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Research Article

The Differentiation-Inducing Effect of a Retinoic Acid Derivative on a Pulmonary Carcinoma Cell Line

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Abstract

Pulmonary carcinoma is a main cause of cancer death internationally. Chemotherapeutic regimens in most solid tumors have limited effect. And in the targeted inhibition of carcinogenic driver mutations with molecular therapies, in a large number of cases, patients acquire resistance to drugs. Therefore, the development of new therapeutic approaches is highly desirable. In the present study, we evaluated the applicability of 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid (Am80), a synthetic retinoid, as a differentiation inducer in pulmonary carcinoma and examined its effect on the viability and differentiation of Calu-6 cells (a human non-small cell lung cancer cell line), with the final goal of establishing a new approach to pulmonary carcinoma therapy. The cell viability of Calu-6 cells after Am80 treatment was assessed by the CellTiter-Glo Luminescent Cell Viability Assay. The early apoptosis of cells was detected by Terminal deoxynucleotidyl Transferase-mediated deoxyuridine triphosphate Nick End Labeling (TUNEL) staining. The differentiation-inducing effect of Am80 on Calu-6 cells was investigated by immunostaining. Am80 induced the differentiation of Calu-6 to alveolar type I and II cells. However, Am80 reduced the viability of Calu-6 cells without inducing apoptosis. In addition, in order to investigate whether Am80 inhibits tumor growth *in vivo*, we conducted an *in vivo* experiment to determine the effect of the intratumoral administration of Am80 in mice harboring sc tumors composed of Calu-6 cells. Am80 significantly influenced the suppression of tumor growth.

Keywords: Am80; Cell differentiation; Pulmonary carcinoma; Retinoid

Abbreviations: Am80: 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid;

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ATRA: All-Trans-Retinoic Acid; AQP5: Aquaporin5; CYP: Cytochrome P450 Enzymes; CRABP: Cellular Retinoic Acid-Binding Protein; NSCLC: Non-small Cell Lung Cancer; SP-A: Surfactant Protein-A; RAR: Nuclear Retinoic Acid Receptor

Introduction

Pulmonary carcinoma is a main cause of cancer death internationally [1]. In particular, non-small cell lung cancer (NSCLC) accounts for about 85% of all pulmonary carcinoma cases. Pulmonary carcinomas are categorized into three histological subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [2]. As the prognosis of pulmonary carcinoma is poor, the development of new therapeutic approaches is highly desirable [3]. Pulmonary adenocarcinoma, which accounts for about 40% of all pulmonary carcinomas, is currently the main histological type and its incidence has increased gradually [4].

Chemotherapeutic regimens in most solid tumors have limited effect and are associated with significant toxic effect. In the last decade, the targeted inhibition of carcinogenic driver mutations with molecular therapies, such as epidermal growth factor receptor and the anaplastic lymphoma kinase, has brought spectacular improvements in the overall survival in defined subsets of patients [5]. Unfortunately, in a large number of cases, patients acquire resistance to these drugs due to secondary mutations and other mechanisms [6].

We became aware of a retinoic acid inductor as a cell-growth inhibitor and differentiation inducer in pulmonary adenocarcinoma. Retinoids reduce the growth and induce the differentiation of a variety of types of tumor cells *in vitro* and *in vivo* [7]. A novel synthetic retinoid, 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carbamoyl]benzoic acid (Am80), has been developed and utilized for the treatment of acute promyelocytic leukemia. Am80 offers a higher differentiation-inducing characteristic and is about ten times more powerful than All-*trans* retinoic acid (ATRA) as an *in vitro* differentiation-inducer in the NB-4 acute myelocytic leukemia and HL-60 human leukemia cell lines [8,9]. Furthermore, Am80 is chemically more stable to light, heat, and oxidation and has higher

receptor selectivity than ATRA. Am80 is a synthetic selective agonist for nuclear retinoic acid receptor (RAR)- α , and RAR- β , and does not bind to RAR- γ , which is the main RAR in the dermal epithelium [10]. In regard to metabolic characteristics, Am80 is also stable in the presence of retinoic acid-metabolizing enzyme CYP26A1 and faintly binds to cellular retinoic acid-binding protein (CRABP), which may lead to ATRA resistance [11-13]. The affinity of Am80 against CRABP is approximately one-twentieth that of ATRA. Although Am80 has defined advantages over ATRA, its differentiation-inducing effect on pulmonary carcinoma has not been investigated. We previously demonstrated the antiproliferative effects of Am80 in A549 cells (a human NSCLC cell line); however, we were unable to confirm whether Am80 induced the differentiation of pulmonary adenocarcinoma [14].

