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Research Paper

Comparison between synthetic retinoid CD437 and acitretin inhibiting melanoma A375 cell *in vitro*☆

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Abstract

Objective: To investigate the effects of synthetic retinoid CD437 and acitretin on cell proliferation, apoptosis, cycle arrest and Bax/Bcl-2 protein expression of melanoma A375 cell. **Methods:**MTT assay was used to determine the anti-proliferative effects of CD437 and acitretin on melanoma A375 cell. Flow cytometry was performed to investigate the influence of CD437 and acitretin on cell cycle and cell apoptosis. SABC immunocytochemistry was employed for detection of Bax/bcl-2 protein expressions. **Results:**10^{-5} mol/L CD437 was more effective than acitretin in inhibiting proliferation and inducing apoptosis of A375 cell after 24 h treatment, growth inhibiting ratio and apoptosis ratio(58.6%vs43.25% and 28.03%vs17.13%, $P < 0.05$ respectively). CD437 promoted G0/G1 arrest in melanoma A375 cell, however acitretin could not. CD437 and acitretin could up-regulate the expression of Bax protein and down-regulate the expression of bcl-2 protein($P < 0.05$). **Conclusion:**CD437 is more effective than acitretin in inhibiting proliferation and inducing apoptosis and cycle arrest on A375 cell. CD437 may have more potentialities than acitretin for subsidiary treatment of melanoma. Mitochondrial apoptosis pathway is partially involved in two drugs inducing apoptosis on A375 cell.

Key words: CD437; acitretin; A375 cell; apoptosis; Bax/bcl-2 protein

INTRODUCTION

The retinoid could inhibit proliferation on many tumor cells in vitro, including human melanoma S91 cell[1-2]. Our early research indicated that acitretin could induce more significant apoptosis, than all-trans retinoid acid (ATRA) and tazarotene on melanoma A375 cell *in vitro*[3], but there is no report that acitretin had treated melanoma in clinic up until now. A new synthetic retinoid CD437, which is a potential anti-tumor drug, induced apoptosis in many tumor cells in the receptor-dependent pathway and non-receptor-dependent pathway[2], But which one is more effective on melanoma A375 cell between CD437 and acitretin is as yet unknown. This research aims to compare the effects of synthetic retinoid CD437 and acitretin on cell proliferation, apoptosis, cycle arrest and Bax/Bcl-2 protein expression of melanoma A375 cell. Meanwhile, the preliminary mechanism of inducing apoptosis was investigated. We hope that we can offer experimental documents for screening more potential anti-tumor drugs.

MATERIALS AND METHODS

Materials

FBS, DMEM and Trypsin were purchased from GIBCO invitrogen corporation. CD437, PI, MTT, RNase and DMSO were obtained from Sigma-aldrich. AnnexinⅤ-FITC kit was offered by JingMei Biotech. Bax/Bcl-2 SABC immunocytochemistry kit was purchased from ZhongShan Goldenbridge Biotechnology Co., Ltd. Acitretin was presented by Huapont Pharm Co., Ltd.

Cell Lines and Culture Conditions

Human melanoma A375 cells(Chinese Type Culture Collection) were routinely cultured in DMEM, supplemented

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with antibiotics and 10% heat-inactivated FBS at 37℃ in a humidified atmosphere with 5% carbon dioxide.

MTT assay

The cells were seeded at densities ranging from 3×10^3 to 6×10^3 cells per well in 96-well tissue culture plates. After 12 h incubation, cells were treated with different concentrations(1,5,10 μmol/L respectively) of the retinoids (acitretin and CD437). After treating 24 h,48 h and 72 h, OD_{490nm} value was measured by an enzyme-linked immunoabsorbent assay reader(DG3022A).The percentage of growth inhibition was calculated by the equation: % growth inhibition ratio=$(1-A_t/A_c)\times100$. At and Ac represent the absorbance in treated and control cultures, respectively. IC50, the drug concentration causing a 50% cell growth inhibition, was determined by interpolation from the dose-response curve.

Apoptosis and Cycle arrest

The cells were seeded at densities ranging from 0.5×10^5 to 1×10^5 cells in per 25 cm^2 culture flask. After 12 h incubation, the cells were treated with 10 μmol/L acitretin and 10 μmol/L CD437 respectively. After 24 h treatment, they were harvested by trypsinization and washed twice with PBS(selection of drugs concentration and time point according to MTT assay results). The medium and PBS were discarded and the cells were left in centrifuge tubes. On the one hand, apoptosis cells were counted according to AnnexinⅤ-FITC kit instruction. On the other hand, the cells were fixed in ice-cold 70% ethanol for 18 h. After washed twice with PBS,the dispersed cells were stained with PropidumIodide(PI) and cell cycle analysis was performed. At least 10^6 cells were analyzed each time. Flow-cytometric analysis was done with a Becton Dickinson FAC Sort and accompanying CellQuest software.

