oncoproteins family, such as RAS-RAF-MEK-ERK and AKT-PI3K-mTOR signaling pathways.[2] In fact, mechanical autophagy is a cellular process through which the cell can degrade and recycle the unnecessary proteins and damaged organelles via delivering the autophagic cargo to lysosomes.[3,4] Numerous studies have reported the regulatory role of autophagy in maintaining cell survival with accumulated evidence, indicating the direct association between autophagy and EGFR signaling in normal and cancer cells.[5-7] In stem cells, the EGFR signaling pathway is activated and may act as a key regulator of the terminal differentiation of distinct populations of the stem cells; however, EGFR activation is controlled and sustained in basic levels.[8] Meanwhile, in cancer cells, the presence of cancer stem cells, overexpression of EGFR, and the mutant downstream effectors, such as KRAS, BRAF, PIK3CA, and PTEN, have been linked to a dynamic change that occurs in cancer cells, leading to drug resistance, distinct oncogenicity alteration, and malignancy transformation.[9,10] Targeting EGFR with anticancer drugs blocks the signal transduction pathways required for controling cancer cell growth, proliferation, and resistance to cell death.[11-13] Among these drugs, we could mention tyrosine kinase inhibitors, such as Sorafenib (SOR), also known as Nexavar, which targets RAF activation and blocks its downstream activity in hepatocellular carcinoma (HCC).[14] Of note, SOR treatment enhances oxidative stress and increases the production of reactive oxygen species (ROS), leading to cell death in HCC cells and human epithelial cells as well.[15] A recent study reported the role of interferon-gamma (IFN-γ) alone and in combination with other anticancer drugs in tumor cell clearance.[16] IFN-γ was initially identified as an antiviral agent that targets two subunits of its receptor, including IFNGR1 and IFNGR2. The ligation of these two subunits receptor by IFN-γ stimulates the Janus kinase (JAK1/JAK2) -STAT1 signaling pathway and interferon regulatory factor-1(IRF-1). JAK/STAT signaling is one of the critical pathways that stimulate the expression of antiviral proteins and the production of pro-inflammatory cytokines.[16] Likewise, Rapamycin (Rap), the mTOR inhibitor and the autophagy inducer agent, has been described as an anticancer agent used alone or in combination with cyclosporine.[17] On the other hand, vitamin B17 is an essential compound, also known as amygdalin, found in nuts and fruit seeds, like apples, red cherries, and apricots. The anticancer activity of vitamin B17 was primarily reported in the 1970s; this was followed by several studies that indicated the antitumor, antioxidant, and antibacterial properties of vitamin B17;[18] for example, a very recent paper reported the effectiveness of the extracted amygdalin from almonds in regulating cell proliferation and inducing apoptosis in oral cancer cell lines.[19] However, the exact mechanism underlying the anticancer properties of vitamin B17 still remains unknown. Therefore, in the present study, we determined the efficiency of IFN-γ, Rap, and amygdalin in the regulation of cancer cell proliferation and compared them to SOR using HepG2 cells and stem cells. We also investigated the potential regulatory role of these indicated candidates in *Raf-1* expression profile, autophagy-related gene expression, and inflammatory cytokines secretion from treated cells.

Materials and Methods

Cell lines and stem cells

The current work was designed in vitro based on the antiproliferation effect and cytotoxicity of some agents, including vitamin B17, Rap, and IFN-γ in HCC. Accordingly, HepG2 cell line and human stem cells were provided from (VACSERA, Giza, Egypt) and were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 4 mM sodium pyruvate, 4

mM L-glutamine, 100 U/mL penicillin/streptomycin, and 10% fetal calf serum albumin (FCS). The cells were incubated at 37°C in a 5% CO_2 incubator and regularly investigated for any mycoplasma contamination using the cell culture contamination detection kit (Invitrogen, USA).[20] The stem cells isolated from the human umbilical cord were kindly provided from the Mansoura University, Faculty of Medicine, Center of the stem cells. The obtained stem cells were maintained in 25 cm^2 flasks and incubated at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with either 10%.[21]

Chemical treatment

To investigate the efficiency of IFN-γ, Rap, vitamin B17, and SOR in cell proliferation, HepG2 cells and stem cells were cultured in 25 cm^2 cell culture flasks with a density of 2×10^5 cells/flask. They were subjected to 10 µM of the indicated agent followed by 48 hrs of incubation. The cells treated with the same concentration of DMSO were severed as control-treated cells. For the ELISA test, the cells seeded in a 96-well plate were subjected to the same concentration of each indicated agent and incubated for different time points (4, 8, 16, 24, and 48 hrs).

Cell viability and toxic effect

Cell viability and cytotoxic effect of each candidate were monitored by calculating the number of surviving cells and measuring the level of produced lactate dehydrogenase (LDH) in the fluid medium of the treated cells. As described previously, assessment of the released LDH from

