

In Vitro Anticancer Effects of All-trans Retinoic Acid in Combination with Dacarbazine against CD117⁺ Melanoma Cells

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ABSTRACT

Background Malignant melanoma is a common form of skin cancer that contains different cell types recognized by various cell surface markers. Dacarbazine-based combination chemotherapy is frequently used for the treatment of melanoma. Despite its potent anticancer properties, resistance to dacarbazine develops in malignant melanoma. Here, we aim to improve response to dacarbazine therapy by pretreatment with all-trans retinoic acid (ATRA) in CD117⁺ melanoma cells.

Methods The CD117⁺ melanoma cells were sorted from A375 malignant melanoma cell line using magnetic-activated cell sorting (MACS). The cell viability was examined by cell proliferation assay (MTT). Apoptosis was determined by acridine orange/ethidium bromide staining. Indeed, we performed flow cytometry to evaluate the cell cycle arrest.

Results Here, the CD117⁺ melanoma cells were incubated with various concentrations of ATRA, dacarbazine, and their combination to determine IC₅₀ values. We found that 20 μM ATRA treatment followed by dacarbazine was found to be more effective than dacarbazine alone. There was an indication that the combination of ATRA with dacarbazine (ATRA/dacarbazine) caused more apoptosis and necrosis in the melanoma cells (P<0.05). Furthermore, ATRA/dacarbazine treatment inhibited the cell at the G0/G1 phase, while dacarbazine alone inhibited the cells at S phase.

Conclusion Collectively, combined treatment with ATRA and dacarbazine induced more apoptosis and enhanced the cell cycle arrest of CD117⁺ melanoma cells. These results suggested that ATRA increased the sensitivity of melanoma cells to the effect of dacarbazine.

Introduction

Human malignant melanoma is a highly metastatic type of skin cancer. Melanoma is markedly resistant to chemotherapy. The mortality rate is high in melanoma [1]. Unfortunately, the prevalence of melanoma has been rising annually [2], but the survival rate of patients diagnosed with melanoma has improved considerably over the past decades [3]. Melanoma is curable at early stages before the cells invade the lower layers of skin. Complete surgical excision used for the treatment of primary lesions of melanoma. The success of the surgery is minimal in malignant metastatic melanoma. Unfortunately, melanoma can remain asymptomatic for a long-time and may invade multiple organs such as lung, bone, brain, and

liver [4]. Dacarbazine, temozolomide, and ipilimumab approved for the treatment of metastatic melanoma. Unfortunately, remission may occur after chemotherapy [5]. Indeed, melanoma is resistant to other drugs such as doxorubicin, etoposide, IL-2, and IFN-α [4, 6]. Therefore, melanoma cells may acquire chemoresistance during or after chemotherapy. P-glycoprotein and multidrug resistant protein MDR1 are upregulated in melanoma. Indeed, down-regulation of topoisomerase II and hyperactivation of DNA repair are the possible mechanisms of chemoresistance in melanoma cells [7].

CD117 (c-KIT), as a member of the tyrosine kinase family, interacts with stem cell factors (SCF). Activation of CD117 increases pro-

liferation, cell survival, migration, and differentiation. The c-kit gain of function mutations has been linked to several cancers such as melanoma, gastrointestinal stromal tumor, acute myeloid leukemia, and testicular cancer [8]. Also, CD117 is expressed in prostate stem cells [9] and hematopoietic stem cells [10]. Previous studies suggested that CD117 plays a critical role in cancer stem cells (CSCs) of non-small lung cancer and ovarian tumor [11]. It may play a role in the progression and metastasis of melanoma. Garcia et al. showed that CD117 was positive in most metastatic melanoma samples; they conclude that CD117 immunoreactivity may be useful in the differential diagnosis of malignant melanoma from clear cell sarcoma [12]. Indeed, it has been shown that CD117 expression increases in metastatic sites of melanoma. Awareness of the expression of CD117 markers in metastatic melanomas may be of importance to identifying novel therapeutic interventions to target CD117⁺ metastatic melanoma cells.