In the present study, in order to evaluate the adaptability of Am80 as a differentiation inducer in pulmonary carcinoma, with the final goal of providing a new approach to pulmonary carcinoma therapy, we examined its effect on the viability and differentiation of a human NSCLC cell line, Calu-6. Calu-6 cells have been identified in anaplastic pulmonary carcinoma [15,16].

Materials and Methods

Ethics statement

All of the animal experiments were conducted with the approval of the Tokyo University of Science ethics committee (Approval Number Y15042). The administration of Am80 was stopped when the average tumor volume reached over 600 mm³ or when necrosis was induced in the tumor. The animals were then euthanized and the tumors were enucleated at the next scheduled date of administration to ameliorate the pain and distress associated with tumor formation.

Materials

Am80, a gift from Itsuu Laboratory (Tokyo, Japan), was dissolved in ethanol and stored at -30°C until used in experiments. Goat anti-human CD90 (Thy-1) polyclonal antibody (#sc-6071) [17], goat anti-human

Figure 1: The inhibition of Calu-6 cell growth by Am80.

The CellTiter-Glo assay of Calu-6 cells. (A) Calu-6 cells that were incubated with or without Am80 for 4 days showed the concentration-dependent inhibition of cell growth with Am80. (B) Calu-6 cells that were incubated with or without Am80 for 2 or 4 days showed the time-dependent inhibition of cell growth with Am80. The results are shown as the means $\pm$ SD (n=3). *P<0.05, **P<0.01 vs. control treatment group for each time point. For the plots of the cell survival rate, the mean basal value (day 0) of each group was set at 100%.

aquaporin5 (AQP-5) polyclonal antibody (#sc-9890) [18], and goat anti-human surfactant protein-A (SP-A) polyclonal antibody (#sc-7699) [19] were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Donkey anti-goat IgG-fluorescein isothiocyanate (FITC) (#AP180F) [20] was purchased from Millipore (CA, USA). 4',6-diamidino-2-phenylindole (DAPI) was purchased from Roche Diagnostics (Mannheim, Germany).

Cell lines

Calu-6 cells, an established human pulmonary

carcinoma cell line (#HTB-56), was purchased from the American Type Culture Collection (Rockville, MD, USA).

Cell culture

Calu-6 cells were cultured with Eagle's minimal essential medium (EMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, and 1 mM sodium pyruvate at 37°C in a humidified 5% CO₂ atmosphere.

Cell viability assay

The cellular viability of Calu-6 cells after Am80 treatment was assessed by a CellTiter-Glo Luminescent Cell Viability Assay (Promega KK, Tokyo, Japan), in compliance with the manufacturer's protocol. Cells were seeded (625 cells/cm²) in 100 μ L of medium in 96-well flat-bottom plates and incubated overnight at 37°C. After exposure to Am80 for 2-4 days, the plates were assayed with an EnVision Plate Reader (PerkinElmer Japan Co., Ltd., Kanagawa, Japan). The medium containing Am80 was replaced every 2 days.

Terminal deoxynucleotidyl Transferase-mediated deoxyuridine triphosphate Nick End Labeling (TUNEL) staining

The cells were seeded (625 cells/cm²) and incubated at 37°C. After exposure to Am80 for 4 days, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). The cells were stained using an In situ Apoptosis Detection Kit, in compliance with the manufacturer's protocol (TaKaRa, Japan). The slides were washed with PBS three times and examined under a fluorescence microscope (Keyence, Japan). The apoptosis rate was determined by the number of TUNEL-positive cells/ 200 DAPI-positive cells.

The differentiation-inducing effect of Am80 *in vitro*

Calu-6 cells were seeded in 8-well plates at a density of 625 cells per cm². After 24 h, cells were treated with various concentrations of Am80. Ethanol treated cells were considered as control cells. After 6 days of treatment, the cells were fixed in 4% paraformaldehyde in PBS and stained with primary antibodies CD90, SP-A, AQP-5, each at a dilution of 1:200 [17-19]. After mounting the sections, the nuclei were stained using DAPI and Donkey anti-goat IgG-FITC at a dilution of 1:200 as a secondary antibody [20]. The samples were then observed by fluorescent microscopy (BZ-9000, KEYENCE, Tokyo, Japan). The ratios of CD90-, AQP-5-, and SP-A-positive cells were then calculated based on their respective numbers of positive cells/200 DAPI-positive cells.