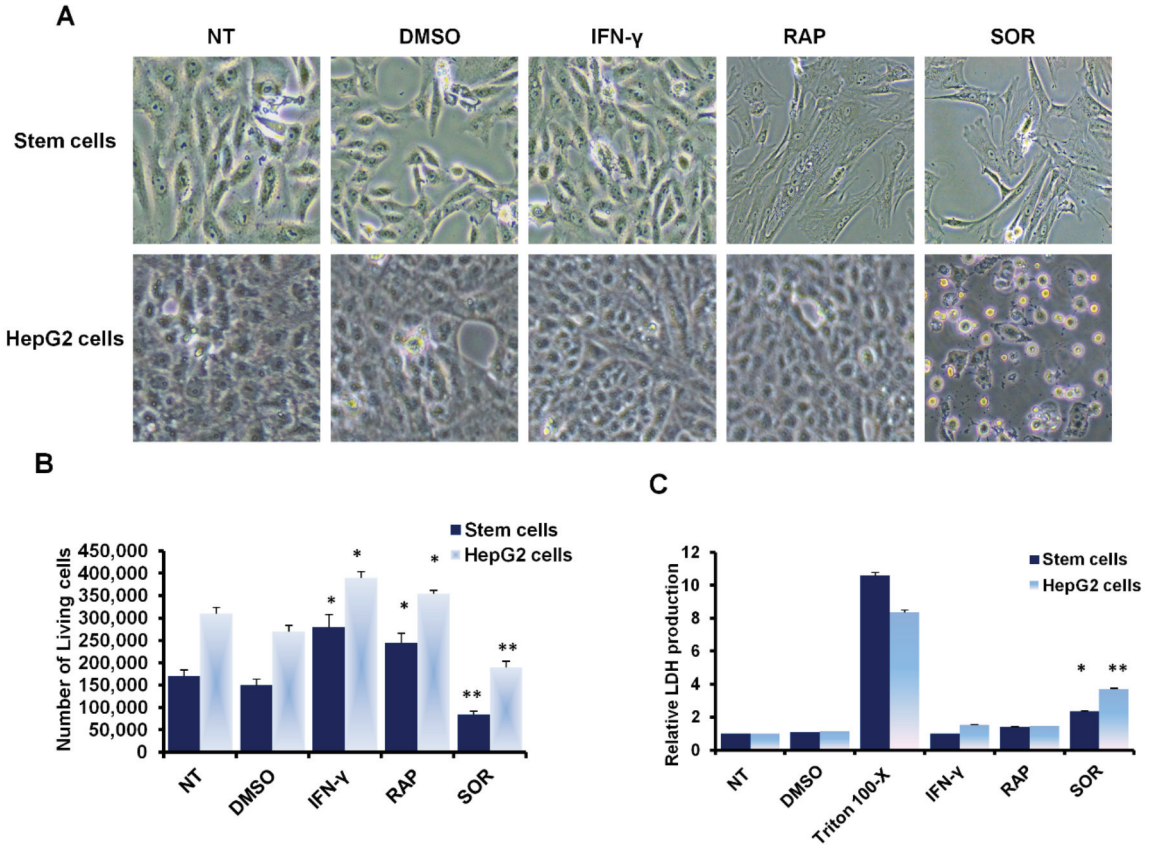

Figure 1. The cytotoxic potential of the indicated anticancer agents on the HepG2 and stem cells: A) The representative inverted microscope images of the stem cells and HepG2 cells 48 hrs after the treatment with 10 µM of the indicated drugs; B) The accounting number of the surviving cells upon 48 hrs following the treatment; C) Relative LDH production from the treated cells in comparison with Triton 100-X treated cells. Error bars reveal the standard deviation of three different replicates. Student's two-tailed t-test was used to determine *P*-values and significance of the accounted number of the surviving cells and LDH production level.

*$P < 0.05$ was considered statistically significant and **$P < 0.01$ as highly significant; NT: Non-treated; DMSO: Dimethyle sulfate; IFN-γ: Interferon-gamma; Rap: Rapamycin; SOR: Sorafenib

Table 1. Oligonucleotides sequences used for quantification analysis of the mRNA for the indicated genes

Description	Primer sequences 5'-3'
Raf-1-sense	TTTCCTGGATCATGTTCCCCT
Raf-1-antisense	ACTTTGGTGCTACAGTGCTCA
LC3B-sense	AGAGTCGGATTCGCCGCCGCA
LC3B-antisense	GACGGCATGGTGCAGGGATCT
P53-sense	GCGAGCACTGCCCAACAACA
P53-anti-sense	GGTCACCGTCTTGTTGTCCT
Casp3-sense	GGACAGCAGTTACAAAATGGATTA
Casp3- anti-sense	CGGCAGGCCTGAATGATGAAG
GAPDH-sense	TGGCATTGTGGAAGGGCTCA
GAPDH-antisense	TGGATGCAGGGATGATGTTCT

Casp3: Caspase 3; GAPDH: Glyceraldehyde 3-phosphate

the treated cells was monitored in a 96-well plate utilizing the LDH production kit (Abcam, USA). Following the manufacturing procedures, the cells were harvested and washed with cold phosphate buffer saline (PBS). The supernatant was reloaded in the ELISA plate-reader and mixed and incubated with 48 µl LDH assay buffer and 2 µl LDH substrate for 30 min, away from the light. The optical density (OD 450) was used for measuring the colorimetric-based LDH activity and the relative LDH production was calculated by dividing the mean absorbance values of the treated cells by the mean absorbance values of the mock indicated by the fold change.[22]

Enzyme-linked immunosorbent assay (ELISA)

ELISA assay was used for the quantification analysis of the released interleukin-4 (IL-4) and IL-6 via human ELISA kits (Abcam 100570 and Abcam 46042, respectively). HepG2 cells and stem cells cultured in 96-well plates were incubated overnight. Subsequently, the cells were treated with 10 µM of each candidate followed by an incubation period of 0, 6, 12, 24, 48, and 72 hrs. At each time point, the cells were lysed using 1X cell lysis buffer (Invitrogen, USA), following which 100 µl of the lysed cells were transferred into the ELISA plate-reader and incubated for 2 hrs at room temperature (RT) with 100 µl control solution and 50µl 1X biotinylated antibody. Afterwards, 100 µl of 1X streptavidin-HRP solution was added to each well and incubated for 30 min in the dark. Additionally, 100 µl of the chromogen TMB substrate was added to each well and incubated for 15 min at RT away from the light. Finally, 100 µl of stop solution was added to each well to cease the reaction. The absorbance of each well was measured at 450 nm.[23]

Flow cytometry assay

Through the use of flow cytometry assay, the expression profiles of phosphorylated RAF and *caspase 3* (*Casp3*) were quantified in the cells treated with vitamin B17 and SOR in comparison with the DMSO-treated ones. As described previously, stem cells and HepG2 cells were cultured for treatment with 10 µM of each candidate for two days.[24,25] For protein staining, the treated cells were trypsinized and fixed using PBS with 2% formaldehyde for 10 min. For permeabilization, the fixed cells were incubated for 3 min in PBS containing Triton-X-100 (0.1%). The staining of phosphorylated RAF protein was assessed by overnight incubation of the cells in PBS containing 1% BSA and 1:500 diluted rabbit monoclonal anti-phospho-RAF (Abcam, USA). After washing, the cells were incubated for 1 hr, away from the light, with the secondary antibody (goat anti-rabbit IgG, Alexa Fluor 488). For phosphorylated casp3 protein staining, the cells were resuspended three times in PBS for washing and incubated for 2 hrs with 1:500 diluted rabbit polyclonal anti-casp3 (phospho S150, Abcam, USA). The cells were then incubated for 1 hr with goat anti-rabbit IgG (Alexa Fluor 594, Abcam, USA) in a dilution of 1:100. Ultimately, the cells were prepared for flow cytometry assay (BD Accuri 6 Plus Flow cytometry).[26]

Quantitative real-time polymerase chain reaction (qRT-PCR)

The quantification analysis of mRNA

expression was detected by using qRT-PCR assay. Acoordingely, total RNA was isolated and purified by using TriZol (Invitrogen, USA) and an RNA purification kit (Invitrogen, USA), respectively. Complementary DNA (cDNA) was synthesized from 1 µg of the total RNA using M-MLV reverse transcriptase (Promega, USA). The quantification analysis of mRNA expression of *Raf-1*, *LC3B*, *TP53*, and casp3 was done utilizing QuantiTect-SYBR-Green PCR Kit (Qiagen, USA) and the specific oligonucleotides listed in table 1. The housekeeping glyceraldehyde 3-phosphate (GAPDH) mRNA level was used for normalization in the real-time PCR data analysis. The PCR reaction was prepared as follows: 10 µl SYBR green, 0.25 µl RNase inhibitor (25 U/µl), 0.2 µM of each primer, 1 µL of synthesized cDNA, and nuclease-free water up to a final volume of 25 µL. The following PCR conditions were applied: 94°C for 5 min, 35 cycles (94°C for 30 sec, 58°C for 15 sec, 72°C for 30 sec).[22,27]

Ethics statement

The Ethics Committee of Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Egypt, approved the study protocol of the present work under the ethical code number 20203001.

