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**Research Paper**

# Apoptotic effect and mechanisms of AHPN on human skin malignant melanoma cell A375☆

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## Abstract

Objective: To study apoptotic effects of synthetic retinoic acid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid(AHPN) on human skin malignant melanoma A375 cells in comparison with the natural ligand all-trans-retinoic acid(ATRA) in vitro and the mechanisms related to the actions of AHPN. Methods: MTT assay was used to determine the anti-proliferative effects of AHPN and ATRA on A375 cells. Flow cytometry was performed to investigate the influence of AHPN and ATRA on cell cycle and cell apoptosis. In addition, transfection and luciferase activity assays were employed to explore the mechanisms of how AHPN executes its proapoptotic function. Results: Firstly, AHPN promoted apoptosis and G1 arrest in A375 cells compared with ATRA. Secondly, the activity of NF-κB in A375 cells treated with AHPN increased 2-3 times compared with solvent DMSO treatment. Conclusion: AHPN, in comparison with ATRA, is a more effective alternative for therapy of malignant melanoma. The potentially proapoptotic function of AHPN requires activation of NF-κB.

**Key words**: 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid(AHPN); ATRA; A375 cell line; apoptosis; NF-κB

## INTRODUCTION

Melanoma is the most aggressive skin cancer with poor prognosis. To date, no agents has promoted a clinically meaningful prolongation of overall survival[1-3]. Thus, novel therapy strategies to melanoma is still to be proposed[4]. Retinoids, a class of natural and synthetic vitamin A analogues, have been shown to be effective in some cancer prevention and treatment in vitro, in animal models, and in clinical trial[5-9]. Recently, a novel synthetic retinoic acid(related to retinoid molecules; RRMs), has raised remarkable enthusiasm because it is associated with selective apoptotic activity on a large variety of leukemic and solid tumor cells, including melanoma. These compounds have a stronger apoptotic potential, lower level of toxicity and a better pharmacokinetic profile than natural ligands[6,10-12]. 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN) is the prototype of this novel family and likely to have a mechanism of action that is rather, if not entirely, different from that of classical cytodifferentiating retinoids. This has prompted efforts towards a further evaluation of its clinical potential as a cancer therapeutic agent. Nuclear factor-κB(NF-κB) is a well-known nuclear transcription factor that regulates the expression of a large number of genes involved in apoptosis, viral replication, tumorigenesis, inflammation, and various autoimmune diseases. It has been found to play a dual role in apoptosis depending on the stimuli utilized and the cell type involved[13-16]. Jin F has reported AHPN could increase NF-κB activity in human prostate cancer cells[17]. However, the association between NF-κB activity and apoptosis induced by AHPN in human melanoma cells remain unclear. In this study, we first investigated the apoptosis effect induced by AHPN in melanoma cell line A375. Next we tested the role of NF-κB in AHPN-induced apoptosis in the certain type of cell, A375.

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## MATERIALS AND METHODS

### Reagents and cell culture

AHPN and all-trans retinoic acid(ATRA; Sigma, USA) were dissolved in Dimethyl sulfoxide(DMSO) at a concentration of 10 mmol/L and stored in the dark at -80℃. Stock solution was diluted to the desired final concentrations with growth medium just before use. Human melanoma cell line A375(CCTCC, Wuhan, China) were grown in DMEM supplemented with 10% fetal bovine serum and 100 U of penicillin and 100 μg of streptomycin(Gibco,USA). The cells were cultivated at 37℃ and 5% $CO_2$ in a humid environment.

### Proliferation inhibition assay

Cells were seeded at a cell density of 1 000 cells/well in 96-well plates and allowed to grow for 24 h before treatment. AHPN was added to the medium to a final concentration of 0.1, 1.0, 5.0 μmol/L separately, which referred to our previous study. ATRA was added to a final concentration of 1, 10, 50 μ mol/L. DMSO replacing AHPN or ATRA was added as control. After treatment for 12, 24, 48 h, cell numbers were estimated by MTT assay. Absorbance(*A*) values at 490 nm in the 96-well plates were determined by a Spectra Microplate Reader. Each assay was independently repeated three times.

### Flow cytometric analysis

Cells were seeded in 6-well plates and treated as described above. After 24 h treatment, cells were harvested at 6 h, 12 h, 24 h and 36 h and stained with FITC-labeled Annexin V and propidium iodide(PI; Sigma, USA.) to explore apoptosis on the FACSCalibur flow cytometer using Cell Quest software.

In addition, the cells were harvested, fixed in 70% ethanol for 12 h at 4℃, and stained with PI in a phosphate buffered saline solution containing RNase(Roche, Basel, Switzerland) for the cell cycle analysis using Modfit software(Variety Software House, Topsham, ME, USA).