ATRA has been suggested to promote apoptosis in melanoma cell lines through cell cycle arrest and dysfunction of mitochondria by binding to retinoic acid receptors (RARs) and retinoic acid × receptors (RXR) [13]. Moreover, Yin et al. showed that ATRA inhibits murine melanoma cell growth by promoting the differentiation and the cytotoxic function of effector CD8⁺ T cells [14]. Although the mechanism of ATRA on regulating apoptosis of melanoma cells is not fully elucidated, it has been suggested that induction of differentiation and apoptosis by retinoic acids may contribute to the treatment of cancers.

Dacarbazine (DTIC) damages DNA and causes cell death. Unfortunately, the response rate for dacarbazine monotherapy is low in malignant melanoma. Dacarbazine can cause common side effects, such as anemia, neutropenia, vomiting, and nausea [15]. This limitation may be overcome by using a combination of lower dosages of chemotherapy agents. Studies have shown that ATRA combination with chemotherapy agents can inhibit the proliferation of gastrointestinal cancers [16], ovarian adenocarcinoma, and squamous head and neck cancers [17].

The importance of CD117 in normal melanocyte and melanoma cell development is well established. The elimination of CD117⁺ cells in malignant melanoma may be an effective therapy. Therefore, we hypothesized that the subtoxic concentration of ATRA might improve the efficacy of dacarbazine in the CD117⁺ melanoma cells.

Material and Methods

Drugs and reagents

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, M2128), dacarbazine (D2390), and ATRA (R2625) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ATRA dissolved in ethanol 96% at 0.01 M and kept frozen (−80) as a stock solution. Dacarbazine dissolved in phosphate saline buffer and diluted in RPMI 1640.

Cell line and cell culture

The human melanoma, A375 cell line, was obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran). The cells were cultured in RPMI 1640 (Gibco, UK) medium supplement-

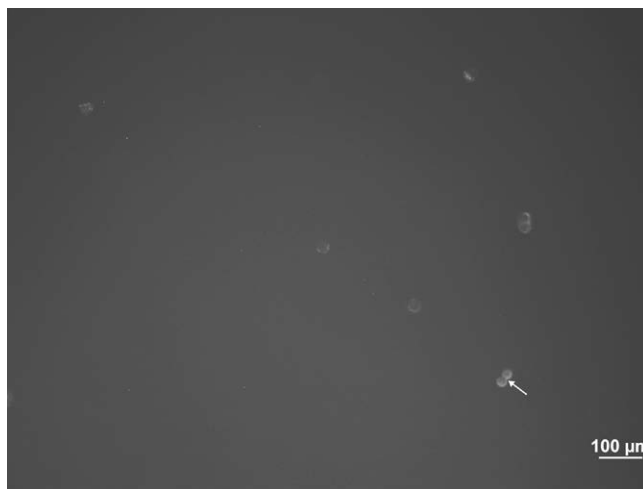
ed with 15% fetal bovine serum (FBS; Cat. Number 10270-106, Gibco, UK) and 1% Penicillin/streptomycin (Gibco: 15140-122). The cells were incubated at 37 °C under a humidified atmosphere.

Separation of CD117⁺ cells by MACS

The CD117⁺ cells were isolated from the A375 melanoma cell line as previously described [18, 20]. The melanoma cells were harvested, prepared into single-cell suspension, and counted. For the selection of CD117 single-positive cells, the cells were stained with a PE-conjugated CD117 antibody (Miltenyi Biotec, 130-099-672) and incubated for 15 min at room temperature. The cells rinsed with wash buffer and incubated with anti-PE Microbeads (Miltenyi Biotec, 130-048-801) for 20 min at 4–8 °C. The cells passed through the MS column. Finally, the retained cells eluted with 500 µl of the cold autoMACS solution. Magnetic separation was performed twice to obtain a higher percentage of CD117⁺ cell populations.

