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# PPARγ-dependent pathway in the growth-inhibitory effects of K562 cells by carotenoids in combination with rosiglitazone

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ABSTRACT

*Background:* Carotenoids have been found to play roles in the prevention and therapy of some cancers which PPARγ was also discovered to be involved in. The present studies were directed to determine the inhibitory effects of carotenoids in combination with rosiglitazone, a synthetic PPARγ agonist, on K562 cell proliferation and elucidate the contribution of PPARγ-dependent pathway to cell proliferation suppression.
*Methods:* The effects of carotenoid and rosiglitazone combination on K562 cell proliferation were evaluated by trypan blue dye exclusion assay and MTT assay. When PPARγ has been inhibited by GW9662 and siRNA, cycle-related regulator expression in K562 cells treated with carotenoid and rosiglitazone combination was analyzed by Western blotting.
*Results:* Rosiglitazone inhibited K562 cell proliferation and augmented the inhibitory effects of carotenoids on the cell proliferation greatly. Specific PPARγ inhibition attenuated the cell growth suppression induced by carotenoid and rosiglitazone combination. GW9662 pre-treatment attenuated the enhanced up-regulation of PPARγ expression caused by the combination treatment. Moreover, GW9662 and PPARγ siRNA also significantly attenuated the up-regulation of p21 and down-regulation of cyclin D1 caused by carotenoids and rosiglitazone.
*Conclusions:* PPARγ signaling pathway, via stimulating p21 and inhibiting cyclin D1, may play an important role in the anti-proliferative effects of carotenoid and rosiglitazone combination on K562 cells.
*General significance:* Carotenoids in combination with rosiglitazone are hopeful to provide attractive dietary or supplementation-based and pharmaceutical strategies to treat cancer diseases.


## 1. Introduction

Considerable epidemiologic evidence has suggested that high consumption of fruit and vegetables can decrease the risk of various types of cancer [1,2]. Among various plant constituents, carotenoids are almost taken daily in significant quantity and hence have been extensively studied. A number of results from experimental studies, animal studies, and observational epidemiologic studies reported that β-carotene and other carotenoids are able to inhibit the growth of some kinds of cancer cells [3–6]. Although some adverse health effects have been observed in human β-carotene intervention studies, the recent studies suggested that the effects of carotenoid supplementation may depend on the individuals' habits and the adverse effects were confined to specific subgroups, e.g., smokers [7,8].

PPARγ, a member of the nuclear hormone receptor superfamily, functions as a transcription factor. It is well known that PPARγ plays crucial roles in numerous physiological and pathological processes, such as fat and glucose metabolism, development of various organs, and the occurrence of several human diseases including diabetes, dyslipidemia, inflammation, and hypertension [9]. PPARγ is expressed in a large number of human tissues such as breast, colon, lung, ovary, prostate, stomach, bladder, and thyroid, where it is demonstrated to regulate cell proliferation, differentiation, and apoptosis. This property makes PPARγ an important target for the development of new and effective anticancer therapies [10–12]. Additionally, various studies in vitro have demonstrated that activation of PPARγ by agonists has a potent anti-proliferative activity against a wide variety of neoplastic cells [13]. PPARγ ligands can regulate cell growth, differentiation, proliferation, and/or apoptosis [14]. Among PPARγ ligands, the antitumorigenic activity of rosiglitazone, a synthetic PPARγ ligand, has been documented in a variety of cancers including colon, breast, prostate, and lung [15]. Liu et al. reported that rosiglitazone (>40 mmol/L) inhibited the growth of human leukemia K562 cells and caused apoptosis in time- and dose-dependent manners [16].

We have shown in previous study that carotenoids (β-carotene, astaxanthin, capsanthin, and bixin) suppressed the proliferation and reduced the viability in dose- and time-dependent manners in MCF-7 and K562 cells, induced apoptosis, and interfered with cell cycle. PPARγ, p21, and cyclin D1 were involved in the anti-proliferative effects of carotenoids [17–19]. However, the inhibitory effects of carotenoid and rosiglitazone combination on K562 cell proliferation have not been

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evaluated well yet. At the moment, no clear-cut data are available to establish that PPARγ-dependent pathway may be directly implicated in the cell growth inhibition by the combination treatment.

The present studies were directed to determine the inhibitory effects of carotenoids in combination with rosiglitazone on K562 cell proliferation and elucidate the contribution of PPARγ-dependent pathway to cell proliferation suppression. Then we explore the PPARγ-mediated cell proliferation regulators in the effects of carotenoid and rosiglitazone combination. Our results demonstrated that carotenoids in combination with rosiglitazone significantly inhibited cell proliferation and increased the expression level of PPARγ as compared with their respective treatments. GW9662 and PPARγ siRNA both remarkably diminished the cell viability loss and the up-regulation of PPARγ expression caused by the combined treatment of carotenoids and rosiglitazone. Furthermore, GW9662 and PPARγ siRNA co-application can significantly attenuate the up-regulation of p21 expression and down-regulation of cyclin D1 expression caused by carotenoids or/and rosiglitazone at the same time.

## 2. Materials and methods

### 2.1. Reagents and antibodies

RPMI 1640 medium was purchased from Gibco BRL (Grand Island, NY, USA). N,N,N′,N′ -Tetramethylethyl-enediamine (TEMED), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT), β-carotene (purity 97%), penicillin, streptomycin, PPARγ siRNA and nonspecific control siRNA were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2-chloro-5-nitro-N-phenylbenzamide (GW9662) and 5-[[4-[2-(methyl-2-pyridinylamino) ethoxy]-phenyl] methyl]-2, 4-thiazolidine-dione (rosiglitazone) were purchased from Cayman Chemicals (Ann Arbor, MI). TurboFect siRNA Transfection Reagent was from MBI Fermentas (Utah, USA), bixin and astaxanthin were purchased from Fluka Chemical Co. (Buchs, Switzerland). β-Actin, PPARγ, p21 and cyclinD1 antibodies were from Santa Cruz Biotechnology, Inc. (Delaware, USA). Chronic myelogenous leukemia K562 cell line was gifted from Physiology Research Center of Basic Medical College of Zhengzhou University (China). Fetal bovine serum was purchased from Tian Hang Biological Engineering Ltd. (Hangzhou, China). Tris(hydroxymethyl)aminomethane (Tris) was the product of Roche Ltd. (Basel, Switzerland).

### 2.2. Cell culture

K562 cells were cultured in RPMI 1640 medium supplemented with decomplemented fetal bovine serum (10%, v/v), penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a humidified incubator under 5% $CO_2$ and 95% air at 37 °C, as described previously [18].

### 2.3. Drug treatment and proliferation assays

Carotenoids (β-carotene, bixin, and astaxanthin) were delivered to the cells using tetrahydrofuran (THF) as a solvent. Rosiglitazone and GW9662 were solubilized with DMSO respectively, and then dissolved in culture media. GW9662 was used to pre-treat K562 cells 3 h before carotenoids and rosiglitazone. The concentrations of THF or DMSO were adjusted to be the same in all experiments and final concentrations were no more than 0.5% (v/v). Control groups received the same amount of THF or DMSO without carotenoids, rosiglitazone, and GW9662.

Drug treatments were performed in 24-well tissue culture plates (growth assays) or 6-well dishes (protein extractions). Drugs were added to the culture medium at a calculated final concentration. The extent of the cell viability and proliferation (cell numbers) was measured by MTT assay and cell counting with the trypan blue dye exclusion assay, respectively, as described before [18].

### 2.4. PPARγ siRNA transfection

K562 cells ($8 \times 10^5$ cells/well) were seeded in 6-well plates. For each well, cells were incubated with or without GW9662 (8 μM) for 24 h before transfection. TurboFect™ siRNA Transfection Reagent and PPARγ siRNA or nonspecific control siRNA were mixed in the 0.15 M solution of NaCl for a final concentration of 15 nM and 1/100 (v/v), respectively, then the mixture was incubated for 25 min at room temperature and added to the corresponding wells. After 4 h of incubation, cells were cultured in the presence or absence of carotenoids and rosiglitazone (20 μM) for up to 48 h for Western blot analysis, MTT assays, and the trypan blue dye exclusion assay, respectively.

### 2.5. Western blot analysis

Western blotting was performed using the protocol described previously [18]. In short, cells were harvested and washed twice with cold 1 × PBS, then lysed in lysis buffer (50 mM Tris–Cl, 150 mM NaCl, 0.02% (w/v) $NaN_3$, 100 μg/mL PMSF, 1 μg/mL aprotinin, 1 μg/mL pepstatin A, 2 μg/mL leupeptin, and 1% (v/v) Triton X-100). Total cellular extracts were quantified by BCA assay (Pierce) as per manufacturer's instructions. Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels, and then transferred to NC membranes (PALL, USA). Blots were blocked in blocking buffer (0.1 M TBS containing 5% no-fat milk, 0.1% Tween 20) for 1 h at room temperature, and then incubated with PPARγ, p21, cyclin D1, and β-actin antibodies, respectively, overnight at 4 °C. After being washed, the membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature. The antigen was detected using the SuperSignal West Pico Chemiluminiscent system (Pierce, USA), and visualized by autoradiography on Kodak-XAR film. The images were digitized and analyzed by an NIH imaging software.

### 2.6. Statistical analysis

All experiments were performed at least in triplicate. One-way ANOVAs were used to estimate overall significance followed by post hoc Tukey's tests that corrected for multiple comparisons. Data are presented as means ± S.D. A probability level of 5% ($P < 0.05$) was considered significant.

