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Short Communication

Anti-Growth Activity of Tanshinone IIA towards Gefitinib. -Sensitive and -Resistant NonSmall Cell Lung Cancer Cells

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Abstract

Lung cancer is the most common fatal malignancy among all cancers worldwide, and non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers. Existing therapies for NSCLC include tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib, which target the epidermal growth factor receptor (EGFR) tyrosine kinase. In this report, we studied the anti-proliferative activity of tanshinone IIA (Tan IIA) from *Radix Salvia miltiorrhiza* against gefitinib-sensitive and -resistant NSCLC cells. Tan IIA effectively inhibited the growth of both gefitinib-sensitive NSCLC cells harboring the EGFR mutation of exon 19 (PC-9) and gefitinib-resistant NSCLC cells (H1650, H358, AY-01, and A549). We also identified another type of NSCLC cells (PC-14), which were resistant to both TKI and Tan IIA. H1650 and AY-01 harbor an EGFR mutation of exon 19, but are resistant to gefitinib. H358 and A549 harbor a KRAS mutation but no EGFR mutation. Therefore, Tan IIA is a candidate compound for the development of anti-cancer drugs targeting a variety of gefitinib-sensitive and -resistant NSCLC.

ABBREVIATIONS

EGFR: Epidermal Growth Factor Receptor; TKI: Tyrosine Kinase Inhibitor; NSCLC: Non-Small Cell Lung Cancer; NAC: *N*-Acetyl-L- Cysteine; ROS: Reactive Oxygen Species; Tanshinone IIA: Tan IIA

INTRODUCTION

Lung cancer is the most common fatal malignancy among all cancers worldwide, and its incidence has gradually increased. Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancers and affects millions of patients each year worldwide. Existing therapies for NSCLC include tyrosine kinase inhibitors (TKIs), including gefitinib and erlotinib, which target EGFR tyrosine kinase [1, 2].

Nearly 90% of the EGFR mutations detected in NSCLC are small in-frame deletions at exon 19 (Del746-750) or the missense mutation L858R in exon 21, replacing leucine with arginine [3]. The use of TKIs in NSCLC patients harboring these EGFR gene mutations has been associated with dramatic response rates and improved progression-free survival.

Activating alterations in a variety of potential oncogenic driver genes other than EGFR have also been identified in NSCLC [4]. Moreover, acquired resistance to gefitinib and erlotinib occurs inevitably in almost all NSCLC patients [5]. Therefore, the intrinsic and acquired forms of resistance to TKIs remain a persistent complication for targeted therapies of lung cancer.

Compounds extracted from natural sources have been introduced into the chemotherapy of various human cancer cells. Among them, the terpenes represent a treasure house of potential agents for cancer treatment. Tanshinone IIA (Tan IIA), an active diterpenoid in the dried root of *Salvia miltiorrhiza*, shows anti-proliferative activity toward various human cancer cells including NSCLC cells [6, 7]. However, the effect of Tan IIA on gefitinib-resistant NSCLC cells is not clearly known. The results of this present study show that Tan IIA is a promising compound for the development of anti-cancer drugs targeting gefitinib-resistant NSCLC.

MATERIALS AND METHODS

Tan IIA

Salvia miltiorrhiza Bunge was cultivated and collected in

high mountainous Sapa, Laocai, Vietnam. The dry roots of *S. miltiorrhiza* (0.3 kg) were extracted at room temperature for 1 week with methanol (2L × 3 times). The combined methanol extract was exhaustively evaporated under reduced pressure to obtain a residue (35.1 g), which was then suspended in water and successively partitioned with *n*-hexane and dichloromethane. Following removal of the solvents *in vacuo*, *n*-hexane (Hx, 7.3 g) and dichloromethane (CH_2Cl_2, 9.6 g) residues were obtained. The CH_2Cl_2 residue (8.0 g) was chromatographed on a silica gel column with *n*-hexane-ethyl acetate (10:1, 9:1, 8:1, ... 1:1) as the eluent to yield 8 fractions (F.1 - F.8). Fraction 2 was then applied to a Sephadex LH20 column and eluted with methanol to yield 5 sub fractions (F.2.1 - F.2.5). The red-colored fraction (F.2.2) was chromatographed on a silica gel column using *n*-hexane-CH_2Cl_2 (1:1) as the eluting solvent to yield Tan IIA (65.1 mg).

Tan IIA: red amorphous powder; mp 202-204°C; UV (MeOH) λ_{max}: 224, 251, 269, 352, 458 nm; IR (KBr) ν_{max}: 1152, 1280, 1460, 1578, 1668, 2935; ESI-MS *m/z* 295 $[M+H]^+$; ^1H- and ^{13}C-NMR spectra were in accordance with previous reports [8, 9].