MTT assay

Briefly, for evaluation of cytotoxicity of the drugs, the CD117⁺ cells were cultured in 96 well plates. The CD117⁺ cells were treated with various concentrations of ATRA, dacarbazine, and ATRA/dacarbazine. The groups include: (i) untreated control (ii) ATRA (4–64 µM) (iii) dacarbazine (0, 800, 1000, 1200, 1400 µg/ml) and (iv) ATRA/



► **Fig. 1** Isolation of CD117⁺ cells from A375 melanoma cell line using a magnetically activated cell sorting method. The cells were identified by fluorescence microscopy. White arrows show CD117 positive cell.

► **Table 1** The CD117⁺ melanoma cells were treated with different concentrations of ATRA, dacarbazine, and ATRA/dacarbazine. IC50 values of the drugs against CD117⁺ melanoma cells were calculated using Sigmaplot (n=3, Mean ± STDEV).

	CD117 ⁺
ATRA	8.09 ± 1.72 µM
dacarbazine	1583.09 ± 133.84 µg/ml
ATRA (5 µM)/dacarbazine	1610.97 ± 210.89 µg/ml
ATRA (10 µM)/dacarbazine	1583.34 ± 45.26 µg/ml
ATRA (20 µM)/dacarbazine	914.21 ± 51.3* µg/ml
* P<0.05 compared to dacarbazine treated cells.	

dacarbazine ; 4 h pretreatment with 5, 10 and 20 μM concentrations of ATRA, then the cells incubated in various concentration of dacarbazine (0, 800, 1000, 1200, 1400 $\mu\text{g/ml}$) for 48 h. Following incubation, 180 μl serum-free medium and 20 μl MTT solution (5 mg/ml, Sigma, M2128) were added and incubated at 37 $^{\circ}\text{C}$ for 4 h. The supernatant was removed and replaced by 200 μl of DMSO (Scharlau Chemie, Barcelona, Spain) to dissolve formazan crystals for 5 min at 37 $^{\circ}\text{C}$. Finally, the survival fraction of the cells was measured at 480 nm by using an ELISA reader (Bio Tek). The inhibitory effect of the drugs at each concentration was expressed as a percentage of the control group (mean OD of treated cells/mean OD of control) \times 100.

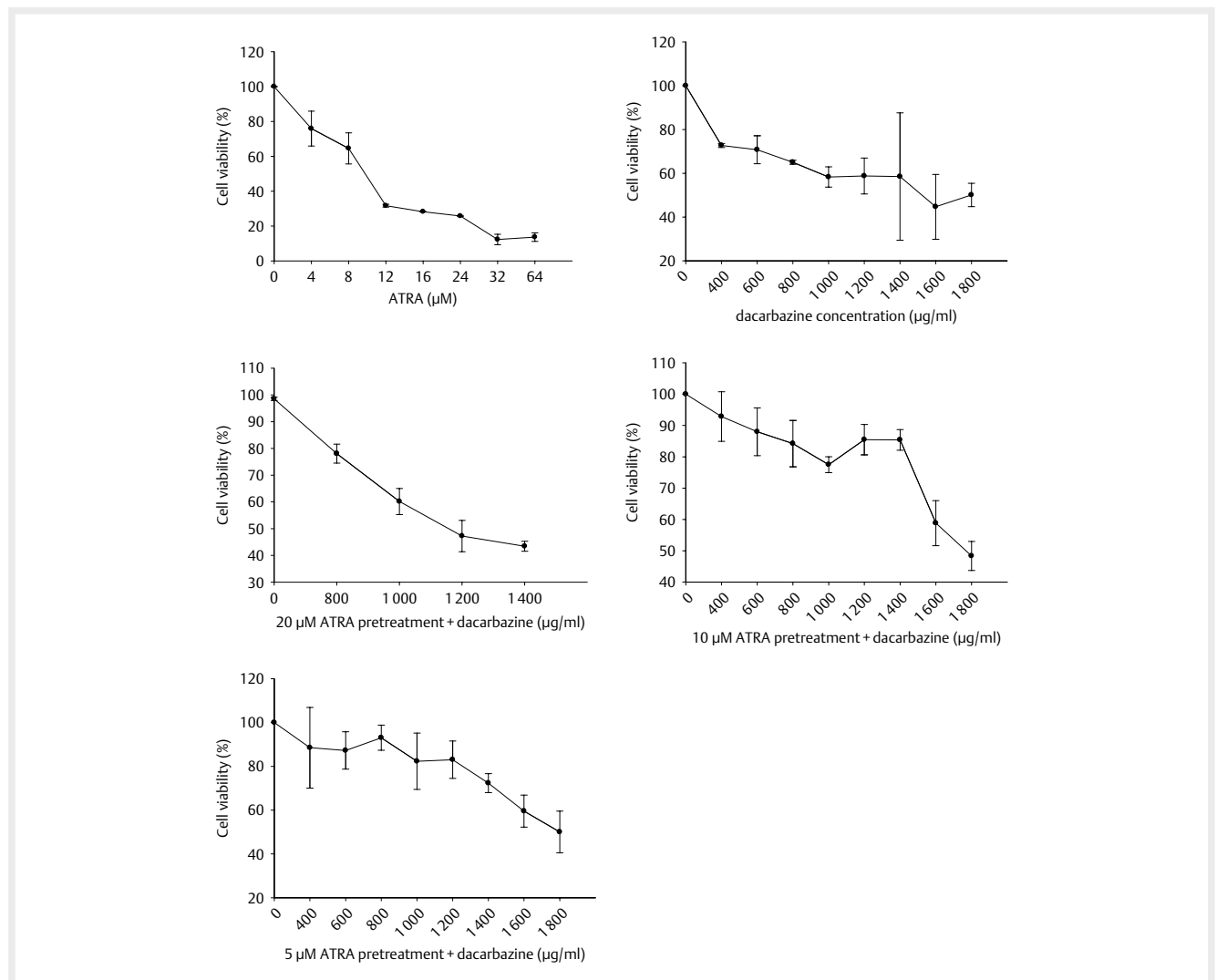
Acridine Orange/Ethidium Bromide (AO/EB) staining