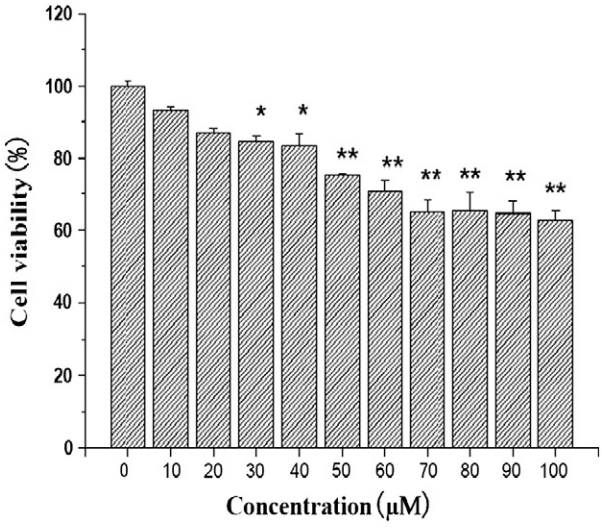


**Fig. 1.** Effect of rosiglitazone on K562 cell viability. The viability of K562 cells treated with different concentrations (10–100 μM) of rosiglitazone for 72 h was measured by MTT assay. Data are presented as the means ± S.D., n = 6, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group.

## 3. Results

### 3.1. Effects of rosiglitazone on the viability of K562 cells

In the first place we treated K562 cells with an increasing concentration (10–100 μM) of rosiglitazone, a PPARγ agonist, for 72 h and measured cell viability, and then investigated the effect of co-application with carotenoids and rosiglitazone on K562 cell growth and viability.

As shown in Fig. 1, rosiglitazone inhibited K562 cell proliferation. The inhibitory effects of low concentrations (10–40 μM) of rosiglitazone were not very significant. The cell viability was reduced obviously when the concentrations up to 50 μM. Over 70 μM, however, rosiglitazone showed very similar inhibitory effects.

### 3.2. Effects of carotenoids in combination with rosiglitazone on K562 cell proliferation

K562 cells were treated with an increasing concentration (5.0–50 μM) of carotenoids (β-carotene, bixin and astaxanthin) in the presence of rosiglitazone (20 μM). As shown in Fig. 2, the inhibitory effects were significantly exacerbated compared with carotenoid treatment groups as measured by the trypan blue exclusion assay. The cell growth curves are close to the straight lines in the presence of 20 μM rosiglitazone. Many dead cells and cellular debris were observed under a microscope after 72 hour treatment. Most of the K562 cells lost their flat, polygonal shape (not shown). In the last two days, cell growth inhibition caused by the combination treatment was more obvious. At 96 h, the cell numbers in the combination treatment groups decreased an approximate of 30% compared with the single carotenoid treatment groups when 5 μM carotenoids were used. With the increase of carotenoid concentrations, a decrease of 30%–50% of the cell numbers can be observed in the combination treatment groups as compared with the carotenoid treatment groups. At 120 h, it was hard to see the live cells. These results indicated that the inhibitory effects of carotenoids on K562 cell proliferation were augmented significantly in the presence of rosiglitazone. It can be seen in Fig. 3 that carotenoids in combination with rosiglitazone had much stronger inhibitory effects on K562 cell viability than their respective treatments as measured by MTT assay. The viability of K562 cells dropped about 30% as compared with the carotenoid treatment groups.

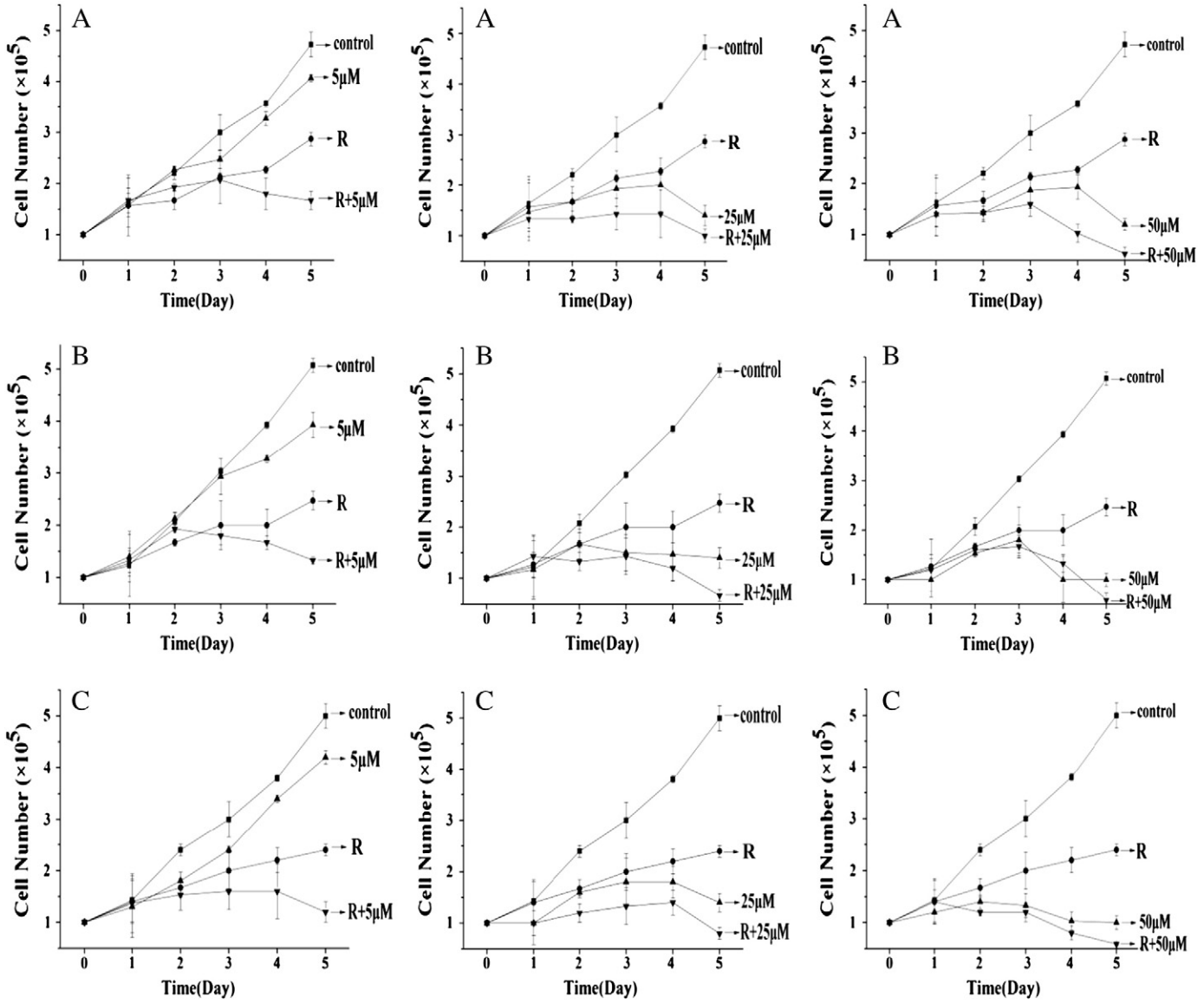


**Fig. 2.** Effect of carotenoids and rosiglitazone (R) on K562 cell growth. K562 cells were harvested at 24 h, 48 h, 72 h, 96 h and 120 h, respectively, after exposure to different concentrations (5–50 μM) of β-carotene (A), bixin (B), astaxanthin (C) and/or rosiglitazone (20 μM). Data are presented as the means ± S.D., n = 6.