### Transfection and luciferase assay

Before the day of transfection, A375 cells($2 \times 10^5$) were plated in each well of 24-well plate. 150 ng NF-κB-luciferase reporter plasmid and 100 ng pRL-TK which was used as internal control were transfected into cells using lipofectamine 2 000 according to the commercial protocol. After 6 h of incubation, the transfection mix was replaced with the fresh medium. AHPN and relative solvent DMSO were added to each well to treat cells for 24 h, Then, the firefly and renilla luciferase activities were analyzed by the dual-luciferase detection kit(promega, USA) according to the commercial manual and the relative activation levels of NF-κB were calculated.

### Statistical analysis

Statistical analysis was performed by one-way ANOVA and the Student's *t* test, using statistical software SPSS 10.0. Statistical significance was set at a level of $P < 0.05$.

## RESULTS

### AHPN was effective in inhibiting proliferation and inducing apoptosis in A375 cells

MTT assay showed AHPN had stronger inhibitory effects on proliferation than ATRA in A375 cells. As an example, A375 exhibited a slightly higher degree of proliferation inhibition when treated with AHPN at 5 μmol/L(Fig 1A) than with ATRA at 50 μmol/L(Fig 1B) for 24 h.

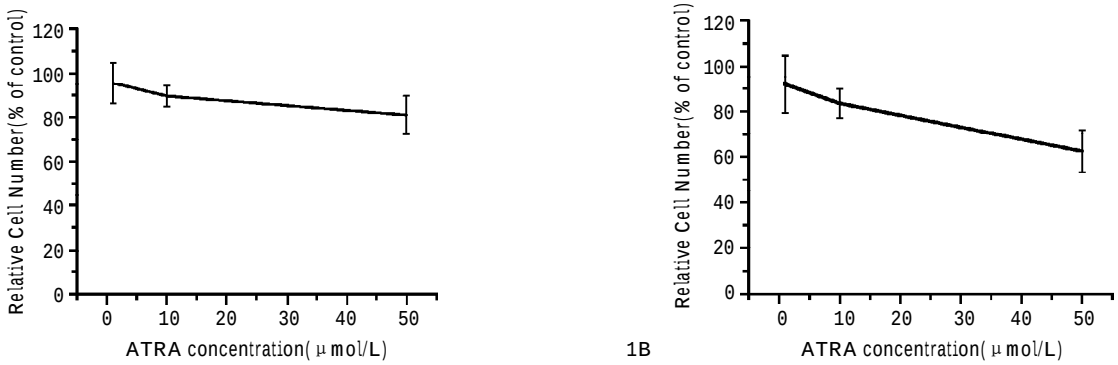


Fig 1 Growth inhibition by AHPN and ATRA in A375 cells

Similarly, apoptosis analysis showed AHPN was far more effective to induce apoptosis than ATRA. 50 μmol/L ATRA was only able to promote apoptotic cells equivalent to approximately 18% of that seen in A375 cells treated with a 10-fold lower concentration of AHPN at 24 h. The percentage of apoptotic cells in the total cell population is indicated in the upper right corner of each panel as the mean of triplicate samples(36.17± 3.14)% vs (6.37 ± 1.12)%; $P < 0.05$; Fig 2).

The appearance of a sub-G1 population, indicating

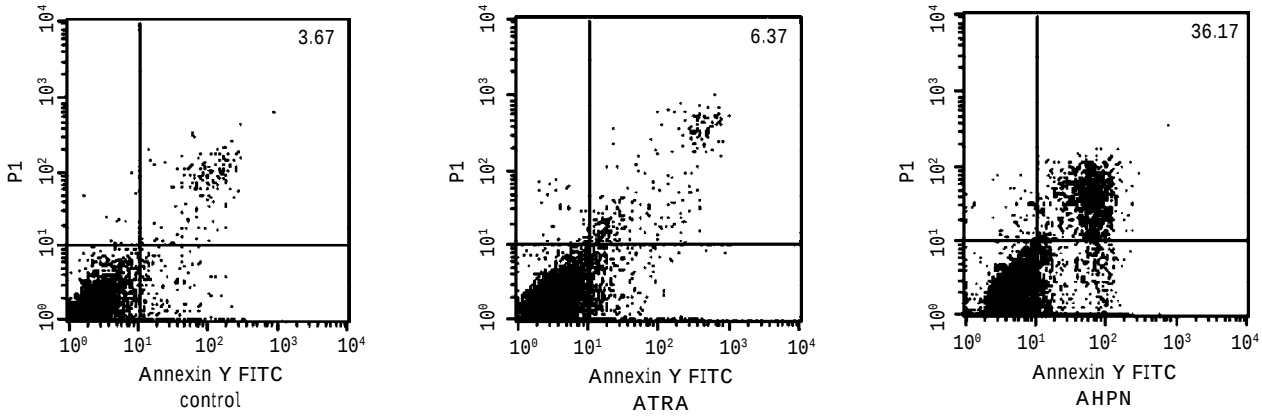


Fig 2 Effects of different treatment with DMSO, 50 μmol/L ATRA and 5 μmol/L AHPN on apoptosis in A375 cells