The CD117⁺ melanoma cells were seeded in 12 well plates. We evaluated the cell proliferation following treatment with dacarbazine (800-1400 $\mu\text{g/ml}$), ATRA (10 μM)/dacarbazine (800 – 1400 $\mu\text{g/ml}$), and ATRA (20 μM)/dacarbazine (800 – 1400 $\mu\text{g/ml}$). Following incubation, 100 μl AO/EB staining solution (100 $\mu\text{g/ml}$ AO and 100 $\mu\text{g/ml}$

EB) was added to each well. The morphological changes were evaluated using fluorescent microscopy. Apoptotic cells revealed nuclei containing condensed and fragmented chromatin. Necrotic cells were stained with EB and detected by uniformly orange cell nuclei. Finally, the cells were analyzed by a fluorescence microscope (DP71, Olympus).

Cell cycle assay

Briefly, after the treatment ((i) control, (ii) ATRA, (iii) dacarbazine, and (iv) ATRA/dacarbazine), the CD117⁺ melanoma cells were trypsinized and suspended in PBS and fixed with cold 70 % ethanol. Then, the fixed cells washed with PBS and stained with 4, 6-diamidino-2-phenylindole dihydrochloride solution (DAPI, 1 $\mu\text{g/ml}$, 1 % Triton X-100 in PBS) for 30 min at 37 $^{\circ}\text{C}$ in a dark room. The stained cells were passed through a 30 μm nylon mesh filter. The distribution of cells in cell cycle phases was assessed by flow cytometry (Partec Cyflow space, Germany).