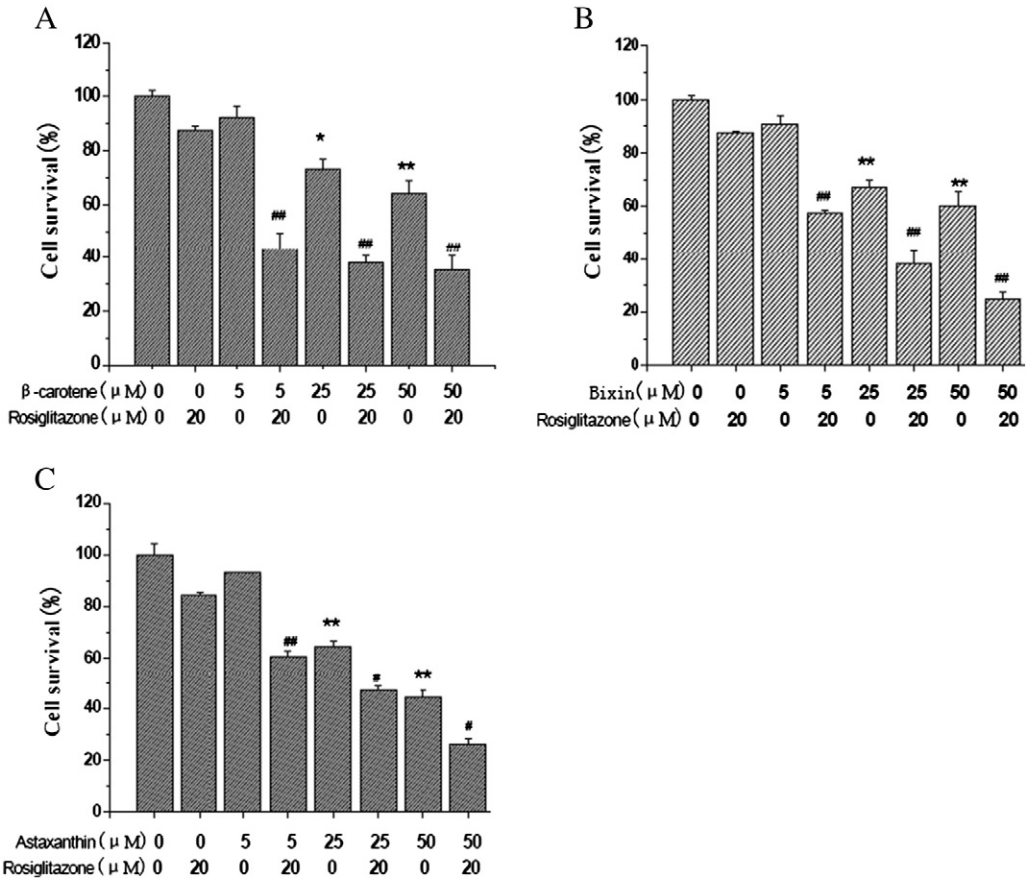


**Fig. 3.** Viability of K562 cells treated with carotenoids and rosiglitazone. The cells were exposed to β-carotene (A), bixin (B), astaxanthin (C), and/or rosiglitazone (20 μM) for 72 h. Data are presented as the means ± S.D., n = 6, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the single carotenoid treatment groups.

### *3.3. Attenuation of carotenoid and rosiglitazone-suppressed K562 cell proliferation by GW9662 and PPARγ siRNA*

To evaluate the relevance of PPARγ-mediated effects on cell proliferation in the suppression of carotenoid and rosiglitazone combination, K562 cells were pre-treated with GW9662, a specific PPARγ inhibitor, for 3 h and then treated with carotenoids and rosiglitazone. As shown in Fig. 4, pre-treatment of GW9662 restored the cell proliferation obviously, especially in the last two days, alive cell numbers were increased more significantly than those in the groups without GW9662. After 96 h, the cell numbers in the GW9662 pre-treatment groups increased an approximate of 75%. When 25 μM and 50 μM carotenoids were used the cell numbers at 120 h increased, respectively, as high as 150% and 250%. Moreover, after the cells were transfected with PPARγ siRNA, their growth suppression by carotenoid and rosiglitazone combination was also attenuated obviously (Fig. 5). These effects can be seen from the first three days. The blockage of PPARγ increased the cell numbers by 50% in both β-carotene and its combination with rosiglitazone treatment groups. In the fifth day of PPARγ inhibition the cell numbers increased an approximate of 65% in the astaxanthin and bixin treatment groups. The blockage of PPARγ increased the cell numbers by almost 85% in the combination of β-carotene or astaxanthin with rosiglitazone groups. It is quite evident that PPARγ siRNA showed the same effects as GW9662 on carotenoid and rosiglitazone combination-suppressed K562 cell proliferation. Fig. 6 shows that GW9662 significantly weakened the suppression of K562 cell proliferation induced by carotenoids and rosiglitazone combination as measured by MTT assay. The viability of K562 cells was increased by more than 20% as compared with co-application treatment groups.

To further understand the relationship between PPARγ and the cell proliferation suppression caused by carotenoids in combination with rosiglitazone, the cells were pre-treated with the combination of GW9662 and PPARγ siRNA. They were firstly incubated in the medium containing 8 μM GW9662 for 24 h, then transfected with PPARγ siRNA. After 52 h PPARγ protein expression was detected by Western blotting. As can be seen in Fig. 7, the expression levels of PPARγ protein were remarkably reduced in the cells treated with the combination of PPARγ siRNA and GW9662. That is to say, in these cases PPARγ in the cells was repressed as fully and specifically as possible. Fig. 8 showed that the cell viability in PPARγ specific repression groups was obviously higher than in the carotenoid and/or rosiglitazone treatment groups. The cell proliferation suppression caused by carotenoids and/or rosiglitazone was diminished greatly. Even if the cells were only exposed to carotenoids and/or rosiglitazone for 48 h, the cell viability in GW9662 and PPARγ siRNA pre-treated groups increased by more than 15% in comparison to the Neg-siRNA pre-treated groups.

### *3.4. Effect of GW9662 on PPARγ expression mediated by carotenoids and rosiglitazone*

Our previous study demonstrated that carotenoids increased PPARγ expression [18]. Rosiglitazone, as a PPARγ agonist, can augment the inhibitory effects of carotenoids on K562 cell proliferation as seen in Figs. 2 and 3. It should activate PPARγ and enhance the up-regulation of PPARγ expression caused by carotenoids. Just as shown in Fig. 9, rosiglitazone treatment increased the expression level of PPARγ protein in K562 cells. Up-regulation of PPARγ expression by carotenoids was further promoted in the presence of rosiglitazone.

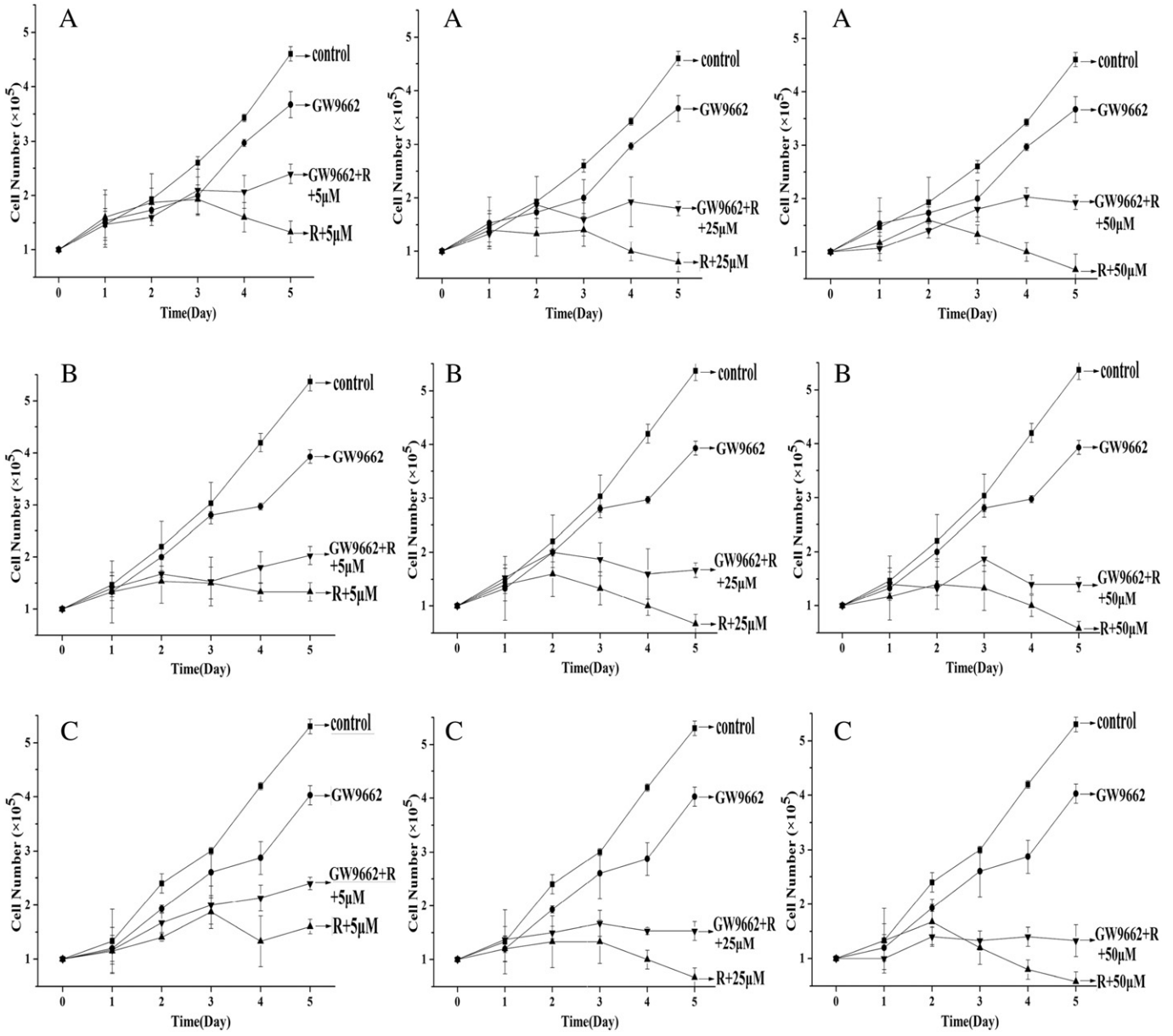


**Fig. 4.** Effects of GW9662 (GW) on carotenoid and rosiglitazone (R)-suppressed cell proliferation. K562 cells were pre-treated with GW9662 (8 μM) for 3 h, and then harvested at 24 h, 48 h, 72 h, 96 h and 120 h, respectively, after being exposed to different concentrations (5–50 μM) of β-carotene (A), bixin (B), astaxanthin (C) and rosiglitazone (20 μM). Data are presented as the means ± S.D., n = 6.