Chemical Reagents

AG1478, [4-(3-chloroanilino)-6,7-dimethoxyquinazoline], was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). RPMI1640 was from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). *N*-acetyl-L-cysteine (NAC) was obtained from Calbiochem, Merck, Darmstadt, Germany. Hoechst 3342 Solution was obtained from Dojindo (Kumamoto, Japan). 2',7'-Dichlorodihydrofluorescein diacetate (DCF-DA) was purchased from Invitrogen Molecular Probes (USA).

Cell culture

Human non-small cell lung cancer (NSCLC) cells PC-9 and PC-14 were obtained from Tokyo Medical University (Tokyo, Japan). Gefitinib-resistant NSCLC cells H358 and H1650 were purchased from ATCC. All these cell lines were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C in a fully humidified atmosphere.

Cloning of AG1478-resistant cell line from PC-9 cells

PC-9 cells were seeded at a density of 1.0 x 10^5 cells/60 mm dish and cultured for 2 days. The cells were then exposed to 500 nM AG1478 for 48 h. The surviving cells were suspended in RPMI1640 medium containing 5% FBS and 500 nM AG1478 and plated at 0.5 cell/well into a 96-well microplate. After 48 h of incubation, the culture medium was replaced with AG1478-free growth medium, and incubation was continued to obtain AG1478-resistant cell line, designated as AY-01.

Growth inhibition assay (WST-1 assay)

PC-9, PC-14, H358, H1650 or AY-01 cells were seeded at a density of 2 x 10^3 cells/well into a 96-well microplate and cultured for 48 h. The cells were then treated with various concentrations of Tan IIA or AG1478. After incubation for 2 days, growth inhibition was quantified by a colorimetric assay using the WST-8 reagent according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan).

ROS level assay

Intracellular ROS in Tan IIA-treated cells was measured by using the oxidation sensitive fluorescent prove DCF-DA as described previously [10]. The intensity of the fluorescence was detected by using a Cellomics Array Scan VTI HCS Reader (Thermo Scientific, Finland) at an excitation wavelength of 515 nm and an emission wavelength of 549 nm.

RESULTS AND DISCUSSION

Tan IIA was earlier shown to have anti-proliferative activity toward various human cancer cells including NSCLC cells and colon cancer cells [6,7]. We first tested the cytotoxic activity of Tan IIA towards 4 human colorectal cancer cell lines and 2 human NSCLC cell lines. The compound inhibited the growth of all of the colorectal cancer cell lines (Caco-2, LS180, LoVo, and HT-29), and its concentrations required to inhibit 50% of the cell growth (IC_{50}) were higher than 30 μM (data not shown). On the other hand, the IC_{50} of Tan IIA against NSCLC cell line PC-9 was less than 1 μM; whereas another NSCLC cell line PC-14 was resistant to Tan IIA up to a concentration of 50 μM (Figure 1).

TKIs gefitinib and erlotinib, which competitively block binding of ATP to the tyrosine kinase domain of EGFR, are effective therapeutic agents for NSCLC treatment. PC-9 cells are a gefitnib-sensitive human cell line with an exon 19 deletion (delE746-A750) in their EGFR, and PC-14 cells are a gefitinib-resistant one without EGFR mutations. Therefore, the sensitivity to Tan IIA in PC-9 cells and PC-14 cells may be associated with the responsiveness of both cell lines to TKIs.

To study whether the responsiveness to TKIs and that to Tan IIA are related to each other, we isolated AG1478-resistant cell line AY-01 from PC-9 cells (Figure 2A). AG1478 is a selective inhibitor of EGFR tyrosine kinase, and its quinazoline-containing structure is closely similar to that of both TKIs gefitinib and erlotinib. Next, AY-01 cells were used together with 2 other TKI-resistant NSCLC cell lines, H358 and H1650, to test the anti-growth activity of Tan IIA. H1650 cells express an exon 19 deletion of their EGFR gene and harbor no known EGFR TKI-resistance mechanism other than functional PTEN loss. On the other hand, H358 cells harbor a heterozygous activating KRAS G12C mutation, but no EGFR mutation. As shown in (Figure 2B), all of the TKI-resistant NSCLC cell lines were sensitive to the growth inhibitory action of Tan IIA; although H1650 cells were much more sensitive to Tan IIA than were the other 2 cell lines. Therefore, there was no association between TKI-sensitivity and growth inhibition by Tan IIA.