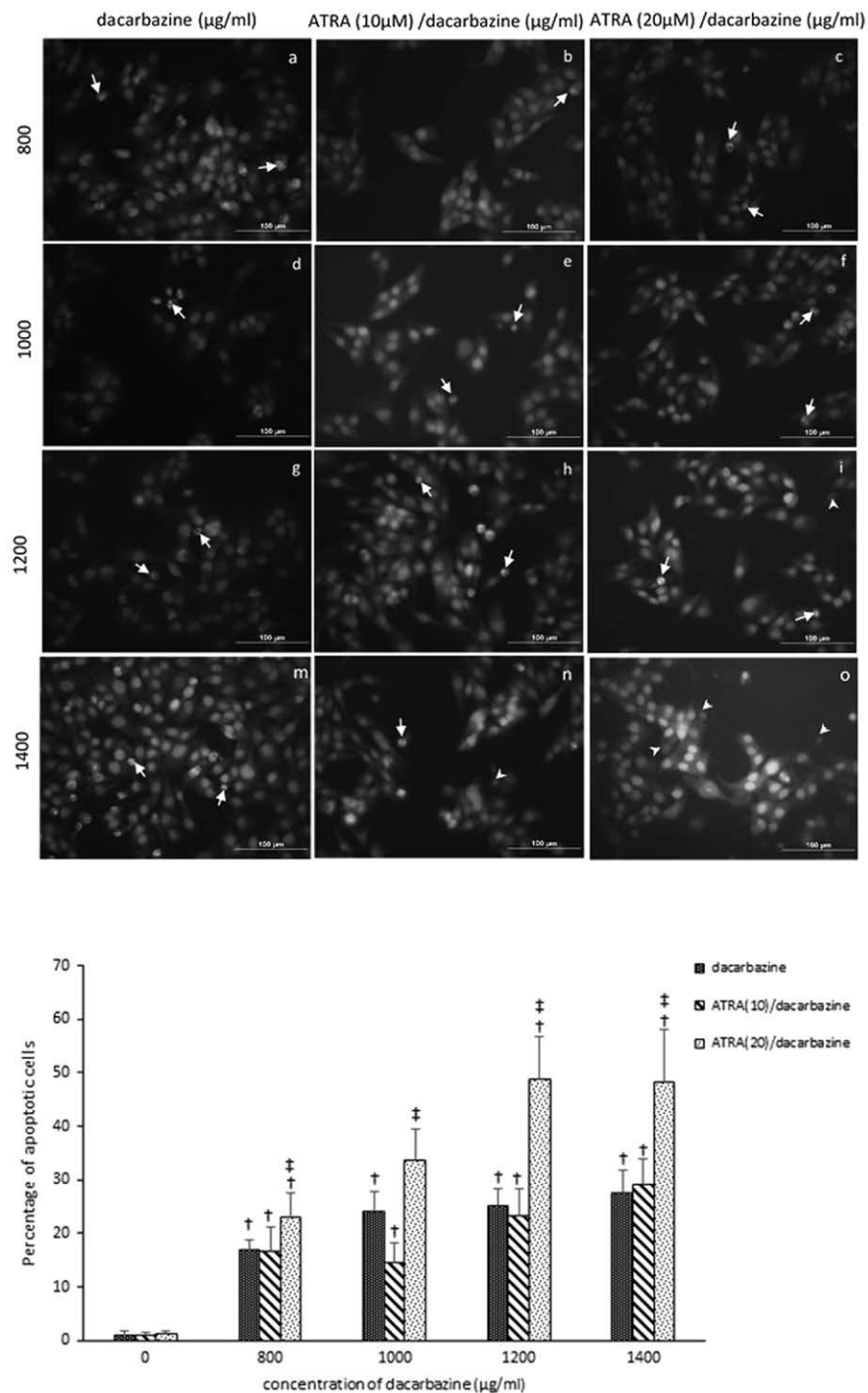


► **Fig. 2** The combination of ATRA and dacarbazine inhibits cell proliferation in the CD117⁺ melanoma cells. Results are as the mean \pm SD (standard deviation) of three independent experiments.

Statistical analysis

The IC_{50} values were calculated by Sigmaplot 12.0 software (Systat Software San Jose, CA). All data were analyzed using SPSS version 21 software (SPSS Inc., USA). The significant differences were

determined using the student's t-test and one-way ANOVA followed by the Tukey post hoc comparison test ($P < 0.05$).