cells with less than G1 DNA content, could be used as an indicator of apoptosis. Evidence showed the sub-G1 population of AHPN-treated cells was more than that of ATRA-treated cells and DMSO-treated cells. Cells in G0/G1 phase are in the first peak, and cells in G2/M phase are in the second peak. Cells in S phase are in the area between the G0/G1 and G2/M phase peaks. The peak of apoptotic cells which were treated for 24 h is indicated in the lower left corner of each panel(Fig 3).

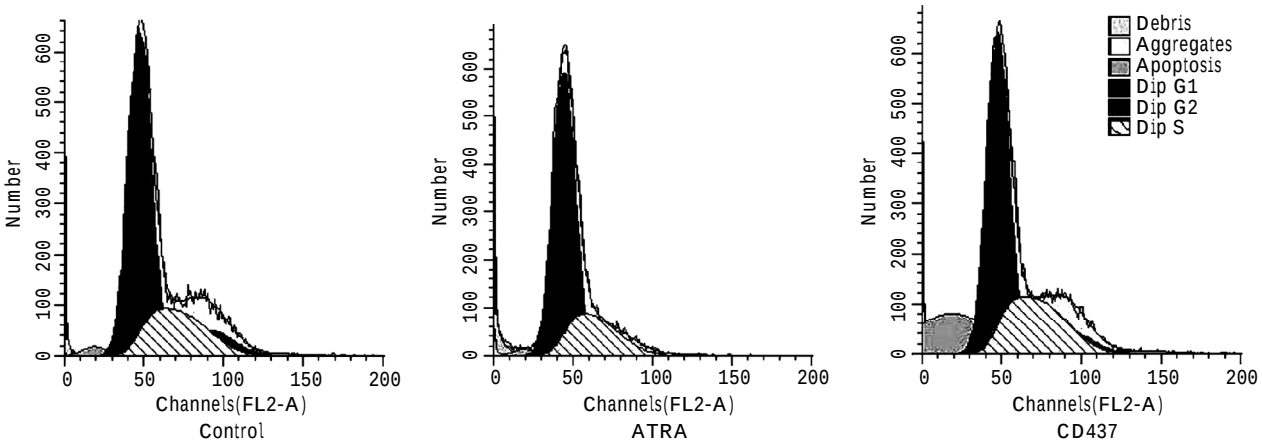


Fig 3 Cell cycle distribution of cells after treatment with DMSO, 50 μmol/L ATRA and 5 μmol/L AHPN

The cell cycle distribution of the ATRA-treated cells did not show specificity to apoptosis induction or to a prominent cell cycle arrest as seen with AHPN treatment in A375 cells. Nonapoptotic S and G2/M cells were present in all A375 cells examined at 24 h following treatment with ATRA(Tab 1). This indicated that ATRA had lower ability to decrease cell number via apoptosis in A375 cells.

Morphological change of A375 cells treated by AHPN was also observed. Results revealed morphological changes(specifically for chromatin condensation and cell shrinkage) were typical of apoptosis. These changes were apparent at 12 h, and became common in approximately 50% or more of the treated A375 cells after 24 h. Exposure to 5 μmol/L AHPN for 24 h caused the majority of A375 cells to shrink, round up, and detach from the culture dish(Fig 4). Instead, A375 cells treated with 50 μmol/L ATRA became flattened and spreaded relative to control cells.

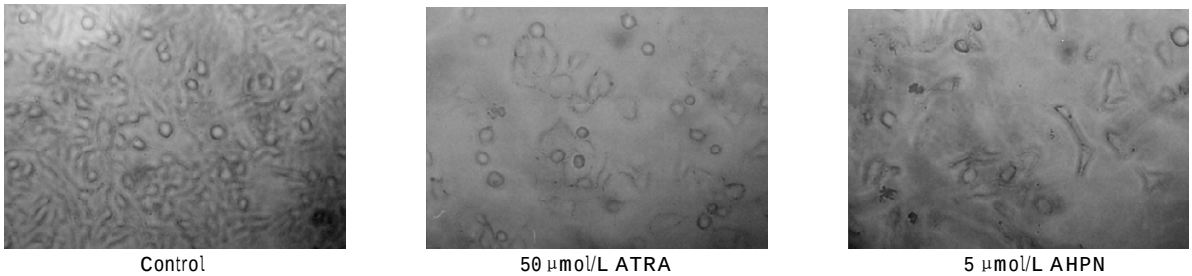


Fig 4 Morphological changes in A375 cells following treatment with DMSO, ATRA and AHPN(inverted microscope, × 40)

### AHPN-induced cell cycle arrest in A375 cells

Continued exposure to AHPN resulted in a time-dependent increase of cell population in the G0/G1 phase in A375 cells while the G0/G1 fraction in DMSO-treated cells was virtually unchanged (66.86% and 75.78% at 12 h and 24 h, respectively; Tab 1). In contrast, ATRA did not markedly change G0/G1 cell population compared to the controls.