Statistical analysis

Delta-delta Ct analysis was used in the quantification analysis of mRNA delivered from qRT-PCR assay based on the following equations: (1) delta-Ct = Ct value for gene - Ct value for GAPDH, (2) (delta–delta Ct) = delta Ct value for experimental – delta Ct for control), (3) Quantification fold change = (2-delta-delta ct).[23,28] We utilized the student's two-tailed t-test for

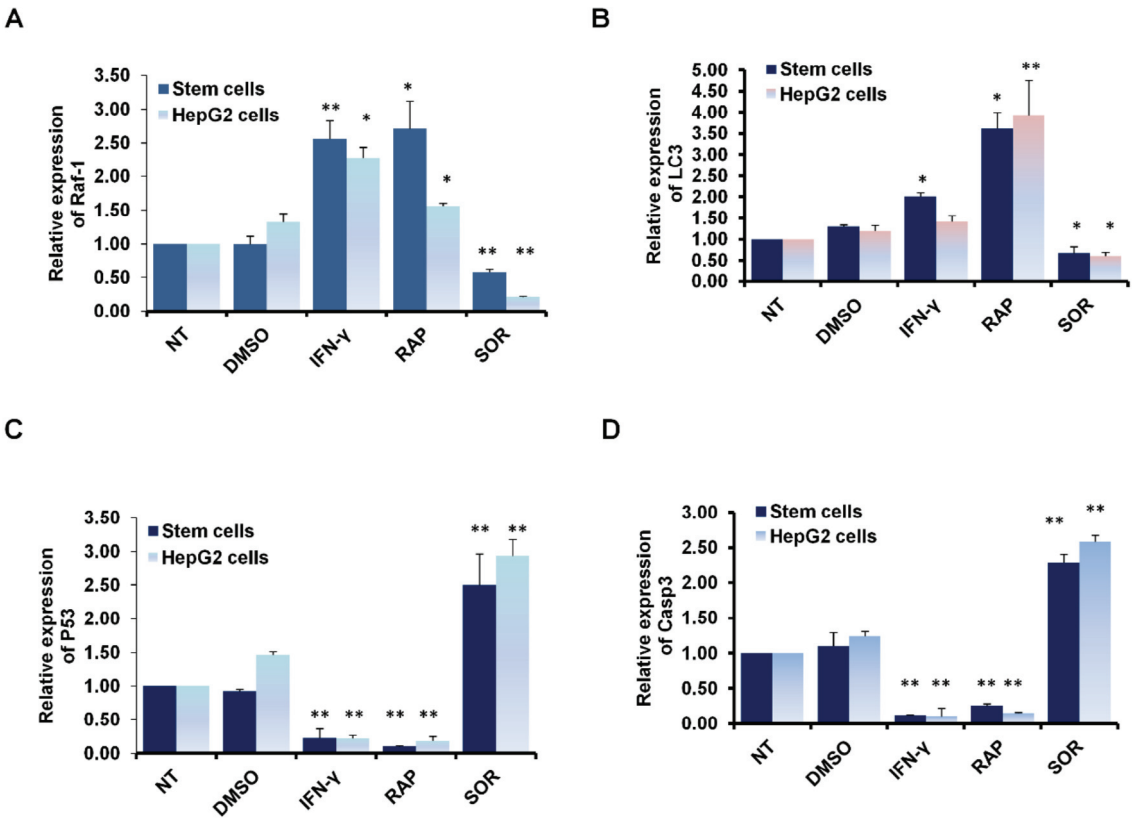

Figure 2. Quantification analysis of the cellular genes involved in cell proliferation and cell death in response to treatment with the indicated anticancer agents: A and B) Quantification analysis of *Raf-1* and *LC3B* mRNA in the HepG2 and stem cells treated with the indicated anticancer agents for 48 hrs in comparison with the control-treated cells using qRT-PCR assay; C and D) Relative gene expression of *TP53* and caps3 in the treated HepG2 and stem cells upon 48 hrs using qRT-PCR. Error bars indicate the SD of three independent experiments. Student two-tailed t-test was used for statistical differences of Ct values in different groups.

* indicates *P*-values ≤ 0.05; ** indicates the *P* ≤ 0.01; SD: Standerd divation; NT: Non-treated; DMSO: Dimethyle sulfate; IFN-γ: Interferon-gamma; Rap: Rapamycin; SOR: Sorafenib; qRT-PCR: Quantitative real-time polymerase chain reaction

Table 2. Statistical analysis of the indicated genes expression in the HepG2 and stem cells treated with anticancer drugs for two days

Genes	Condition	Fold changes		Standard deviation		Student two-tails t-test		*P*-values	
		Stem cells	HepG2 cells	Stem cells	HepG2 cells	Stem cells	HepG2 cells	Stem cells	HepG2 cells
Raf-1	DMSO	0.99	1.3	0.12	0.13	0.93	0.07	>0.05	>0.05
	IFN-γ	2.5**	2.28**	0.27	0.16	0.015	0.007	≤0.01	≤0.01
	Rap	2.7*	1.56**	0.42	0.04	0.028	0.002	≤0.05	≤0.01
	SOR	0.58**	0.21**	0.05	0.01	0.005	0.001	≤0.01	≤0.01
LC3B	DMSO	1.3	1.19	0.04	0.14	0.08	0.18	>0.05	>0.05
	IFN-γ	2.02**	1.42*	0.09	0.14	0.003	0.05	≤0.01	≤0.05
	Rap	3.62**	3.94*	0.39	0.81	0.01	0.03	≤0.01	≤0.05
	SOR	0.67*	0.60*	0.15	0.09	0.08	0.02	≤0.05	≤0.05
P53	DMSO	0.92*	1.47*	0.03	0.05	0.05	0.05	≤0.05	≤ 0.05
	IFN-γ	0.23**	0.22**	0.14	0.05	0.016	0.003	≤0.01	≤ 0.01
	Rap	0.10**	0.19**	0.01	0.06	0.001	0.002	≤0.01	≤ 0.01
	SOR	0.25*	2.94**	0.46	0.24	0.044	0.007	≤0.05	≤ 0.01
casp3	DMSO	1.10	0.25	0.19	0.07	0.53	0.35	>0.05	>0.05
	IFN-γ	0.12**	0.11**	0.01	0.11	0.016	0.008	≤0.01	≤0.01
	Rap	0.26**	0.15**	0.03	0.01	0.007	0.001	≤0.01	≤0.01
	SOR	2.29**	2.58**	0.11	0.09	0.003	0.001	≤0.01	≤0.01

DMSO: Dimethyle sulfate; IFN-γ: Interferon-gamma; Rap: Rapamycin; SOR: Sorafenib; * : significant *P*-values ≤ 0.05; **: highly significant *P*-values ≤0.01

statistical analysis. Data are presented as mean ± S.D. *P*-values below 0.05 were considered as the level of significance, while *P*-values below 0.01 were considered to be highly significant.