Bax/Bcl-2 Immunocytochemistry analysis

In order to test the Bax/bcl-2 proteins, the cells were plated into the cover glasses. After the cells treated with acitretin and CD437(concentration 10 μmol/L, time point 24h), Bax/bcl-2 proteins were tested by SABC immunocytochemistry method according to manufacture's protocol. Gray scale values were evaluated by Motic Med 6.0 (A) Digital Medical Image Analysis System.

Statistical analysis

Data were expressed as mean ± SD. Independent-samples T test was used for statistical analysis by SPSS 12.0. $P<0.05$ indicated significant difference.

RESULTS

Growth inhibition

Both CD437 and acitretin could inhibit growth of human melanoma A375 cell in a dose-dependent manner(***Tab 1*** and ***Fig 1***). CD437 was more significant in inhibiting growth than acitretin after 10 μmol/L drugs treated A375 cell for 24h. Growth inhibition ratio was 58.6% vs 43.25%(t_{OD}=4.053,$P<0.05$, $n=6$). Growth inhibition of acitretin on A375 cell achieved its peak after 24h treatment, but growth inhibition of CD437 on human melanoma A375 cell was in a slow ascent stage after 24 h drug intervention(***Fig 1***). $IC50_{acitretin}=13.49\times10^{-6}$mol/L, $IC50_{CD437}=6.23\times10^{-6}$mol/L.

Apoptosis and cycle arrest

Tab 1 **Growth inhibition of drugs on human melanoma A375 cell** (A_{490nm}, $\bar{x}\pm s, n=6$)

Drug	12 h		24 h			48 h			
(10^{-6}mol/L)	1×10^{-6}	5×10^{-6}	10×10^{-6}	1×10^{-6}	5×10^{-6}	10×10^{-6}	1×10^{-6}	5×10^{-6}	10×10^{-6}
Acitretin	0.881 ± 0.018	0.783 ± 0.023*	0.618 ± 0.127*	0.952 ± 0.039	0.821 ± 0.025*	0.554 ± 0.088*	1.027 ± 0.022	0.958 ± 0.051	0.799 ± 0.13*
CD437	0.886 ± 0.053	0.786 ± 0.049*	0.698 ± 0.028*	0.837 ± 0.084*#	0.502 ± 0.065*#	0.404 ± 0.023*#	0.983 ± 0.03#	0.888 ± 0.045*#	0.406 ± 0.019*#
control	–	0.894 ± 0.011	–	–	0.976 ± 0.037	–	–	1.061 ± 0.104	–

*$P<0.05$ *vs* control; #$P<0.05$ *vs* acitretin; A_{490nm}:absorbance in 490nm wavelength

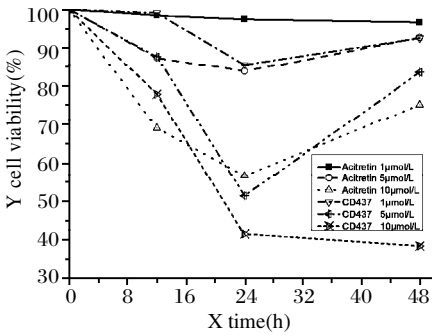

Fig 1 Effects on A375 cell viability by different concentrations of the retinoids(%)

Compared with the control group, both 10 μmol/L CD437 and 10 μmol/L acitretin could significantly induce A375 apoptosis after 24h treatment. CD437 was more significant in inducing apoptosis than acitretin (apoptosis ratio 28.03% *vs* .17.03%,$t=15.38$,$P<0.05$,$n=3$, ***Fig 2***). CD437 obviously promoted G0/G1 phase arrest in A375 cell, however, acitretin could not(***Tab 2***).

Bax/Bcl-2 immunocytochemistry analysis

Compared with the control group, both 10 μmol/L CD437 and 10 μmol/L acitretin can significantly up-regulate the expression of Bax protein and down-regulate