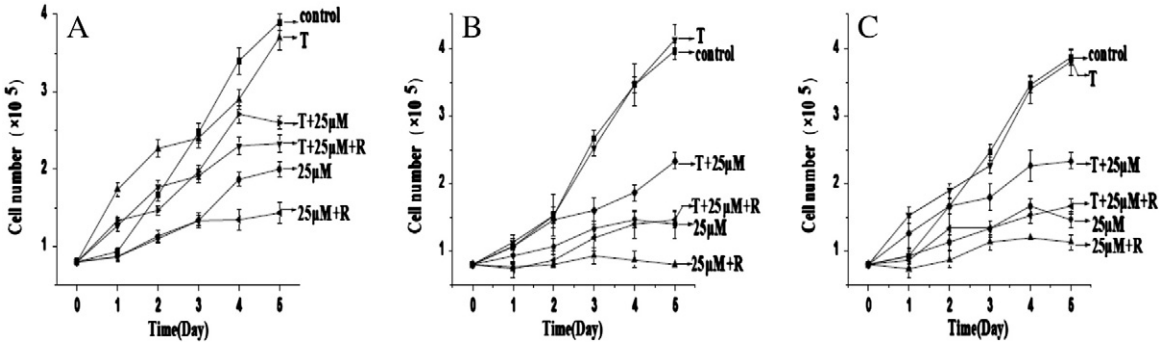


**Fig. 5.** Effects of PPARγ siRNA transfection treatment on the cell proliferation suppression by carotenoids and rosiglitazone (R). The transfected K562 cells with PPARγ siRNA (T) or with negative siRNA were treated by 25 μM of β-carotene (A), bixin (B), astaxanthin (C), and rosiglitazone (20 μM) 4 h after transfection for 5 days, respectively, and in the third day they were transfected again in the same way. Data are presented as the means ± S.D., n = 4.

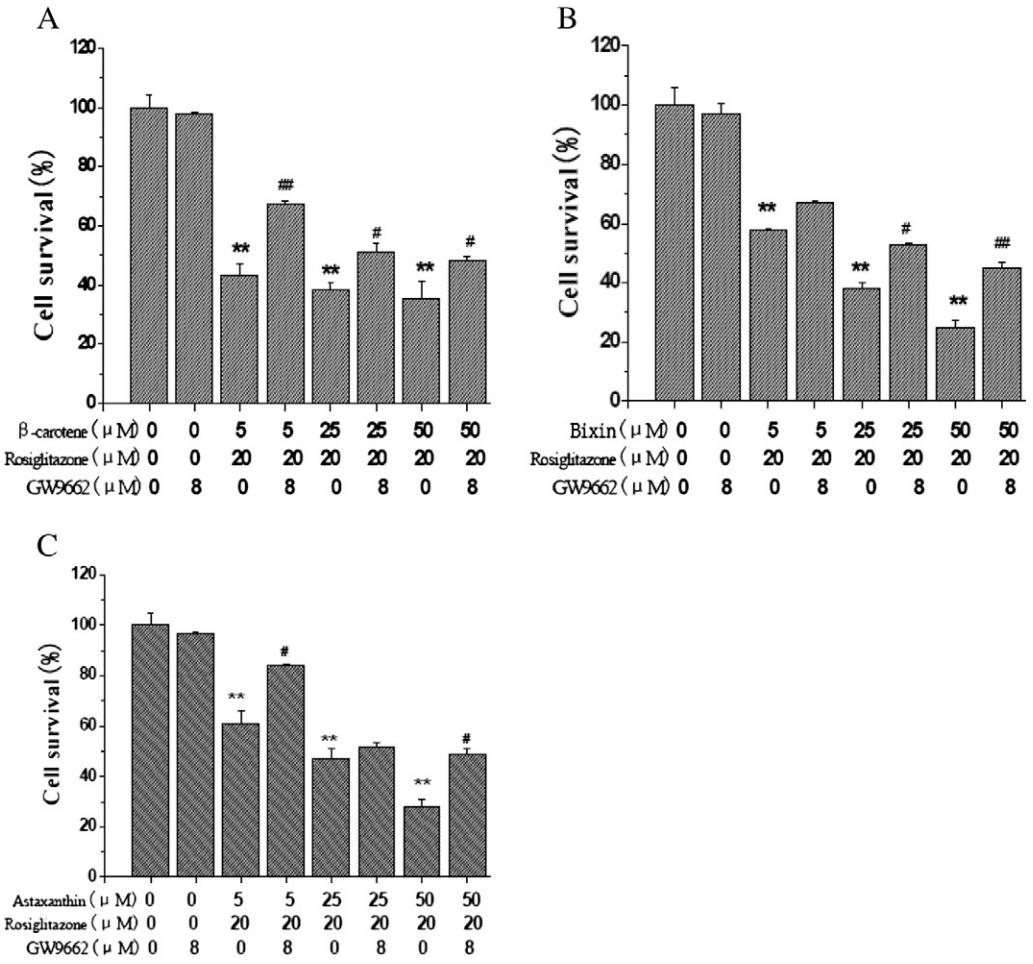


**Fig. 6.** Effects of GW9662 on the reduction of cell viability caused by carotenoid and rosiglitazone combination. K562 cells were exposed to different concentrations (5–50 μM) of β-carotene (A), bixin (B), astaxanthin (C) and rosiglitazone (20 μM) for 72 h after pre-treatment with GW9662 (8 μM) for 3 h. Data are presented as the means ± S.D., n = 6, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control groups; $^{\#}P < 0.05$ compared with the combination-treated groups in the absence of GW9662.

The effects of GW9662 on up-regulation of PPARγ expression caused by carotenoids and/or rosiglitazone were examined to further investigate the role of PPARγ signaling pathway in the anti-cancer activities of carotenoids and their co-application with rosiglitazone. It can be seen from Fig. 10 that β-carotene, bixin, and astaxanthin up-regulated the expression levels of PPARγ protein in K562 cells in a dose-dependent manner after 72 h. However, after GW9662 pre-treatment the up-regulation of PPARγ expression was obviously reduced. Especially it is evident when the concentrations of carotenoids reached 25 μM and 50 μM.

Furthermore, we studied the effect of GW9662 on the up-regulation of PPARγ expression by the combined treatment with carotenoids and rosiglitazone. As shown in Fig. 11, GW9662 pre-treatment significantly suppressed the up-regulation of PPARγ expression by carotenoids in combination with rosiglitazone. PPARγ protein expression levels decreased by more than 15%.

### *3.5. Effects of PPARγ repression on p21 and cyclin D1 expression mediated by carotenoids and rosiglitazone*

The above results showed that GW9662 reduced the up-regulation of PPARγ caused by carotenoids and rosiglitazone. Moreover, our former study demonstrated that carotenoids can also mediate the expression of the cell cycle control gene cyclin D1 and the cyclin-dependent kinase inhibitor p21. In order to further evaluate whether in the cell proliferation suppression these two cell cycle regulators are implicated in PPARγ signaling pathway, we inhibited the PPARγ expression through the co-application of siRNA and GW9662, then treated the cells with carotenoids and rosiglitazone, and measured cyclin D1 and p21 expression. The results in Figs. 12 and 13 showed that the expression levels of cyclin D1 and p21 changed along with the inhibition of PPARγ. For cyclin D1, its down-regulation induced by carotenoids and rosiglitazone was diminished to some extent in the PPARγ specific inhibition groups as compared with in Neg-siRNA treatment groups. For p21, its expression levels were increased obviously by the three kinds of carotenoids and rosiglitazone in the PPARγ normal expression groups. But as the PPARγ being inhibited, the increased trend was obviously weakened.

## 4. Discussion

The growth-inhibitory effects of carotenoids have been observed in numerous tumors and cell lines. Our previous studies demonstrated that β-carotene inhibited breast cancer MCF-7 cell growth, induced apoptosis, and carotenoids (β-carotene, astaxanthin, capsanthin, and bixin) suppressed leukemia K562 cell proliferation and induced apoptosis. Rosiglitazone, one of the thiazolidinedione derivatives, is the most potent synthetic ligand of PPARγ. In addition to its antidiabetic effects, numerous studies have shown that rosiglitazone can induce cell growth arrest and apoptosis in tumor cells, such as adrenocortical cancer [20], colorectal cancer [21], granulose cells [22], lung cancer [15], malignant melanoma cells [23], leukemia cells [16,24], and so on. Our results showed that rosiglitazone suppressed K562 cell growth, which is in agreement with Liu et al.'s observation [16]. Moreover, the growth-

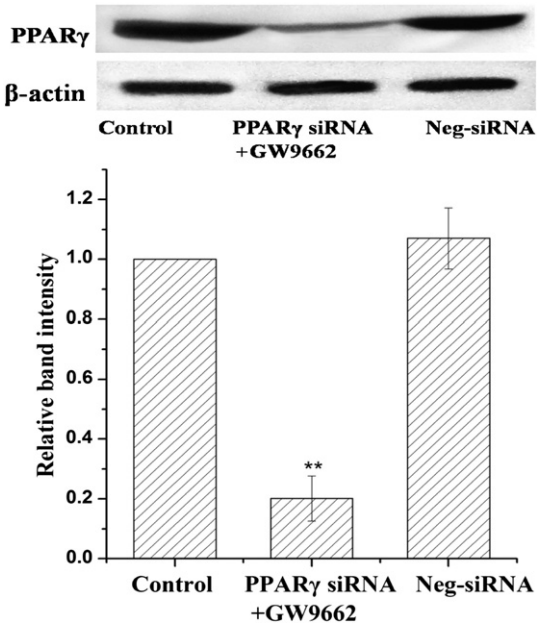


**Fig. 7.** PPARγ expression of K562 cells treated with the combination of PPARγ siRNA and GW9662. Western blot analysis was performed using whole cell lysate from K562 cells incubated in the medium with or without 8 μM GW9662 for 24 h, and then transfected with PPARγ siRNA or Neg-siRNA for 52 h. The expression levels of samples were normalized to the corresponding levels of β-actin. Data are presented as percent of control and the bars are the means ± S.D. values for three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control groups.

inhibitory effects of carotenoids in combination with rosiglitazone on K562 cells were greatly enhanced while at 20 μM rosiglitazone alone had very weak growth-inhibitory effects. It is interesting to note that cells were hardly visible after 120 h combination treatment, the combination of carotenoids and rosiglitazone significantly augmented their anti-proliferative effects on malignant leukemia cells in comparison with their single agent application, which suggested their combined application to be a promising tool for further therapeutic studies.