Results

Cytotoxicity comparison of IFN-γ, Rap, and SOR on stem cells and HepG2 cells

To compare the cytotoxic effect of IFN-γ, Rap, and SOR on stem cells and HepG2 cells, the cells cultured in 25 cm^2 cell culture flasks were exposed to 10 µM of each candidate for 48 hrs. The cell morphology was monitored through the use of inverted microscopy whereas the number of surviving cells was manually calculated. The relative production of LDH in the fluid medium of the treated cells was measured in order to determine the cytotoxic potential on them. Of note, the cell morphology revealed a disturbing proliferation in both stem cells and HepG2 cells treated with SOR when compared with cell morphology of other treated cells (Figure 1A). The number of the surviving cells significantly decreased in response to SOR treatment in both stem cells and cancer cells, while IFN-γ and Rap treatment showed an increasing number of surviving cells (Figure 1B). Likewise, the relative LDH production significantly increased upon SOR treatment on both treated cells (Figure 1C). These findings indicated the harmful effect of SOR treatment on the stem cells as well as cancer cells. In contrast, IFN-γ or Rap treatment stimulated cell proliferation without any detectable toxic effects.

Assessing proliferation signaling and apoptotic-related factors in treated cells

To investigate the inhibitory effect of SOR, IFN-γ, and Rap on cell proliferation-related signals, the relative gene expressions of *Raf-1* and *LC3B* were detected in the treated cells. Expectedly, the relative expressions of *Raf-1* and *LC3B* significantly decreased in the treated cells with SOR, while markedly increasing in the cells treated with IFN-γ and Rap in comparison with the control-treated cells (DMSO-treated cells) (Table 2) (Figures 2A and B). On the contrary, the relative gene expressions of *TP53* and casp3 dramatically increased in response to SOR treatment, whereas their expression decreased pronouncedly in response to IFN-γ and Rap treatment (Table 2) (Figures 2C and D). These findings further confirmed the anticancer activity of SOR, while highlighting the potent cytotoxic effect of SOR on the stem cells. Furthermore, the indicated response to IFN-γ and Rap treatment in cancer cells suggested the insufficient role of both IFN-γ and Rap in treating cancer cells.

Regulation of anti-inflammatory cytokines secretion by IFN-γ and Rap in treated cells

The comparison between the stem cells and

HepG2 ones based on the production of inflammatory cytokines upon treatment was assessed using ELISA assay. Accordingly, the concentration of the produced IL-4, as an anti-inflammatory cytokine, and IL-6, as a pro-inflammatory cytokine, was measured in the cells subjected to 10 µM of each candidate. We found that SOR treatment markedly decreased the production of IL-4 in both treated cells. Nonetheless, IFN-γ treatment positively regulated the production of IL-4 from stem cells, but not in the treated HepG2 cells. Meanwhile, R treatment showed an increased level of IL-4 in the treated HepG2 cells, but not in the treated stem ones (Figures 3A and B). In contrast, the level of released IL-6 strongly increased in both treated cells with SOR; however, its level significantly decreased in both treated cells with IFN-γ and Rap (Figures 3C and D). Overall, these data revealed that SOR treatment increases the production level of IL-6 accompanied by a low production level of IL-4 in cancer cells and stem cells as well. Furthermore, treatment with IFN-γ or Rap was found to increase the production of IL-4 accompanied with the minimum production of IL-6 in HepG2 and stem cells.

Selective regulation of cancer cell proliferation by vitamin B17 treatment

To figure out the regulatory role of vitamin B17 on cancer cell proliferation and its cytotoxic effect on stem cells, the cultured cells under the same conditions as described previously were exposed to 10 µM of vitamin B17 for 48 hrs. In comparison with SOR treatment, vitamin B17 sufficiently regulated HepG2 cell proliferation indicated by cell morphology and the number of