A secondary mutation in EGFR (T790M) and amplification of MET have been identified as major mechanisms of acquired resistance to TKIs [11]. Mutational activation of KRAS is also associated with primary resistance toward gefitinib [12]. KRAS encodes a small GTP-binding protein involved in many cellular processes including proliferation, differentiation, and apoptosis. Its mutations are detected in more than 25% of lung adenocarcinomas, and activating mutations in EGFR and KRAS seem to be mutually exclusive. Since H358 cells harboring a KRAS mutation were sensitive to the growth inhibitory action of Tan IIA (Figure2B), it is of importance to examine the effect of Tan IIA on A549 lung adenocarcinoma cells, which harbor a homozygous

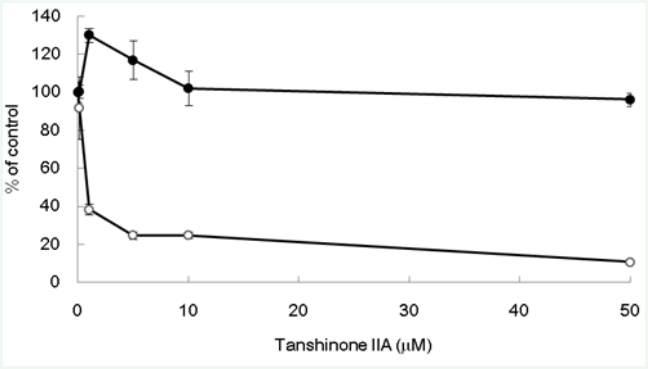

Figure 1 Growth-inhibitory action of Tan IIA against gefitini b-sensitive and -resistant NSCLC cells. PC-9 (○) and PC-14 (●) cells were incubated with the indicated concentrations of Tan IIA. After 2 d of incubation, their growth was estimated by means of the WST-1 assay as described in Materials and methods. The error bar denotes SD (n=4).

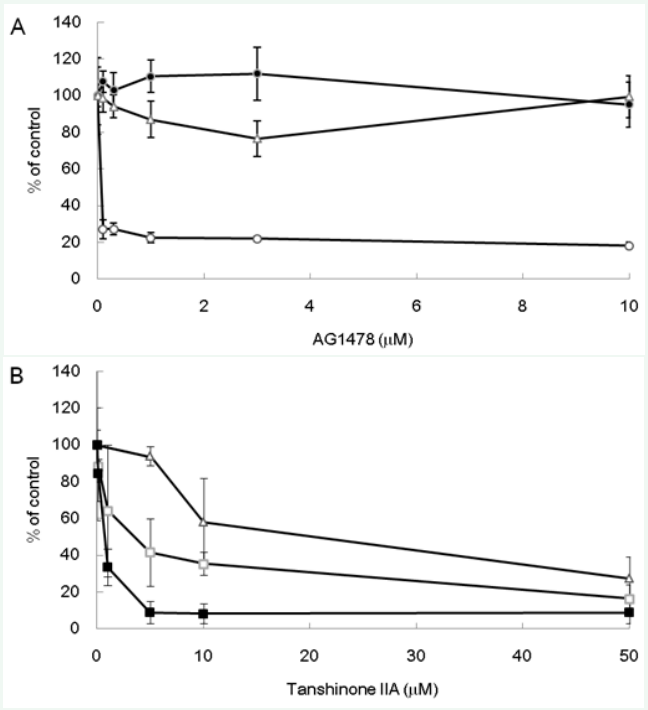

Figure 2 Growth inhibitory effect of Tan IIA on gefitinib-resistant cell lines. (A) PC-9 (○), PC-14 (●), and AY-01 (△) were treated with the indicated concentrations of AG1478. (B) H358 (□), H1650 (■), and AY-01 (△) cells were treated with the indicated concentrations of Tan IIA. After a 2d incubation with AG1478 or Tan IIA, their growth was assayed by means of the WST-1 assay as described in Materials and methods. The error bar denotes SD (n=4).

activating KRAS G12S mutation and wild-type EGFR. Microscopic observation revealed that 10 μM Tan IIA completely inhibited the growth of A549 cells (data not shown). Thus, Tan IIA exerted anti-growth activity against NSCLCs carrying not only EGFR mutations but also KRAS mutations. Although we identified another type of NSCLC cells (PC-14), which were resistant to both TKI and Tan IIA, Tan IIA should be considered as a potential agent for the chemotherapeutic treatment of a variety of gefitinib-sensitive and -resistant NSCLC.

NAD (P)H: quinone oxidoreductase catalyzes the two-electron reduction of a variety of quinone substrate, using both NADH and NADPH as electron donors. Tan IIA is a phenanthrene quinone derivative. So, to explore if the growth of NSCLCs was inhibited by Tan IIA through the generation of reactive oxygen species (ROS), we treated PC-9 and PC-14 cells with Tan IIA. As shown in (Figure 3A), the compound increased the intensity of the fluorescence in PC-9 cells, but not in PC-14 cells. Therefore, it is likely that DCF-DA was oxidized and converted into fluorescent 2', 7'-dichlorofluorescein by ROS in Tan IIA-sensitive PC-9 cells. Next, we used *N*-acetyl cysteine (NAC), ROS scavenger, to counteract the production of ROS and examined its effect on the Tan IIA-induced growth inhibition. Treatment of PC-9 cells with 5 mM NAC abolished the Tan IIA -induced growth inhibition (Figure 3B). Chiu et al. [13] previously demonstrated