PPARγ is widely expressed in multiple tumors and cell lines, and this receptor has become the most promising target for anti-cancer therapies. The efficacy of PPARγ agonists as anticancer agents has been examined in various cancers, including colon, breast, lung, ovarian, and prostate [25]. A great deal of evidence suggests that PPARγ agonists can cause cell growth inhibition and apoptosis in a PPARγ-dependent manner though they may also play roles in a PPARγ-independent manner. In the present experiment, either GW9662 or PPARγ siRNA pre-treatment significantly attenuated K562 cell proliferation inhibition induced by carotenoids and rosiglitazone although their pre-treatment did not restore the cell proliferation to the control levels. Their combination pre-treatment also significantly diminished cell viability loss caused by carotenoids and/or rosiglitazone. Meanwhile, GW9662 remarkably reduced the up-regulation of PPARγ protein level caused not only by carotenoids but also by carotenoid and rosiglitazone combination. It is quite evident that PPARγ may participate in the growth-inhibitory effects of carotenoids in combination with rosiglitazone on K562 cells. Our previous studies proposed that PPARγ activation may be associated with the anti-proliferative effects of β-carotene in MCF-7 cells and that the up-regulation of PPARγ expression at least partly contributed to the anti-proliferative effects of carotenoids in K562 cells [17–19]. Yang et al reported that lycopene significantly inhibited the proliferation of androgen-dependent human prostate LNCaP cancer cells, increased the protein and mRNA expression of PPARγ and Liver X receptor alpha (LXRα) at 24 and 48 h, and increased the expression of ATP-binding cassette transporter A1 (ABCA1) and apoA1 protein at 96 h. Incubation of LNCaP cells with lycopene in the presence of GW9662 and LXRα antagonist GGPP restored the cell proliferation to

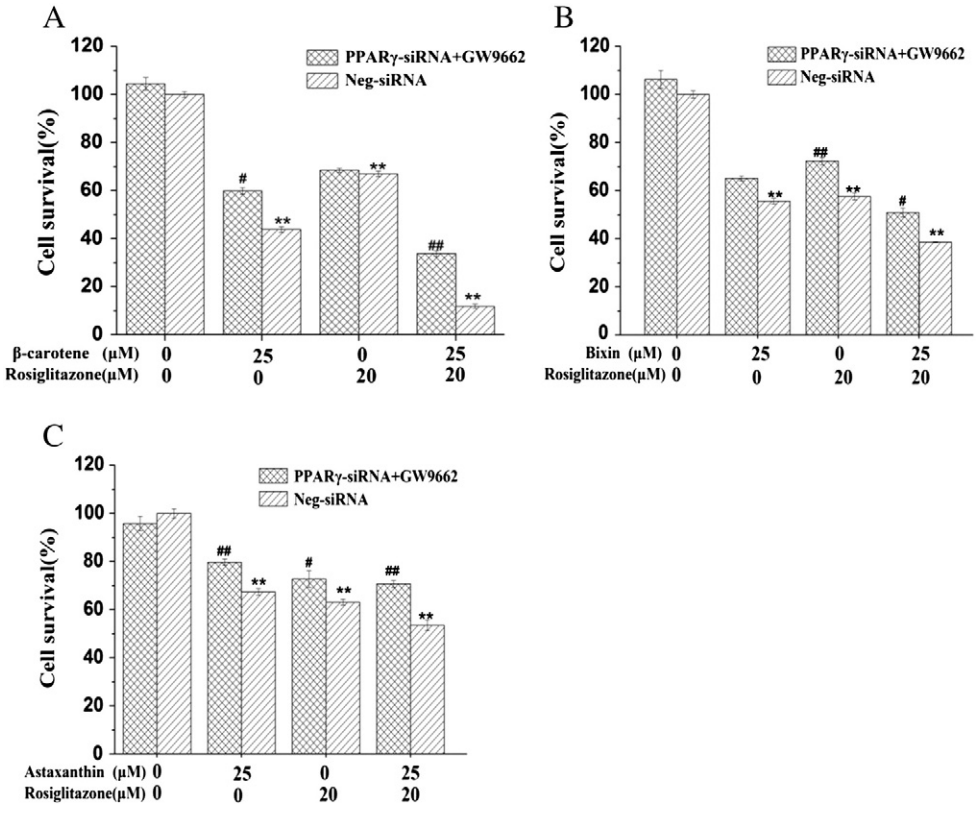


**Fig. 8.** Effects of PPARγ siRNA and GW9662 on the viability of K562 cells treated by carotenoids and rosiglitazone. 8 μM GW9662 was added to the cell medium 24 h prior to transfection. K562 cells were exposed to 25 μM β-carotene (A), bixin (B), astaxanthin (C) and/or 20 μM rosiglitazone 4 h after transfection for 48 h. Data are presented as the means ± S.D., n = 4, $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control groups; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the Neg-siRNA treatment groups.

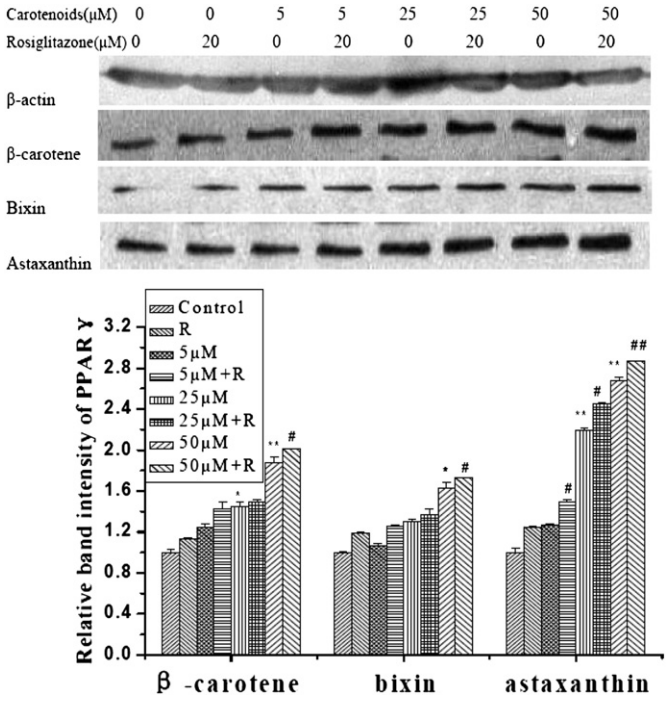


**Fig. 9.** Effects of rosiglitazone on PPARγ expression mediated by carotenoids. K562 cells were exposed to 5–50 μM carotenoids and/or 20 μM rosiglitazone for 72 h. Data are presented as percent of control and the bars are the means ± S.D. values for three independent experiments, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control groups, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the carotenoid treatment groups.

the control levels and significantly suppressed protein expression of PPARγ and LXRα. The similar results were observed in androgen-independent prostate cancer DU145 cells. They proposed that the anti-proliferative effect of lycopene on LNCaP and DU145 cells involves

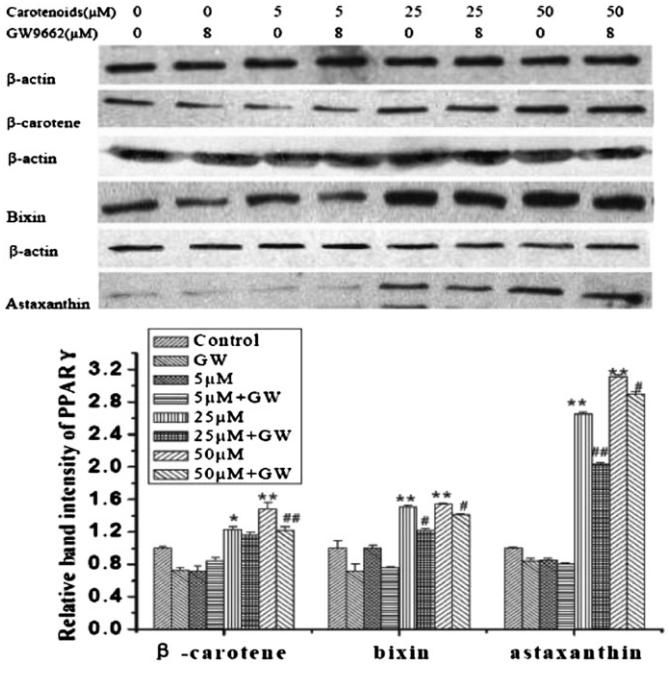


**Fig. 10.** Effects of GW9662 (GW) on PPARγ expression in K562 cells treated with carotenoids. K562 cells were exposed to carotenoids (5–50 μM) for 72 h after pre-treating with GW9662 (8 μM) for 3 h. The expression levels of the samples are normalized to the corresponding levels of β-actin. Data are presented as percent of control and the bars are the means ± S.D. values for three independent experiments, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control groups, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with carotenoid treatment groups.