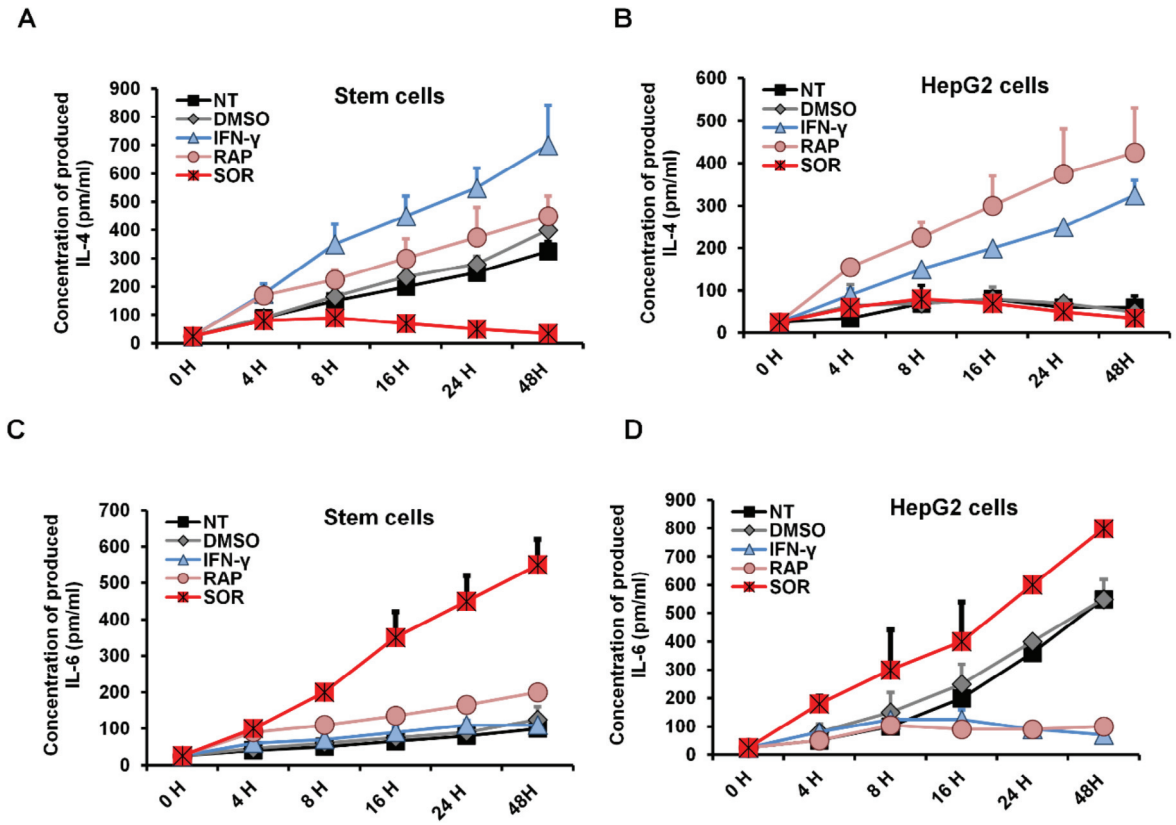

Figure 3. Levels of the produced IL-4 and IL-6 from the HepG2 and stem cells in response to treatment with the anticancer agents: A and B) The concentration of IL-4 (pm/ml) produced in the fluid media of the stem cells and HepG2 cells treated with 10 µM of the anticancer drugs and incubated for the indicated time points compared with the control-treated cells; C and D) The concentration of IL-6 (pm/ml) released in the fluid media of the stem cells and HepG2 cells treated with 10 µM of the anticancer agents and incubated for the indicated time points compared with the control-treated cells. Error bars indicate the SD of four different replicates.

SD: Standerd divation; NT: Non-treated; DMSO: Dimethyle sulfate; IFN-γ: Interferon-gamma; Rap: Rapamycin; SOR: Sorafenib

Table 3. Statistical analysis of the indicated gene expression in the HepG2 and stem cells treated with vitamin B17 or SOR for two days

Genes	Condition	Fold changes		Standard deviation		Student two-tails t-test		*P*-values	
		Stem cells	HepG2 cells	Stem cells	HepG2 cells	Stem cells	HepG2 cells	Stem cells	HepG2 cells
Raf-1	DMSO	1.36	1.09	0.22	0.09	0.14	0.28	>0.05	>0.05
	SOR	0.29**	0.17**	0.13	0.03	0.015	0.006	≤0.01	≤0.01
	B17	1.75	0.46**	0.35	0.01	0.09	0.004	>0.05	≤0.01
LC3B	DMSO	1.5	1.31	0.17	0.39	0.06	0.37	>0.05	>0.05
	SOR	1.12	0.32*	0.24	0.21	0.54	0.043	>0.05	≤0.05
	B17	1.81**	0.16**	0.16	0.06	0.019	0.002	≤0.01	≤0.01
P53	DMSO	1.15	1.26	0.17	0.44	0.35	0.40	>0.05	>0.05
	SOR	3.25*	3.51	0.68	1.26	0.043	0.10	≤0.05	>0.05
	B17	1.11*	2.22**	0.04	0.10	0.058	0.003	≤0.05	≤0.01
Casp3	DMSO	0.92	1.15	0.28	0.33	0.07	0.59	>0.05	>0.05
	SOR	3.46**	3.67**	0.07	0.30	0.004	0.006	≤0.01	≤0.01
	B17	0.72**	2.81**	0.02	0.28	0.003	0.001	≤0.01	≤0.01

DMSO: Dimethyle sulfate; IFN-γ: Interferon-gamma; Rap: Rapamycin; SOR: Sorafenib; * : significant *P*-values ≤ 0.05; **: highly significant *P*-values ≤ 0.01

surviving cells with a protective consequence on stem cell proliferation (Figures 4A and B). Similarly, the relative production of LDH significantly increased in the fluid media of HepG2 cells subjected to vitamin B17, while the treated stem cells showed comparable levels of the produced LDH compared with the control-treated cells (Figure 4C). Therefore, the current observation initially indicated the selective regulatory role of vitamin B17 in cancer cell proliferation without any detectable cytotoxic effects on the stem cells.

Stimulation of programmed cells death by vitamin B17 treatment through inhibiting Raf-1 expression

The relative gene expression of *Raf-1* and *LC3B* were addressed in response to vitamin B17 treatment in both stem and HepG2 cells. Similar to SOR, vitamin B17 markedly reduced gene expression of both *Raf-1* and *LC3B* in HepG2 cells compared with the DMSO-treated cells. However, unlike SOR, vitamin B17 treatment demonstrated a negligible effect on gene expression of both *Raf-1* and *LC3B* in the stem cells (Table 3, Figures 5A and B). Similarly, vitamin B17 treatment revealed a significantly increased level of both *TP53* and casp3 in the HepG2 cells, whereas the expression of these apoptotic indicators was comparable in the stem cells treated with vitamin B17 compared with the control-treated cells (Table 3, Figures 5C and D). Moreover, based on the percentage of positive cells, which reveals the fluorescent intensity of Alexa Fluor signals, the quantification of phosphorylated *Raf-1* and caps3 proteins revealed almost 40% and 10% of B17-treated stem cells, respectively. Meanwhile, SOR treatment showed an increased level of phosphorylated casp3 indicated by more than 90% of the stained cells (Figure 5E). In the HepG2 cells, both vitamin B17 and SOR indicated a decreasing level of phosphorylated RAF (below 10% of stained cells) as well as an increasing level of phosphorylated casp3 indicated by over 80% of the stained cells (Figure 5F). These findings strongly suggested that the treatment with vitamin B17 can selectively regulate the expression profile of *Raf-1* and its related factors in cancer cells, but not in stem cells. Furthermore, the current data highly recommended vitamin B17 as an anticancer candidate without affecting stem cell proliferation.

The biological influence of vitamin B17 in IL-4 and IL-6 production in cancer cells

To address the correlation between vitamin B17 treatment and produced cytokines from the treated cells, IL-4 and IL-6 were measured in both treated stem cells and HepG2 ones in a time-course experiment using ELISA assay. Compared with SOR, vitamin B17 treatment showed a high production level of IL-4 in both treated cells in a time-dependent manner (Figures 6A and B). However, the production level of IL-6 was dramatically reduced in the stem cells and HepG2 cells treated with vitamin B17 in a time-dependent

manner (Figures 6C and D). In sum, these data further confirmed the positive impact of vitamin B17 on regulating the production of anti-inflammatory cytokines from treated cells and revealed its protective role against the production of pro-inflammatory cytokines from cancer cells.