Tab 1 Cell cycle distribution of cells after treatment with DMSO, 50 μmol/L ATRA and 5 μmol/L AHPN

| groups | 12 h | | | 24 h | | |
|---|---|---|---|---|---|---|
| | G0/G1 | S | G2/M | G0/G1 | S | G2/M |
| control | 52.43 | 39.07 | 8.49 | 65.56 | 24.08 | 10.36 |
| ATRA | 50.74 | 43.46 | 5.80 | 62.16 | 29.31 | 8.53 |
| AHPN | 66.86 | 28.05 | 5.09 | 75.78 | 24.22 | 0.00 |

### AHPN increased nuclear factor-κB activity

To clarify if NF-κB plays a crucial role in AHPN-induced apoptosis in the certain type of cell A375, we checked the activity of NF-κB in the cells following treatment with AHPN. NF-κB-luciferase plasmid, which was commonly used to detect the activation of NF-κB, and pRL-TK, which was used as an internal control, were transfected to A375 cells 6 h before AHPN treatment(final concentration was 1 μmol/L). The cell lyses were analyzed to obtain the firefly and renilla luciferase values 24 h after AHPN treatment. The results showed the activity of NF-κB in A375 cells treated with AHPN was increased 2-3 times compared to solvent DMSO treatment($P < 0.05$; Fig 5).

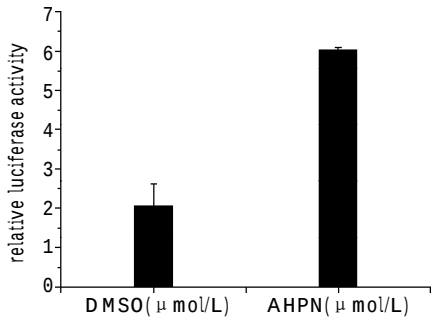


Fig 5 The activity of NF-κB in A375 cells treated with AHPN

## DISCUSSION

Many antineoplastic agents inhibit tumor cell proliferation by promoting apoptosis[18-20]. In this study, data illustrated that the synthetic retinoid AHPN had stronger ability to inhibit proliferation of A375 cells than the natural ligand ATRA(Fig 1). Furthermore, evidence showed that AHPN can efficiently trigger apoptosis in A375 cells(Fig 2). The results supported above findings from different aspect(Fig 3-4, Tab 1). This corresponds to other studies showing the ability of AHPN to promote similar activity in a variety of tumor cell lines and transformed hematopoietic cells in which AHPN reacted apparently in a nuclear receptor-independent fashion[17,21-23]. However, the mechanisms of how AHPN achieve their nuclear receptor-independent fashion remain unclear.

In our present study, two pieces of evidence supported that NF-κB activation had played a critical role in AHPN-mediated apoptosis in A375 cells. Firstly, apoptosis analysis showed that AHPN could effectively induce apoptosis(Fig 2). Secondly, luciferase assay(Fig 5) indicated the activity of NF-κB in A375 cells treated with AHPN was increased 2-3 times when compared with DMSO solvent treatment. That is, NF-κB activation may lead to pro-apoptotic response in the specific cell type A375 and the stimulus AHPN involved. Above results were similar to the observation on human prostate cancer cells[17]. Therefore, we concluded that NF-κB activation might be one of the mechanisms with which AHPN achieve their nuclear receptor-independent fashion.

It is well known that most commonly, NF-κB activation inhibits programmed cell death(PCD), as evidenced by several knockout mouse models[24]. However, it has been shown that under certain circumstances NF-κB activation may promote apoptosis[24-27]. For instance, NF-κB was required for anti-CD3-induced apoptosis of double-positive thymocytes[28]. Apoptosis in HL-60 cells induced by etoposide or 1-β-d-arabinofuranosylcytosine correlated with NF-κB activation[29]. These results were coincident with our observation.

In summary, this study has shown for the first time the effect of apoptosis induction of synthetic retinoid AHPN in human melanoma cell line A375 when compared to ATRA. From this, we demonstrated that apoptosis of A375 cells induced by AHPN is related to NF-κB activation. We look forward to exciting research in this area not only to uncover and understand the intricate signaling networks involved in NF-κB biology, but also to better design therapeutic strategies that could possibly unleash the pro-apoptotic ability of NF-κB in human melanoma cells.

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