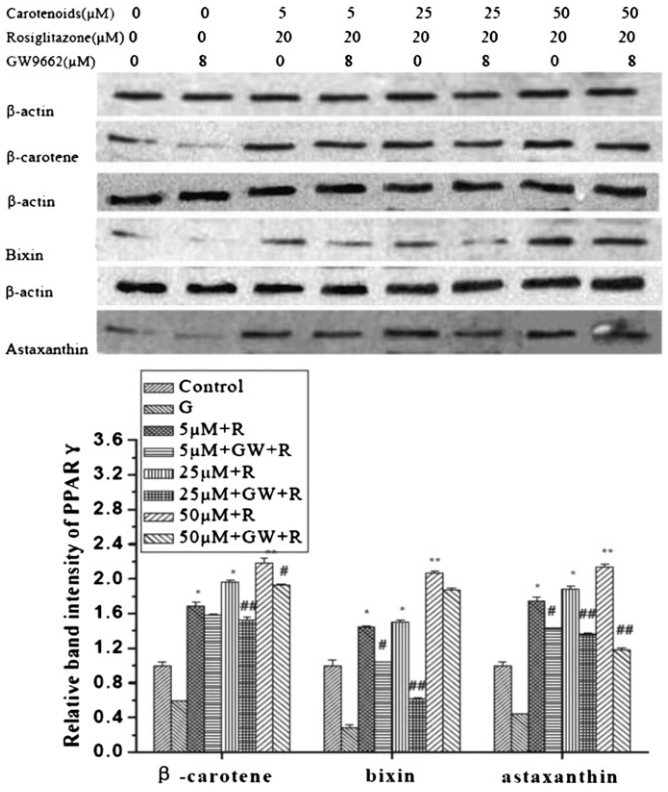


**Fig. 11.** Effects of GW9662 (GW) on PPARγ expression in K562 cells treated with carotenoids and rosiglitazone (R). K562 cells were exposed to 5–50 μM carotenoids and 20 μM rosiglitazone for 72 h after pre-treating with GW9662 (8 μM) for 3 h. The expression levels of samples are normalized to the corresponding levels of β-actin. Data are presented as percent of control and the bars are the means ± S.D. values for three independent experiments, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control groups, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the carotenoid and rosiglitazone combination treatment groups.

the activation of the PPARγ–LXRα–BCA1 pathway [26,27]. In the present experiment GW9662 and/or PPARγ siRNA pre-treatment did not eliminate the proliferation-inhibitory effects by carotenoids in combination with rosiglitazone completely and did not bring about the total recovery of the cell growth and viability, which suggested the involvement of PPARγ-independent pathways in the effects of carotenoids and rosiglitazone. Without a doubt, PPARγ-dependent pathway is a very important part in the growth-inhibitory effects of carotenoids and rosiglitazone on cancer cells.

It has been reported that the expression of cyclin-dependent kinase inhibitors plays important roles in the control of cell cycle progression in many cell types, including tumor cells [28]. $p21^{WAF1/Cip1}$ inhibits cell cycle progression leading to G1 arrest. Cyclin D1 is a key factor in the transition of cell from G1 to S phase. Proliferation inhibition of tumor cells by PPARγ ligands is associated with the alteration in the expression of p21 and cyclin D1. These two cell cycle regulators have been proposed to be potential PPARγ candidate target genes identified in other cancer cell lines [29–32]. It was demonstrated in melanoma MM96L cells that down-regulation of cyclin D1 and up-regulation of the p21 cell cycle proteins following a 24-h PPARγ agonist (rosiglitazone) treatment were noted with a more pronounced regulation after 96 h of treatment [33]. Our previous results showed that carotenoids increased the cell number in G0/G1 phase and up-regulated p21 and down-regulated cyclin D1 expression in K562 cells, and β-carotene up-regulated the expression of p21 in MCF-7 [17,18]. However, the association between the two cell cycle regulators and PPARγ has not been previously examined. Our present study showed here that concomitant with the up-regulation of PPARγ protein levels, down-regulation of cyclin D1 and up-regulation of p21 expression were observed in response to the treatment of carotenoids and/or rosiglitazone after 48 h and 72 h.

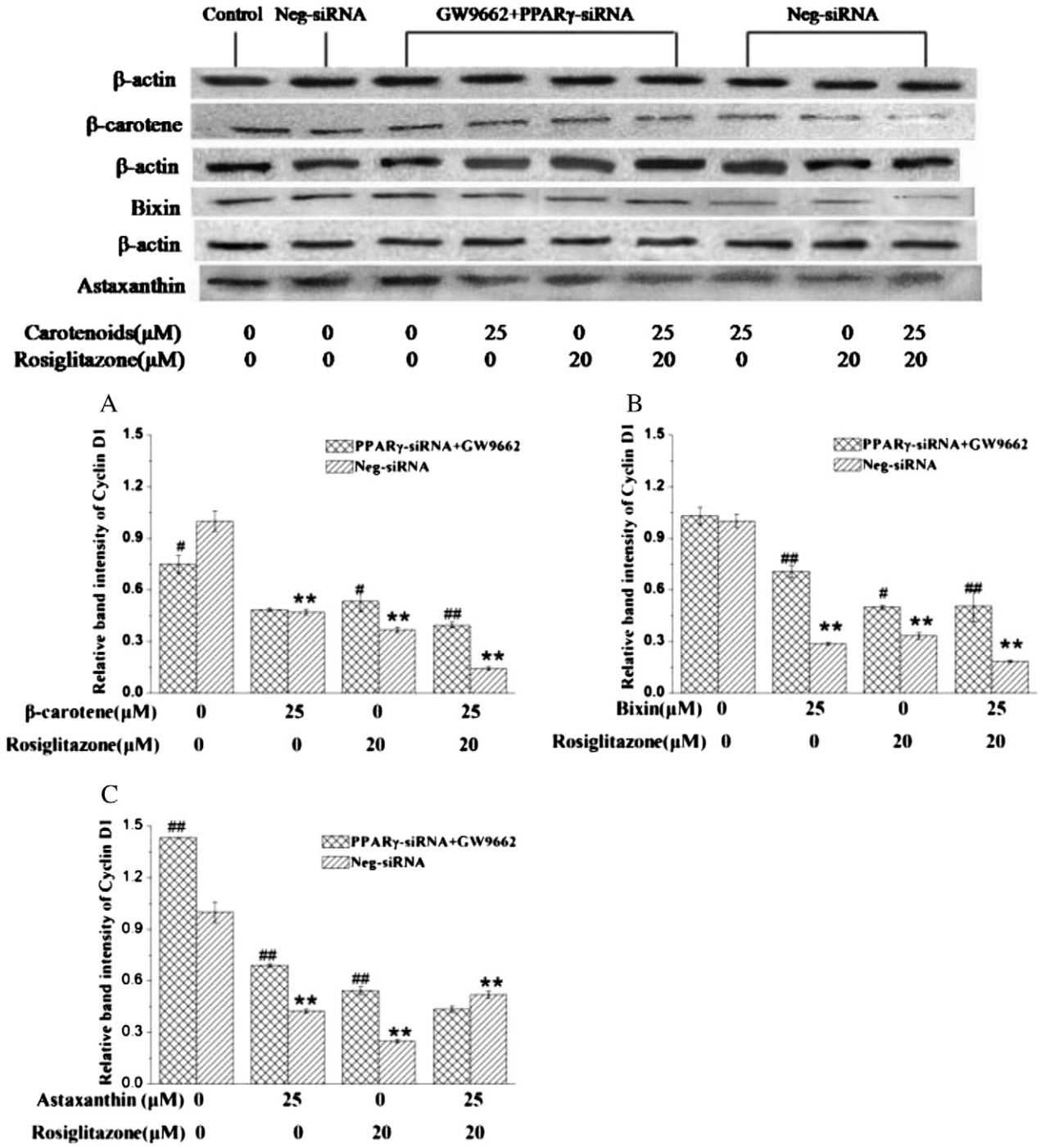


**Fig. 12.** Effects of PPARγ siRNA and GW9662 on cyclin D1 expression in K562 cells treated with carotenoids and rosiglitazone. K562 cells were incubated in the medium with or without 8 μM GW9662 for 24 h, and then transfected with PPARγ siRNA or Neg-siRNA. Each carotenoid was added 4 h after transfection. After 48 h the whole cell lysate was used for Western blot analysis. The expression levels of samples were normalized to the corresponding levels of β-actin. Data are presented as percent of control and the bars are the means ± S.D. values for three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control groups. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the Neg-siRNA treatment groups.

PPARγ siRNA and GW9662 significantly weakened the expression changes of cyclin D1 and p21 caused by carotenoids and rosiglitazone. This suggested that p21 and cyclin D1 were involved in PPARγ-dependent pathway in the cell growth-inhibitory effects. Carotenoids in combination with rosiglitazone might prevent K562 cells from entering S phase by stimulating p21 and inhibiting cyclin D1 through activating PPARγ, which in turn inhibit the cell proliferation.