Discussion

In the current work, we provided a comparative study that considered the biological activity of a number of recommended anticancer agents, including IFN-γ, Rap, vitamin B17, and SOR, on stem cells, as precursor cells, and HepG2 cells, as a cancer cell line. Stem cells isolated from human umbilical cords were used in our study on account of their immunosuppressive response and anti-inflammatory properties, in addition to the regular and constant activation of cell proliferation signals, such as mitogen-activated protein kinase (MAPK) and autophagy.[29] Actually, in cancer cells, the cell proliferation signals, such as MAPK and autophagy, are over-stimulated accompanied by overexpression of several oncoproteins and high secretion levels of the pro-inflammatory cytokines, like IL-6 and TNF-γ.[30,31] Our findings further confirmed the regulatory role of SOR on cancer cell proliferation via reducing the expression profile of RAF and autophagy-related *LC3B* parallel with overexpression of the tumor suppressor gene *TP53* and the apoptotic transcriptional factor casp3 in treated HepG2 cells; this result is in agreement with a previous study by Yousef et al.[32] The RAF/MEK/ERK pathway has key roles

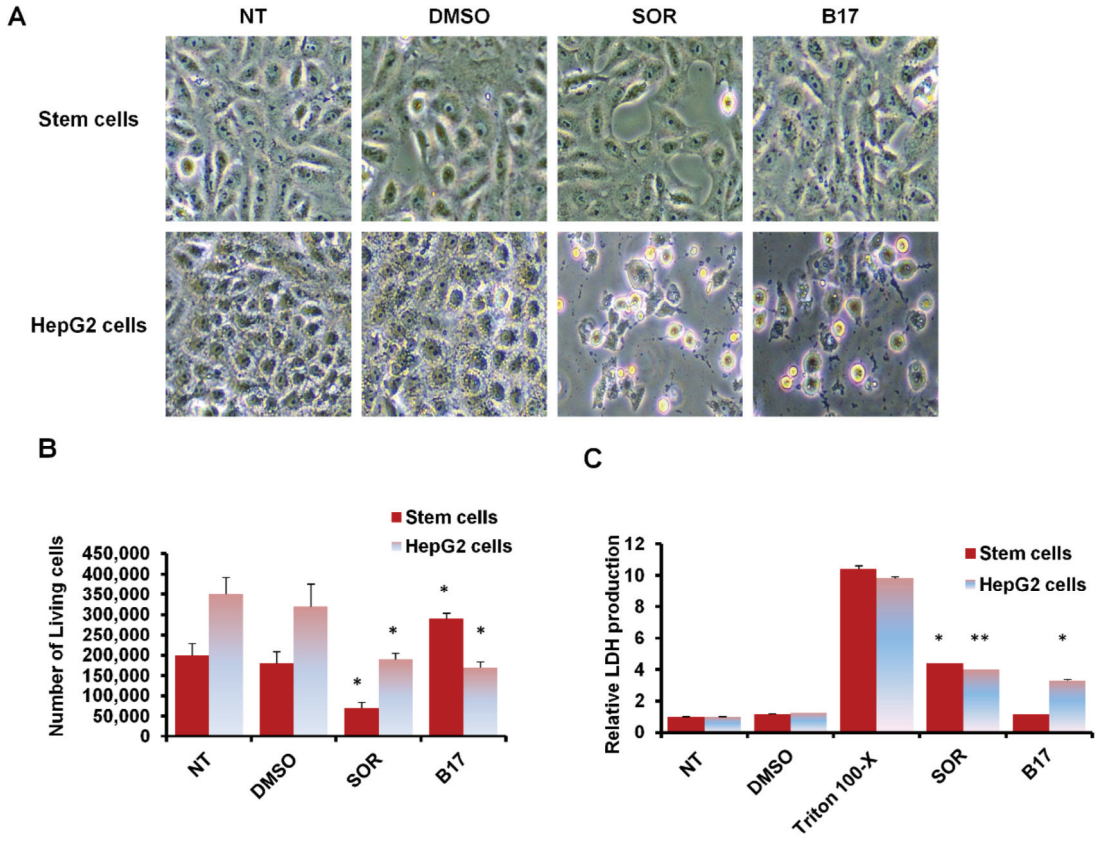

Figure 4. The cytotoxic potential of vitamin B17 on the HepG2 and stem cells: A) The representative inverted microscope images of the stem cells and HepG2 cells 48 hrs after the treatment with 10 µM of vitamin B17 or SOR; B) The accounting number of the surviving cells upon 48 hrs following the treatment; C) Relative LDH production from the treated cells in comparison with Triton 100-X treated cells. Error bars reveal the SD of three different replicates. Student's two-tailed t-test was used to determine *P*-values and significance of the accounted number of the surviving cells and LDH production level.

*$P < 0.05$ was considered statistically significant and **$P < 0.01$ as highly significant; SD: Standerd divation; NT: Non-treated; DMSO: Dimethyle sulfate; IFN-γ: Interferon-gamma; Rap: Rapamycin; SOR: Sorafenib

in regulating cell survival, cell cycle progression, and differentiation. Evidence has indicated that aberrant RAF/MEK/ERK activation increases cellular levels of autophagy-related effectors *LC3B*-I and *LC3B*-II, and SQSTM1.[33] Furthermore, the RAF/MEK/ERK pathway can modulate the activity of many proteins involved in the apoptotic signal, such as Bim, Bax, casp9, casp3, and P53 which in turn regulates autophagosomes formation resulting in programmed cell death.[34,35] Mechanistically, the effect of SOR on casp3 mRNA may be related to activation of mitochondrial-mediated apoptotic pathway via increasing the expression of Bax (Bcl-2 associated x protein), which in turn stimulates the expression of casp9 and casp3 on mRNA and protein level.[36] Typically, the same effect of SOR has been observed on treated stem cells, indicating the potential toxic effect of SOR on stem cells during cancer therapy. Such cytotoxic effect of SOR on stem cells may be due to overproduction of IL-6, as a pro-inflammatory cytokine accompanied by the low production level of IL-4, as an anti-inflammatory cytokine. Great body of evidence has indicated that the high dose of SOR enriched the inflammatory response and malignancy transformation via stimulating IL-6 expression in breast cancer and liver cancer cells.[37,38]

As opposed to SOR, IFN-γ herein showed significant upregulation of both *Raf-1* and *LC3B* in the treated cells, including stem cells and