► **Fig. 3** Morphological changes in nuclei of CD117⁺ melanoma cells following treatment with dacarbazine (a, d, g, and m), ATRA (10 μM)/dacarbazine (b, e, h, and n), and ATRA (20 μM)/dacarbazine (c, f, i, and o) evaluated by AO/EB staining. The arrows show apoptotic cells and the arrowheads show necrotic cells. The apoptotic cells significantly increased in a dose-dependent manner after treatment with dacarbazine and ATRA/dacarbazine (p). † $P < 0.05$ compared to control group. ‡ $P < 0.05$ compared to dacarbazine alone.

Results

ATRA enhanced dacarbazine anticancer effect against CD117⁺ melanoma cell line

We isolated CD117⁺ cells from the A375 melanoma cell line using MACS, which was confirmed by immunofluorescence microscopy (► Fig. 1). MTT assay was used to evaluate the cytotoxicity of ATRA, dacarbazine, and ATRA/dacarbazine. These drugs exerted a dose-dependent manner against CD117⁺ melanoma cells following 48 h treatment. According to dose-response curves, dacarbazine had a weak growth-inhibitory effect on the CD117⁺ cells, while ATRA had a more cytotoxic effect. The IC₅₀ values of dacarbazine and ATRA were 1583.09 µg/ml and 8.09 µM, respectively. Furthermore, IC₅₀ values of combination treatment with low concentrations of ATRA (5 and 10 µM) and dacarbazine were not statistically significant compared to dacarbazine alone. Pretreatment with high-dose of ATRA (20 µM) before dacarbazine was more effective than dacarbazine alone. The IC₅₀ values of the combination of ATRA (5 µM and 10 µM) and dacarbazine were 1610.97 ± 210.89 and 1583.34 ± 45.26 µg/ml, respectively. The IC₅₀ value of the combination of 20 µM ATRA and dacarbazine (914.21 ± 51.3 µg/ml.) was 1.7 fold less than dacarbazine alone (► Table 1 and ► Fig. 2).

Morphological changes

The morphological properties of the cells were determined by AO/EB staining (► Fig. 3). The results showed that combination treatment with ATRA and dacarbazine leads to a series of apoptotic morphological changes such as nuclear condensation, apoptotic bodies, and cell shrinkage, while viable cells exhibit green nuclei appearances in the control group. Dacarbazine treatment in all concentrations (800, 1000, 1200, 1400 µg/ml) caused apoptosis in the CD117⁺ melanoma cells. Pretreatment with 10 µM ATRA had not considerable changes in cell death compared to dacarbazine alone. In contrast, more apoptotic cells were seen after incubation of CD117⁺ melanoma cells with a combination of 20 µM ATRA and dacarbazine.

Effects of ATRA, dacarbazine and their combination treatment on the distribution of cell cycle

In the next step, we used DAPI staining and flow cytometry to detect the effect of ATRA, dacarbazine, and their combination treatments on the cell cycle progression. The distribution of the cells within the cell cycle differs between ATRA-, dacarbazine-, and ATRA/dacarbazine- treated cells. The results showed that ATRA significantly increased the percentage of cells at the G₀/G₁ phase, while dacarbazine inhibited the cells at S phase. Furthermore, the

distribution of the cell population significantly increased in the G₀/G₁ phase following treatment with 20 µM ATRA/dacarbazine (► Table 2 and ► Fig. 4).

Discussion

The main chemotherapies for melanoma are dacarbazine, temozolomide, and ipilimumab. Unfortunately, response rates are low and relapse occurs due to chemoresistance in melanoma. Although, there have been made many attempts to find novel medicine that might serve as more effective therapy to inhibit cancer growth, the efficacy is still unsatisfactory. ATRA exhibits anticancer activity through modulating melanoma cell growth and invasion [19]. It is commonly used to treat acute myeloid leukemia [20]. Xu et al. also revealed that ATRA inhibits the growth of squamous cell carcinoma cells by inducing the expression of RARβ [21]. In fact, due to inducing differentiation of cancer stem cells, it is possible that ATRA used in combination with other chemotherapy agents to increase their efficacy. It has been reported that ATRA combinations with other drugs has cytotoxic effects on several cancer cell lines [22, 23]. In the previous study, we showed that ATRA was more effective to inhibit proliferation of CD117⁺ cells than CD44⁺ cells in the A375 melanoma cell line [18]. This study extended prior work by revealing the response of CD117⁺ cells to short-term ATRA pretreatment and dacarbazine. An interesting finding in our study was a significant decrease in IC₅₀ value following treatment with the combination of a high dose of ATRA and dacarbazine. We further demonstrated that a high dose of ATRA and dacarbazine strongly inhibited the CD117⁺ cells at the G₀/G₁ phase. Indeed, the combination treatment significantly increased apoptosis in the CD117⁺ melanoma cells.