A growing body of evidence including our results in K562 and MCF-7 cells has shown that natural carotenoids have anti-carcinogenic activity. One of the ways of their anti-proliferative effects on cancer cells is the transcriptional modulation of the important gene expression concerned, such an important transcription factor as PPARγ. PPARγ has been proven to be involved in anti-proliferation, anti-obesity, inflammation modification, and cholesterol homeostasis modulation of carotenoids [17–19,26,27,34–41]. Moreover, thiazolidinediones (including rosiglitazone, ciglitazone, pioglitazone, and troglitazone), synthetic PPARγ ligands, have been found to inhibit cancer cell growth in vitro and in animal models besides anti-diabetes, anti-inflammation, and cholesterol homeostasis modulation [11–14,29–31,33,42–45]. The present experiment showed that carotenoids in combination with rosiglitazone

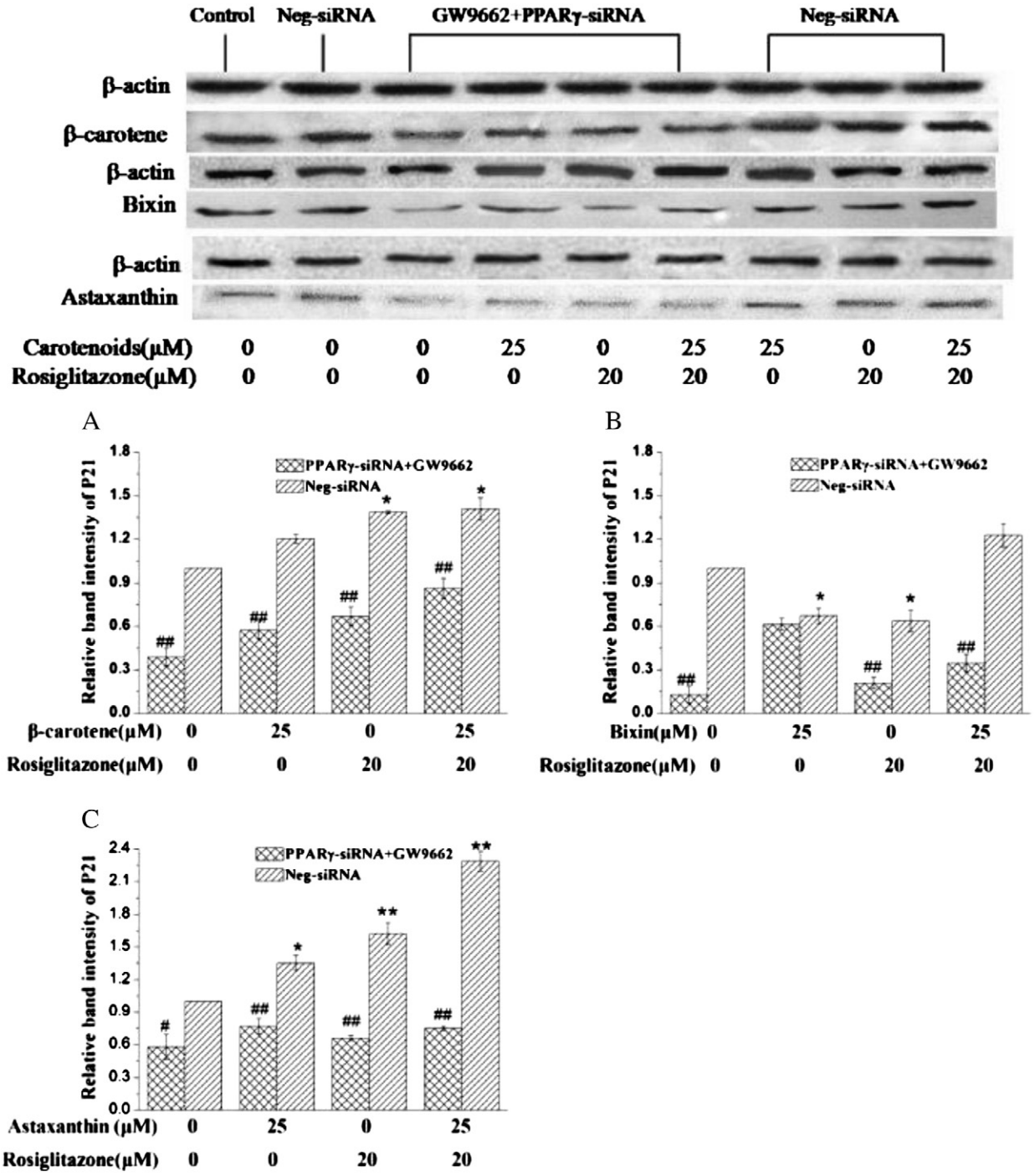


**Fig. 13.** Effects of PPARγ siRNA and GW9662 on p21 expression in K562 cells treated with carotenoids and rosiglitazone. K562 cells were incubated in the medium with or without 8 μM GW9662 for 24 h, and then transfected with PPARγ siRNA or Neg-siRNA. Each carotenoid was added 4 h after transfection. After 48 h the whole cell lysate was used for Western blot analysis. The expression levels of samples were normalized to the corresponding levels of β-actin. Data are presented as percent of control and the bars are the means ± S.D. values for three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control groups. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the Neg-siRNA treatment groups.

had much stronger inhibitory effects on leukemia K562 cell proliferation than their respective treatments. It is imaginable that carotenoids in combination with thiazolidinediones may be attractive dietary or supplementation-based and pharmaceutical strategies to treat such chronic diseases as cancers. Further studies are needed to determine the types of cancer disease and specific populations suited by these strategies. Furthermore, understanding of the PPARγ-dependent signaling pathway will be helpful to offer new insights into the biological activities and molecular mechanisms of dietary bio-active compounds including carotenoids, thereby contributing to the design and application of future intervention and therapeutic approaches in tumorigenesis using PPARγ as a potential target.

## 5. Conclusions

In summary, we found that the combination of carotenoids and rosiglitazone resulted in a more obvious anti-proliferative effect on K562 cells and the PPARγ-dependent signaling pathway played an important role in it via the regulation of p21 and cyclin D1.