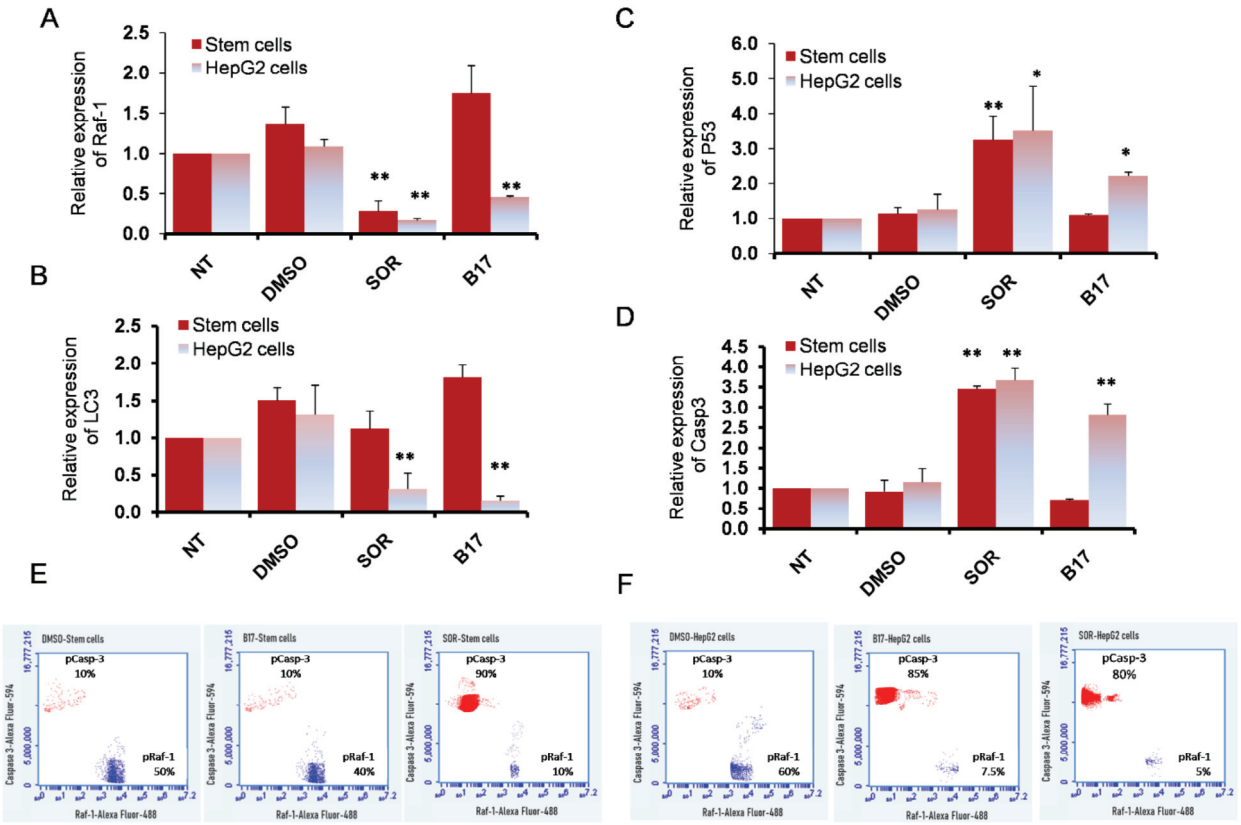

Figure 5. Quantification analysis of *Raf-1*, *LC3B*, *TP53*, and casp3 in response to treatment with vitamin B17 or SOR: A and B) Quantification analysis of *Raf-1* and *LC3B* mRNA in the HepG2 cells and stem cells treated with vitamin B17 for 48 hrs in comparison with the SOR-treated cells and control-treated ones using qRT-PCR assay; C and D) Relative gene expression of *TP53* and caps3 in the treated HepG2 cells and stem cells upon 48 hrs using qRT-PCR. Error bars indicate the SD of three independent experiments. Student two-tailed t-test was used for statistical differences of Ct values in different groups. (*) indicates $P \leq 0.05$ and (**) indicates $P \leq 0.01$; E and F) Quantification of the kinetic profile of phosphorylated *Raf-1* and casp3 proteins in the stem cells (E) and HepG2 cells (F) in response to vitamin B17 or SOR treatment indicated by flow cytometry assay and compared with DMSO-treated cells).

SD: Standerd divation; NT: Non-treated; DMSO: Dimethyle sulfate; IFN-γ: Interferon-gamma; Rap: Rapamycin; SOR: Sorafenib

HepG2 cells, while the expressions of *TP53* and casp3 were significantly reduced in the treated cells. This observation was supported by the positive effect of IFN-γ in cell proliferation, in addition to the independent production of IL-4 and low levels of IL-6 in the treated cells. This finding suggested the insufficient impact of IFN-γ as a potential anticancer agent at least for short-term treatment despite previously reported results indicating the pro-apoptotic and anticancer progress of IFN-γ. More importantly, recent study suggested the dual function of IFN-γ in cell proliferation and programmed cell death.[39] In addition to its potential antitumor proprieties, a great deal of study has shed light on the pro-tumorigenic effect of IFN-γ via stimulation of autophagic process. Mechanistically, activation of autophagy by IFN-γ is due to targetting of serine/threonine kinase (mTOR) pathways and cell proliferation signaling, including RAF/MEK/ERK pathway in liver cancer cells.[39]

In this way, the mammalian target of rapamycin, Rap, is known to stimulate the maturation of autophagosomes via targeting mTOR signaling pathway and stimulating the serine/threonine protein kinases AMP-activated protein kinase.[40] The anticancer properties of Rap were investigated in our study through the expression of *Raf-1* and *LC3B* in the treated HepG2 cells, on top of the statement of cell proliferation, programmed cell death, and cytokines production in the treated cells. Notably, cell proliferation indicated by the number of surviving cells significantly increased in the treated cells. Simultaneously, the relative expression of both *Raf-1* and *LC3B* significantly increased in