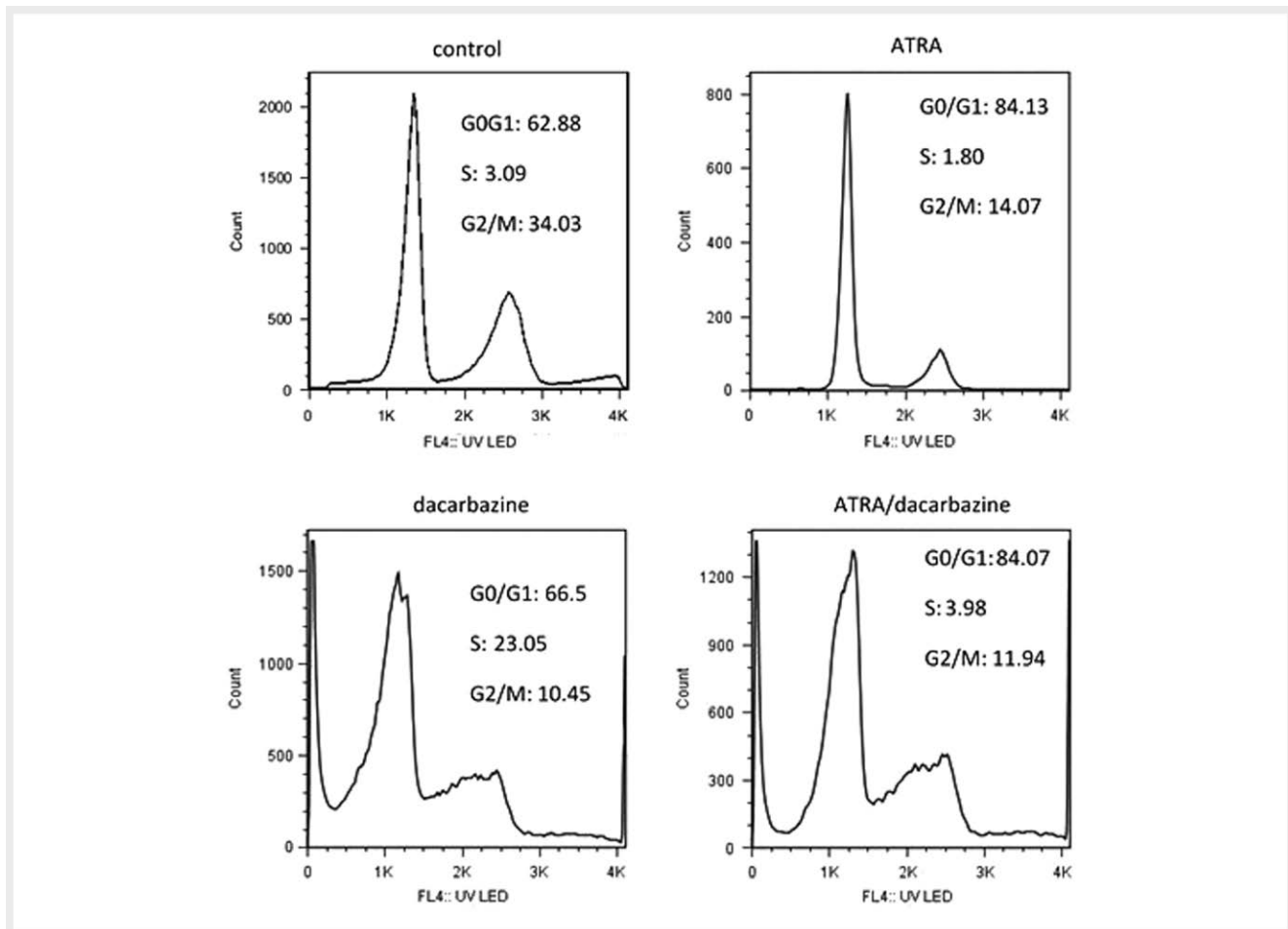
These data are consistent with those obtained from several studies. Recently, Li et al. encapsulated ATRA and dacarbazine in lipid nanoformulations. They showed that the simultaneous delivery of ATRA and dacarbazine resulted in significant inhibition of colony formation and cell proliferation in B16F10 melanoma cells. Similar to our study, they showed that the combination of ATRA and dacarbazine inhibited the melanoma cells at the sub-G₀ phase [24].