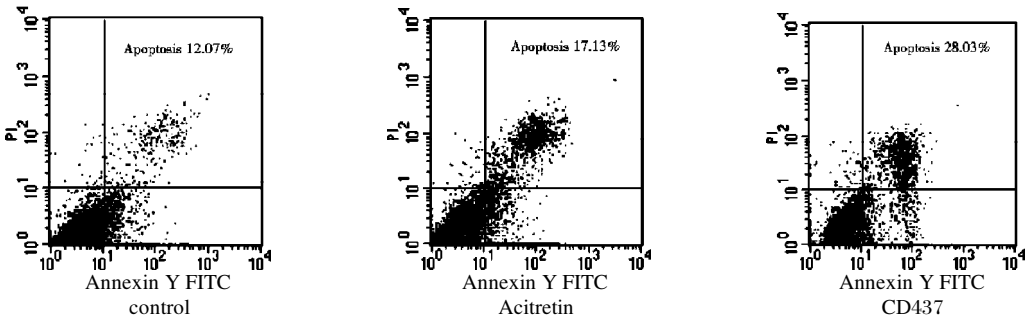

Fig 2 Apoptosis signal map after 10 μmol/L drugs treating A375 cell for 24 h

the expression of bcl-2 protein after 24h treatment. (Bax: $t\text{-}_{cd437}$ *vs.* $t\text{-}_{control}$=5.03, $P < 0.05$, $t\text{-}_{acitretin}$ *vs.* $t\text{-}_{control}$ = 7.113, $P < 0.05$; bcl-2: $t\text{-}_{cd437}$ *vs.* $t\text{-}_{control}$=-5.69, $P < 0.05$, $t\text{-}_{acitretin}$ *vs.* $t\text{-}_{control}$ =-6.63, $P < 0.05$, $n = 6$). But there was no significant difference between CD437 group and acitretin group(***Tab 2, Fig 3***).

Tab 2 **G0/G1 ratio and gray scale values of Bax/bcl-2 protein expression**

group	G0/G1(%, $n = 3$)	Bax(values, $n = 6$)	bcl-2(values, $n = 6$)
Acitretin	62.84 ± 0.61	176.94 ± 2.96*	181.85 ± 4.75*
CD437	77.86 ± 0.31*	180.63 ± 3.17*	177.94 ± 4.35*
control	61.23 ± 1.11	190.45 ± 3.59	157.68 ± 7.56