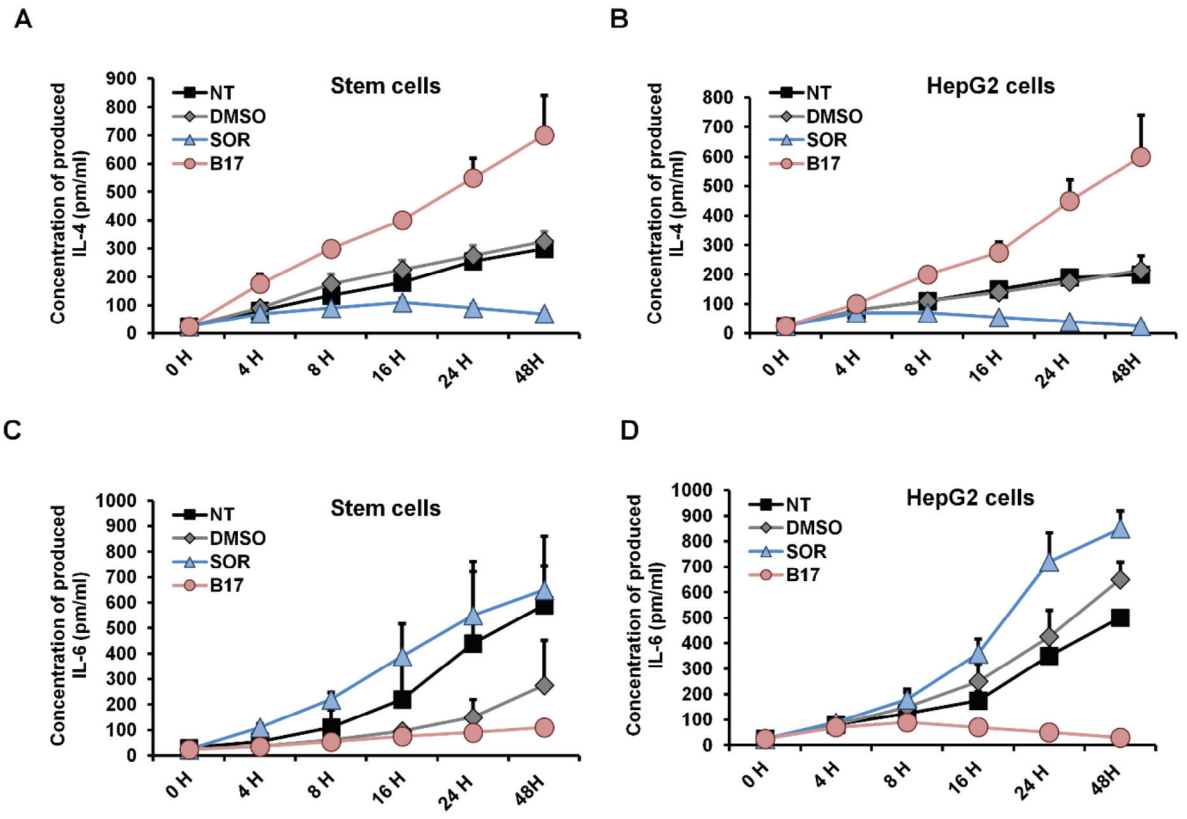

Figure 6. Levels of the produced IL-4 and IL-6 from the HepG2 and stem cells in response to treatment with vitamin B17 or SOR: A and B) The concentration of IL-4 (pm/ml) produced in the fluid media of stem cells and the HepG2 cells treated with 10 µM of either SOR or vitamin B17 and incubated for the indicated time points compared with the control-treated cells; C and D) The concentration of IL-6 (pm/ml) released in the fluid media of the stem cells and HepG2 cells treated with 10µM of SOR or vitamin B17 and incubated for the indicated time points compared with the control-treated cells. Error bars indicate the SD of four different replicates.
SD: Standerd divation; NT: Non-treated; DMSO: Dimethyle sulfate; IFN-γ: Interferon-gamma; Rap: Rapamycin; SOR: Sorafenib

both stem cells and HepG2 ones, while the expression of P53 and casp3 was significantly reduced in both treated cells. Furthermore, IL-4 production markedly increased in the treated cells accompanied by a low production level of IL-6; however, the mechanism through which Rap mediates the production of IL-4 and IL-6 needs further investigation. This observation indicated the deficient role of Rap in regulating cancer cell division. This insufficient role of Rap in cancer therapy may be due to the fact that Rap can inhibit only one side of mTOR efficiency, while other mTOR functions and properties are still active.[41] Most likely, the anticancer activity of vitamin B17 or amygdalin has been reported in several studies; nevertheless, its exact mechanism still remains poorly understood.[42] Sufficient evidence has suggested that vitamin B17 can be used as an anticancer drug in human colon cancer via the inhibition of cell cycle-related genes in SNU-C4 human colon cancer cells.[43] In bladder cancer cell lines, vitamin B17 was found to inhibit cell proliferation, clonal growth, and cell cycle progress via targeting the phosphorylated Akt and Rictor and blocking of Cdk and cyclin components.[42] Likewise, herein, we firstly reported the downregulation of RAF and *LC3B* proteins in response to vitamin B17 treatment in HepG2 cells. Furthermore, our findings revealed the efficiency of vitamin B17 in the activation of the programmed cell death through overexpression of P53 and casp3 on the treated cancer cells without any detectable toxic effects on the treated stem cells. A previous work indicated that amygdalin exerts a cytotoxic effect on estrogen receptors-positive breast cancer cell lines, downregulates B-cell lymphoma 2 (Bcl-2), and upregulates Bax, and casp3.[44] Moreover, vitamin B17 treatment stimulated the production of IL-4 and reduced the production of IL-6 in both stem cells and cancer cells. Interestingly, an in vitro examination showed the stimulatory effect of amygdalin on apoptotic signaling as a result of increasing levels of Bax protein and casp3.[45] To the best of our knowledge, these data firstly indicate the possible interaction between vitamin B17 and *Raf-1* gene expression profile, as an indicator of MAPK signaling and the autophagy-related *LC3B* gene as potential anticancer properties of amygdalin in HepG2 cells. On the other hand, herein, we demonstrated the insufficient anticancer activity of both IFN-γ and Rap in the short-term treatment compared with SOR and amygdalin.

Overall, the current work revealed the anticancer properties of amygdalin in HCC treatment. However, further investigation in mice xenograft models are required to confirm the molecular interaction involved in the anticancer activity of amygdalin in mice with HCC.

Conclusion

Concerning cancer, the most common factors involved in its initiation and development include epidermal growth factor (EGF) and mitogen-activated protein kinase (MAPK). As a supportive mechanism, the autophagic machinery plays a crucial role in cell survival and recycling the damaged and un-needed cytosolic components. In the present work, we investigated the potential anticancer properties of certain recommended drugs, such as IFN-γ, Rap, and vitamin B17, and compared them to SOR using HepG2 cells and stem cells as a precursor cells. Interestingly, vitamin B17 showed competitive inhibition of HepG2 cell proliferation, while IFN-γ and Rap demonstrated insufficient roles in regulating cell proliferation compared to SOR treatment. Notably, the expressions of both *Raf-1* and *LC3B* were significantly reduced in HepG2 cells treated with vitamin B17, whereas their expressions were markedly upregulated in the treated stem cells. In contrast, the expressions of P53 and casp3 were significantly upregulated in the treated cancer cells and downregulated in the treated stem cells. Furthermore, in both treated cells, vitamin B17 stimulated the expression of IL-4 and blocked the secretion of IL-6. On the contrary, vitamin B17 revealed a negligible effect on cell viability and proliferation in stem cells compared to SOR and DMSO treatment.

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Conflict of Interest

None declared.

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