## References


[1] F. Baldrick, J. Woodside, J. Elborn, I. Young, M. McKinley, Biomarkers of fruit and vegetable intake in human intervention studies: a systematic review, Crit. Rev. Food Sci. 51 (2011) 795–815.
[2] H. Boeing, A. Bechthold, A. Bub, S. Ellinger, D. Haller, A. Kroke, E. Leschik-Bonnet, M.J. Muller, H. Oberritter, M. Schulze, P. Stehle, B. Watzl, Critical review: vegetables and fruit in the prevention of chronic diseases, Eur. J. Nutr. 51 (2012) 637–663.
[3] L. Gallicchio, K. Boyd, G. Matanoski, X. Tao, L. Chen, T.K. Lam, M. Shiels, E. Hammond, K.A. Robinson, L.E. Caulfield, J.G. Herman, E. Guallar, A.J. Alberg, Carotenoids and the risk of developing lung cancer: a systematic review, Am. J. Clin. Nutr. 88 (2008) 372–383.
[4] C.S. Huang, J.W. Liao, M.L. Hu, Lycopene inhibits experimental metastasis of human hepatoma SK-Hep-1 cells in athymic nude mice, J. Nutr. 138 (2008) 538–543.
[5] F. Lian, K. Hu, R.M. Russell, X. Wang, β-Cryptoxanthin suppresses the growth of immortalized human bronchial epithelial cells and non-small-cell lung cancer cells and up-regulates retinoic acid receptor β expression, Int. J. Cancer 119 (2006) 2084–2089.
[6] R.M. Tamimi, G.A. Colditz, S.E. Hankinson, Circulating carotenoids, mammographic density, and subsequent risk of breast cancer, Cancer Res. 69 (2009) 9323–9329.
[7] P. Palozza, R. Simone, M.C. Mele, Interplay of carotenoids with cigarette smoking: implications in lung cancer, Curr. Med. Chem. 15 (2008) 844–854.
[8] M. Touvier, E. Kesse, F. Clavel-Chapelon, M.C. Boutron-Ruault, Dual association of beta-carotene with risk of tobacco-related cancers in a cohort of French women, J. Natl. Cancer Inst. 97 (2005) 1338–1344.
[9] M. Lehrke, M.A. Lazar, The many faces of PPAR gamma, Cell 123 (2005) 993–999.
[10] I. Sainis, K. Vareli, V. Karavasilis, E. Briasoulis, PPARγ: the portrait of a target ally to cancer chemopreventive agents, PPAR Res. 2008 (2008) 294–304.
[11] S. Han, J. Roman, Peroxisome proliferator-activated receptor [gamma]: a novel target for cancer therapeutics, Anti Cancer Drugs 18 (2007) 237–244.
[12] F. Ondrey, Peroxisome proliferator-activated receptor gamma pathway targeting in carcinogenesis: implications for chemoprevention, Clin. Cancer Res. 15 (2009) 2–8.
[13] H.P. Koeffler, Peroxisome proliferator-activated receptor gamma and cancers, Clin. Cancer Res. 9 (2003) 1–9.
[14] S. Han, J. Roman, Anticancer actions of PPARγ ligands: current state and future perspectives in human lung cancer, World J. Biol. Chem. 1 (2010) 31–40.
[15] C.M. Lyon, D.M. Klinge, K.C. Do, M.J. Grimes, C.L. Thomas, L.A. Damiani, T.H. March, C.A. Stidley, S.A. Belinsky, Rosiglitazone prevents the progression of preinvasive lung cancer in a murine model, Carcinogenesis 30 (2009) 2095–2099.
[16] J. Liu, T. Hu, X. Wu, C. Wang, Y. Xu, Y. Zhang, R. Xiao, D. Lin, R. Huang, Q. Liu, Peroxisome proliferator-activated receptor-gamma agonist rosiglitazone-induced apoptosis in leukemia K562 cells and its mechanisms of action, Int. J. Toxicol. 28 (2009) 123–131.
[17] Y. Cui, Z. Lu, L. Bai, Z. Shi, W. Zhao, B. Zhao, β-Carotene induces apoptosis and up-regulates peroxisome proliferators-activated γ expression and reactive oxygen species production in MCF-7 cancer cells, Eur. J. Cancer 43 (2007) 2590–2601.
[18] X. Zhang, W. Zhao, L. Hu, L. Zhao, J. Huang, Carotenoids inhibit proliferation and regulate expression of peroxisome proliferators-activated receptor gamma (PPARγ) in K562 cancer cells, Arch. Biochem. Biophys. 512 (2011) 96–106.
[19] W. Zhao, X. Zhang, P. Guo, Carotenoids inhibit cell proliferation and up-regulate PPARγ expression in K562 cells, Acta Biophys. Sin. S1 (2009) 420.
[20] L. Cerquetti, C. Sampaoli, D. Amendola, B. Bucci, L. Masuelli, R. Marchese, S. Misiti, A.D. Venanzi, M. Poggi, Rosiglitazone induces autophagy in H295R and cell cycle deregulation in SW13 adrenocortical cancer cells, Exp. Cell Res. 317 (2011) 1397–1410.
[21] R. Miao, T. Xu, L. Liu, M. Wang, Y. Jiang, J. Li, R. Guo, Rosiglitazone and retinoic acid inhibit proliferation and induce apoptosis in the HCT-15 human colorectal cancer cell line, Exp. Ther. Med. 2 (2011) 413–417.
[22] T. Araki, M. Varadinova, M. Goldman, Z. Rosenwaks, L. Poretsky, D.S. Young, Rosiglitazone and pioglitazone alter aromatase kinetic properties in human granulosa cells, PPAR Res. 2011 (2011) 1–5.
[23] C. Freudlsperger, A. Dahl, J. Hoffmann, S. Reinert, U. Schumacher, Mistletoe lectin-I augments anti-proliferative effects of the PPAR gamma agonist rosiglitazone on human malignant melanoma cells, Phytother. Res. 24 (2010) 1354–1358.
[24] J. Liu, X. Yan, Y. Zhang, R. Xiao, D. Lin, Peroxisome proliferator-activated receptor gamma (PPAR-gamma) agonist rosiglitazone (RGZ) inhibits HL-60 cell growth by induction of apoptosis, Lab. Med. 40 (2009) 297–302.
[25] A. Heath, S.Y. Elrod, Sun, PPARγ and apoptosis in cancer, PPAR Res. 2008 (2008) 641–653.
[26] C.M. Yang, I.H. Lu, H.Y. Chen, M.L. Hu, Lycopene inhibits the proliferation of androgen-dependent human prostate tumor cells through activation of PPARγ–LXRα–ABCA1 pathway, J. Nutr. Biochem. 23 (2012) 8–17.
[27] C.M. Yang, I.H. Lu, H.Y. Chen, M.L. Hu, Lycopene and the LXRα agonist T0901317 synergistically inhibit the proliferation of androgen-independent prostate cancer cells via the PPARγ–LXRα–ABCA1 pathway, J. Nutr. Biochem. (Nov 30 2011).
[28] S.S. Chellappan, A. Giordano, P.B. Fisher, Role of cyclin dependent kinases and their inhibitors in cellular differentiation and development, Curr. Top. Microbiol. Immunol. 227 (1998) 57–103.
[29] C. Grommes, G.E. Landreth, M.T. Heneka, Antineoplastic effects of peroxisome proliferator-activated receptor gamma agonists, Lancet Oncol. 5 (2004) 419–429.
[30] M. Toyota, Y. Miyazaki, S. Kitamura, Y. Nagasawa, Peroxisome proliferator-activated receptor γ reduces the growth rate of pancreatic carcinoma cells through the reduction of cyclin D1, Life Sci. 70 (2002) 1565–1575.
[31] S. Han, N. Sidell, P.B. Fisher, J. Roman, Up-regulation of p21 gene expression by peroxisome proliferator-activated receptor γ in human lung carcinoma cells, Clin. Cancer Res. 10 (2004) 1911–1919.
[32] T.H. Chang, E. Szabo, Enhanced growth inhibition by combination differentiation therapy with ligands of peroxisome proliferator-activated receptor-γ and inhibitors of histone deacetylase in adenocarcinoma of the lung, Clin. Cancer Res. 8 (2002) 1206–1212.
[33] A.G. Smith, K.A. Beaumont, D.J. Smit, A.E. Thuiber, A.L. Cook, G.M. Boyle, P.G. Parsons, R.A. Sturm, G.E.O. Muscat, PPARγ agonist attenuate proliferation and modulate Wnt/β-catenin signaling in melanoma cells, Int. J. Biochem. Cell Biol. 41 (2009) 844–852.
[34] H. Kameji, K. Mochizuki, N. Miyoshi, T. Goda, β-Carotene accumulation in 3 T3-L1 adipocytes inhibits the elevation of reactive oxygen species and the suppression of genes related to insulin sensitivity induced by tumor necrosis factor-alpha, Nutrition 26 (2010) 1151–1156.
[35] A. Harari, D. Harats, D. Marko, H. Cohen, I. Barshack, Y. Kamari, A. Gonen, Y. Gerber, A. Ben-Amotz, A. Shaish, A 9-*cis* β-carotene-enriched diet inhibits atherogenesis and fatty liver formation in LDL receptor knockout mice, J Nutr. 138 (2008) 1923–1930.
[36] J. Amengual, E. Gouranton, Y.G.J. van Helden, S. Hessel, J.R. Kramer, B. Kiec-Wilk, U. Razny, G. Lietz, A. Wyss, A. Dembinska-Kiec, A. Palou, J. Keijer, J.F. Landrier, M.L. Bonet, J. von Lintig, Beta-carotene reduces body adiposity of mice via BCMO1, PLoS One 6 (2011) e20644.
[37] N. Takahashi, T. Goto, A. Taimatsu, K. Egawa, S. Katoh, T. Kusudo, T. Sakamoto, C. Ohyane, J.Y. Lee, Y.I. Kim, T. Uemura, S. Hirai, T. Kawada, Bixin regulates mRNA expression involved in adipogenesis and enhances insulin sensitivity in 3 T3-L1 adipocytes through PPAR, Biochem. Biophys. Res. Commun. 390 (2009) 1372–1376.
[38] S. Zaripheh, T.Y. Nara, M.T. Nakamura, J.W. Erdman Jr, Dietary lycopene down-regulates carotenoid 15,15′-monooxygenase and PPAR-γ in selected rat tissues, J. Nutr. 136 (2006) 4932–4938.
[39] R.E. Simone, M. Russo, A. Catalano, G. Monego, K. Froehlich, V. Boehm, P. Palozza, Lycopene inhibits NF-kB-mediated IL-8 expression and changes redox and PPARγ signalling in cigarette smoke-stimulated macrophages, PLoS One 6 (2011) e19652.
[40] P. Palozza, R. Simone, A. Catalano, G. Monego, A. Barini, M.C. Mele, N. Parrone, S. Trombino, N. Picci, F.O. Ranelletti, Lycopene prevention of oxysterol-induced proinflammatory cytokine cascade in human macrophages: inhibition of NF-κB nuclear binding and increase in PPARγ expression, J. Nutr. Biochem. 22 (2011) 259–268.
[41] P. Palozza, R. Simone, A. Catalano, N. Parrone, G. Monego, F.O. Ranelletti, Lycopene regulation of cholesterol synthesis and efflux in human macrophages, J. Nutr. Biochem. 22 (2011) 971–978.
[42] K.K. Ryan, B.E. Grayson, K.R. Jones, A.L. Schneider, S.C. Woods, R.J. Seeley, J.P. Herman, Y.M. Ulrich-Lai, Physiological responses to acute psychological stress are reduced by the PPARgamma agonist rosiglitazone, Endocrinology 153 (2012) 1279–1287.
[43] S. Han, J. Roman, Rosiglitazone suppresses human carcinoma cell growth through PPARγ-dependent and PPARγ-independent signal pathways, Mol. Cancer Ther. 5 (2006) 430–437.
[44] M. Li, T.W. Lee, A.P.C. Yim, T.S.K. Mok, G.G. Chen, Apoptosis induced by troglitazone is both peroxisome proliferator-activated receptor γ and ERK-dependent in human non-small lung cancer cells, J. Cell. Physiol. 209 (2006) 428–438.
[45] M. Saiki, Y. Hatta, T. Yamazaki, T. Itoh, Y. Enomoto, J. Takeuchi, U. Sawada, S. Aizawa, T. Horie, Pioglitazone inhibits the growth of human leukemia cell lines and primary leukemia cells while sparing normal hematopoietic stem cells, Int. J. Oncol. 29 (2006) 437–443.