*$P < 0.05$ *vs.* control

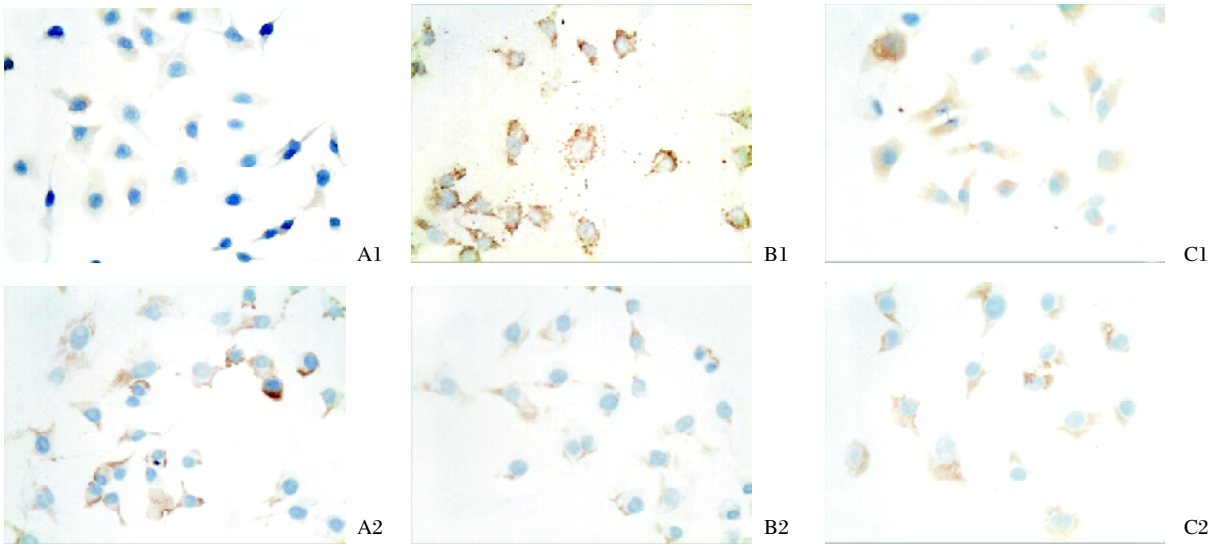

Fig 3 Expression of Bax/bcl-2 protein

DISCUSSION

Acitretin is a second-generation non-selective retinoid receptor agonist, which is mainly employed for the treatments of psoriasis, keratosis dermatosis and some skin tumors. Our early research indicates that acitretin can induce more significant apoptosis than ATRA and tazarotene in human melanoma A375 cell in vitro[3], but there is no report that acitretin has treated melanoma in clinic. Screening more effective anti-melanoma drugs is always the popular in research owing to the characteristics of high malignancy and rapid invasion of melanoma. The synthetic retinoid CD437(6-[3-(adamantly)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid or AHPN) is the prototype of a promising class of cytotoxic compounds, known as adamantly-retinoids or retinoid-related molecules(RRMs), which was originally developed as an RAR-γ-elective agonist[4-6]. This agent has recently been reported to induce apoptosis on several different malignant cells *in vitro*[7]. The essential pharmacophoric elements in the AHPN scaffold necessary for apoptosis induction are the hydrophobic 1-adamantyl group, its adjacent hydrogen-donating hydroxyl group, and a distant carboxylic acid group[8](***Fig 4***) CD437 as a potential alternative anti-cancer drug for clinical application could induce melanoma S91 cell cycle arrest and apoptosis *in vitro* rapidly, CD437 causes DNA adduct-formation, resulting in induction of a p53-independent DNA damage response, and subsequent growth-arrest and apoptosis on melanoma S91cell. p21 is likely to be responsible for early S-phase growth-arrest of CD437-treated cells, whereas ei24 is a critical mediator of CD437-induced apoptosis on S91

cells[1], CD437 also has a significant anti-proliferative effect on human melanoma cell lines WM1341 and MeWo[9]. However, there has been no report about CD437-induced apoptosis on A375 cells and which provides more significant apoptosis on A375 cell between CD437 and acitretin.

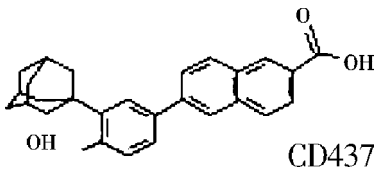

Fig 4 CD437 framework

We compared the effects on inhibiting A375 cell between CD437 and acitretin in vitro and investigated the preliminary mechanism of inducing apoptosis. In this research, both CD437 and acitretin could dose-dependently inhibit A375 cell proliferation. Growth inhibition ratio was 58.6% vs 43.25%. Growth inhibition of acitretin on A375 cell achieved its peak after 24 h treatment. However, growth inhibition of CD437 on A375 cell was in a slow ascent stage after 24h drug intervention. In a word, CD437 inhibited proliferation more effectively than acitretin on the A375 cell. Furthermore, CD437 induced A375 cell apoptosis more significantly than acitretin. Gray scale analysis manifested both CD437 and acitretin could up-regulate Bax protein expression and down-regulate bcl-2 protein expression, which was identical to other researches[2,3,10]. Although gray scale values between, CD437 group and acitretin group showed no significant difference, mitochondrial apoptosis pathway is partially involved in the apoptosis induction of two drugs on A375 cell. It was confirmed that bcl-2 and Bax belonged to anti-apoptosis gene and pre-apoptosis gene respectively. Bax/bcl-2 proteins were the important regulators by stimulating Caspase pathway in mitochondrion mediating apoptosis. CD437 obviously promoted G0/G1 phase arrest and reduced S phase of A375 cell cycle, however acitretin could not. CD437 produced a noticeable effect on A375 cell by inducing apoptosis and cell cycle arrest. However, acitretin had an effect on A375 cell only by inducing apoptosis. This could partially explain why CD437 had more effective growth inhibition than acitretin on melanoma A375 cell *in vitro*.

It was reported that acitretin could activate RAR-α, RAR-β and RAR-γ receptor without binding receptor[11]. How acitretin achieved this effect still needs further research. CD437 could induce apoptosis on many kinds of cancer cell *in vitro*[2]. Surprisingly, when RAR antagonist blocked retinoic acid receptor, CD437 still induced apoptosis on prostate cancer cell, ovarian cancer cell and human lung cancer cell in vitro, which was interpreted that CD437 could induce apoptosis by non-receptor-dependent pathway *in vitro*[12-14]. It was reported that the regulation of differentiation-associated cell-type-specific genes was related to the RARγ -dependent pathway and apoptosis induction relied on non-receptor-dependent pathway[10,15]. CD437 stimulated the mitochondrial apoptosis pathway and activated apoptosis-regulative protein including p53、p21、Bax/bcl-2,PTPC,NF-B, Nur77/TR3 etc[1,10,12,16-20]. How it is possible to activate mitochondrial apoptosis pathway is still under investigation. Recently, there have been some reports that CD437 induced apoptosis in human respiratory epithelial cells via caspase-independent mitochondrial and caspase-8-dependent pathways both up-regulated by JNK signaling pathway[18] and in ovarian adenocarcinoma cells via endoplasmic reticulum(ER) stress signaling[21].

In conclusion, we have demonstrated for the first time that CD437 inhibited proliferation more effectively than acitretin on melanoma A375 cell. In addition, we found that the mitochondrial apoptosis pathway is partially involved in CD437 inducing apoptosis on human melanoma A375 cells. CD437 is a potential alternative antitumor drug for clinical application on melanoma.

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