In vivo tumorigenesis assay

Five-week-old male congenital athymic BALB/C nude (nu/nu) mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). A total of 6 $\times$ 10⁵ Calu-6 cells were suspended in 100 μ L of PBS (50% matrigel; Becton Dickinson & Co., San Jose, CA) and injected subcutaneously into both the left and right flanks of the nude mice. When the tumors exceeded 50 mm³ in volume, the mice were divided randomly into control and Am80 groups (day 0). The twice weekly intratumoral administration of Am80 (0.2 mg/kg), dissolved in isotonic saline with DMSO at a concentration of 1%, was then initiated (day 0). The dose of Am80 was selected based on previous reports [21,22]. The mice were weighed and the tumor volume was determined using the standard formula $L \times W^2 \times 0.5$, where L is the longest diameter and W is the shortest diameter, as previously described [23].

Statistics

The normally distributed data are shown as the mean $\pm$ standard deviation (SD) or standard error (SE). Student's *t*-test was used for the comparisons of two groups. P-values of <0.01 or <0.05 were considered to indicate statistical significance

Results

In vitro cell-growth inhibition by Am80

Calu-6 cells were treated with different concentrations of Am80 for 2 and 4 days, and the cell viability determined by a CellTiter-Glo assay (Figure 1). Am80-treated cells exhibited reduced cell viability in a dose- and time-dependent manner. The inhibition ratios of 10, 50, 100, and 200 μ M Am80 for 4 days were approximately 5%, 61%, 64%, and 95%, respectively (Figure 1A). Am80 at a concentration greater than 100 μ M reduced the viability of Calu-6 cells at 2 days of treatment (Figure 1B).

The induction of apoptosis in Calu-6 cells

The rate of TUNEL-positive cells in 200 DAPI-positive

Figure 2: The induction of apoptosis in Calu-6 cells.

TUNEL staining in Calu-6 cells. Calu-6 (625 cells/cm²) cells were treated with (B) or without (A) 100 μM Am80 for 4 days. (C) Percent of TUNEL-positive cells in 200 DAPI-positive cells. Results represent the means ±SE (n=3). Scale bar, 200 μm.

cells was $1.7 \pm 1.6\%$ in the control group and $1.6 \pm 1.2\%$ in the Am80 group. In this study, we could not confirm the induction of apoptosis by Am80 by TUNEL staining (Figure 2). This finding is consistent with a previous report which showed that Am80 may not induce apoptosis, or that growth inhibition may not be caused by apoptosis directly [14].

The *in vitro* differentiation-inducing effect of Am80

We investigated whether Am80 had a differentiation-inducing effect on Calu-6 cells, using specific markers (CD90 for anaplastic pulmonary cells, AQP-5 for type I alveolar epithelial cells, and SP-A for type II alveolar epithelial cells). CD90 is a specific marker of human

alveolar epithelial progenitor cells [24], and Calu-6 cells were positively stained for CD90. The culturing of Calu-6 cells with 100 μM Am80 for 6 days resulted in significant increases in the number of AQP-5- and SP-A-positive cells (Figure 3). Taken together, these findings reveal that Am80 induced the differentiation of Calu-6 cells to alveolar type I and II cells.

Am80-induced the growth inhibition of Calu-6 xenografts *in vivo* in nude mice

To evaluate the antitumor effect of Am80 on Calu-6 cells, we conducted an *in vivo* experiment using mice which harbor sc tumors composed of Calu-6 cells. The mean tumor volume was effectively inhibited after treatment with Am80 and the differences between the

Figure 3: Differentiation is induced in Calu-6 cells after treatment with Am80.

(A) Calu-6 cells were stained for CD90, AQP-5, and SP-A. Scale bar, 25 μm. (B) The percentage of positively-stained cells in 200-DAPI positive cells. The results are shown as the mean ± SD (n=4-7). *P<0.05, **P<0.01 vs. the control treatment group, Student's *t*-test.

control and Am80 groups were significant from day 7 (Figure 4A). At the termination of the experiment, all of the mice were euthanized and the tumors were weighed. We also observed that the average weight of the tumors in the Am80 treated group was significantly reduced in comparison to that in the Control group (Figure 4C). We showed the antitumor effects of Am80 by the gross examination of the mice and tumors on day 31 (Figures 4B and D). The results showed that Am80 significantly

influenced the suppression of tumor growth.