In a study, Najafzadeh et al. planned a survey to determine the combination of ATRA as potent anticancer and chemotherapeutic drugs such as cisplatin and 5-fluorouracil to increase the efficacy of the drugs. They pretreated the cancer cells at low dose ATRA for 7 days [16], but in the present study, we pretreated the melanoma cells for a short time (4 h). Our results elucidated that a high dose of ATRA (for 4 h) highly promotes the apoptotic effects of dacarbazine, which may be due to apoptosis and cell cycle arrest. Similarly, Zhang et al.

► **Table 2** The CD117⁺ melanoma cells were treated with ATRA, dacarbazine, and ATRA/dacarbazine. Then, the cell cycle distribution was analyzed by flow cytometry.

	G ₀ /G ₁ %	S %	G ₂ /M %
control	64.755 ± 2.65	3.54 ± 0.63	31.705 ± 3.29
ATRA	82.28 ± 5.6 ^a	2.6 ± 2.6	15.11 ± 3.6
dacarbazine	69.41 ± 4.12	19.29 ± 5.31 ^a	11.29 ± 1.19
ATRA/dacarbazine	84.28 ± 5.6 ^{ab}	3.31 ± 0.95	12.355 ± 0.59

^aP < 0.05 compared to control. ^bP < 0.05 compared to dacarbazine alone.



► **Fig. 4** Effects of ATRA, dacarbazine, and ATRA/dacarbazine treatments on the cell cycle of CD117⁺ melanoma cells. The percentage of cells in each phase of the cell cycle was obtained by flow cytometry analysis.

showed that at high concentrations ATRA causes cytotoxicity against melanoma cells after a long exposure time (3 days) [13]. In another study, Neumann et al. showed that long-term treatment with retinoid can upregulate CD117 expression and increases sensitivity for the imatinib mesylate in neuroblastoma [25]. Lindner et al. proved that the combination of retinoic acid and interferon-beta suppresses the growth of MCF-7 and NIH-OVCAR-3 tumors [26]. Also, in another similar study, Fang et al. (2010) demonstrated that ATRA stimulates cell cycle arrest at the G0/G1 phase through the upregulation of ubiquitin mRNA and increases 20S proteasome activity in the APL cells [27].

Indeed, several studies showed that ATRA alone or in combination with chemotherapeutics could not promote the cytotoxicity against some cancers. Demary et al. reported that the A375 melanoma cell line is resistant to ATRA, lowering intracellular ROS may increase the sensitivity of the cells to ATRA by enhancing RAR activity [28]. Retinoic acid receptors are not usually mutated in many cancers. Nevertheless, several mechanisms reported on the resistance to ATRA. Degradation of retinoids, downregulation of CRABP II, and efflux of retinoids from cells are possible mechanisms that affect the sensitivity of cancer cells to treatment with retinoids [29].

We conclude that the combination treatment of short-term low dose ATRA did not significantly enhance the cytotoxic effects of dacarbazine on melanoma CD117⁺ cells. Furthermore, the combination of ATRA and dacarbazine can inhibit the cell cycle progression at the G0/G1 phase.

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Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest

All authors declare that they have no conflict of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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