Discussion

We previously identified Am80 as an effective inhibitor of cell growth in a pulmonary adenocarcinoma cell line [14]. However, we could not confirm the antiproliferative mechanism of Am80 in the previous study. We therefore investigated the effect of Am80 in Calu-6 cells, an anaplastic pulmonary carcinoma cell

Figure 4: The anti-tumor effect of Am80.

(A) Time after treatment initiation (days). Tumor volume was shown up to the day 28 after administration of Am80. From day 7, the differences between control and Am80 groups were significant. (B) The gross examination of the mice harboring sc tumor. Representative photographs of the mice receiving Control (left) and Am80 (0.2 mg/kg) (right). (C) The average tumor weight at the endpoint of study. (D) The gross examination of enucleated Calu-6-tumors. Representative photographs of the tumors of the Control (left) and Am80 (0.2 mg/kg) (right) groups. The results are shown as the mean $\pm$ SD (n=8-13). *P<0.05, **P<0.01 vs. the control treatment group, Student's *t*-test.

line. We evaluated the effects of Am80 on growth in the pulmonary carcinoma cell line and revealed that Am80 reduced the viability of Calu-6 cells (Figure 1). Am80 also inhibits the cell growth in other cancer cell lines, such as human breast cancer cells and the human glioblastoma cell line [25,26].

Next, as mentioned in our previous report, and in order to investigate the antiproliferative mechanism of Am80, we performed TUNEL staining to detect early cellular apoptosis. The result was that we were unable to confirm the induction of apoptosis by Am80 (Figure 2). In a publication that elucidated the basic biological mechanisms of ATRA resistance in A549 human pulmonary carcinoma cells, ATRA was found to bind to RAR- α and promote phosphoinositide 3-kinase (PI3K)/Akt pathway activation, which is associated with cellular survival and invasion. It was found to suppress the transcription of genes associated with apoptosis, such as RAR- β 2 and p53 by Akt activation [27]. Thus, Am80 may also inhibit growth without inducing apoptosis. Am80 has been shown to stimulate the differentiation of acute promyelocytic leukemia cells into neutrophils and induce neuronal differentiation in a human neuroblastoma NH-12 cell line [28,29]. Based on the results of our investigation into whether Am80 had a differentiation-inducing effect on Calu-6 cells, we suggest that Am80 is a novel inducer of differentiation in anaplastic pulmonary carcinoma Calu-6 cells, which induces their differentiation into alveolar type I and II cells (Figure 3). It is reported that Am80 induces differentiation via the PI3K/AKT and mitogen-activated protein kinase (MAPK) /extracellular signal-regulated kinase (ERK) 1/2 pathways in the NB-4 acute myelocytic leukemia cell line [30]; thus, differentiation may be induced via a similar pathway in anaplastic pulmonary carcinoma. On the other hand, it is reported that the induction of RAR- β expression is significant for the inhibition of tumor cell growth [31]. Furthermore, Am80 also induced differentiation in alveolar epithelial type II progenitor cells and inhibited the expression of Wnt1 and induced neurexin3 (NRXN3), which contribute to differentiation [32,33]. Thus, to elucidate the relationship between those molecular mechanisms and Am80 activity, it is important to understand the antiproliferative and differentiation-inducing effect of Am80.

Am80 inhibits rat collagen arthritis and ameliorates experimental autoimmune myositis [34,35]. The antitumor activity of Am80 against human leukemic monocyte lymphoma cell U937 tumor xenografts has also been demonstrated *in vivo* [36]. That said, there are no reports to indicate that Am80 is effective against the growth of pulmonary carcinoma cells. In the present study, in order to investigate whether Am80 inhibits tumor growth *in vivo*, we conducted experiments in mice harboring sc tumors composed of Calu-6 cells. The mice were treated with Am80 (0.2 mg/kg) and the tumor volume measured twice per week. The results revealed that Am80 treatment reduced the tumor volume and weight (Figure 4). We revealed the antitumor effects of Am80 on Calu-6 tumors; however, the relationship between the mechanism of tumor volume reduction and the differentiation effect *in vitro* were not clearly elucidated. Thus, in the future, further study will be needed to investigate the contribution of the differentiation effect of Am80 to the inhibition of tumor growth, including the molecular mechanisms.

Conclusions

Am80 had a significant influence on the suppression of tumor growth. We revealed that Am80 induced the differentiation of Calu-6 cells and that it can be used as an effective cell-growth inhibitor and differentiation inducer in pulmonary adenocarcinoma.

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Competing Interests

The authors declare no conflict of interest.

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