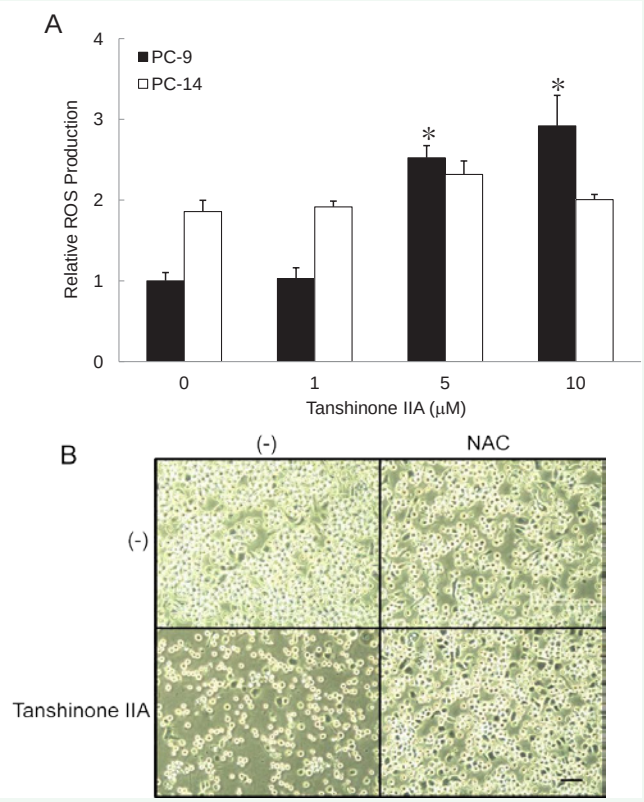

Figure 3 Involvement of ROS in Tan IIA-induced inhibition of cell growth. (A) PC-9 and PC-14 cells were treated with the indicated concentrations of Tan IIA for 24 h. The cells were then incubated with DCF-DA (100 μM) and Hoechst 33342 (200 ng/ml) for 30 min. Relative ROS production is shown as fold change compared to that in Tan IIA untreated-PC-9 cells. *p<0.01 compared with the control (no treatment with Tan IIA). (B)PC-9 cells were treated with 1 mM NAC for 2 h prior to the addition of 10 μM Tan IIA. The phase-contrast photomicrographs were taken after 24-h incubation with Tan IIA. Scale bar, 100 μm.

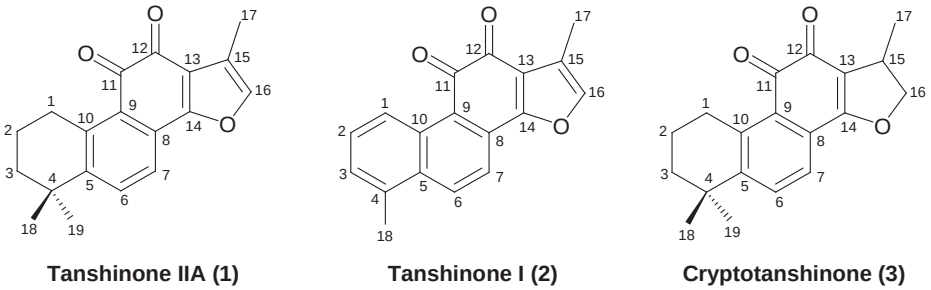

Figure 4 The structures of Tanshinones.

that Tan IIA-induced apoptosis of A549 cells is associated with ROS production. Furthermore, Liu et al. [6] reported that Tan IIA induces cell death of A549 NSCLC cells via a NAD(P)H:quinone oxidoreductase-initiated and ROS-mediated apoptotic pathway. Taken together, all evidence suggests that Tan IIA is likely to exert its inhibitory activity towards a variety of NSCLC cells via increased ROS production.

Celestrol, a triterpene derived from the Chinese herb *Trypterygium wilfordii*, has been reported to induce apoptosis in 3 gefitinib resistant NSCLC cells lines, H1650, H1975, and H2228 [14]. As described above, H1650 has an activating deletion on exon 19 of the EGFR gene, but is resistant to gefitinib. H1975 harbors a secondary mutation (T790M) in EGFR in addition to the L858R activating mutation. On the other hand, H2228 contains wild-type EGFR and EML4-ALK fusion mutation. Therefore, natural compounds with a terpene structure, such as Tan IIA and celestrol, could serve as lead candidates for the development of therapeutic agents against a variety of gefitinib-resistant NSCLC.

In a previous study, we characterized the molecular mechanism by which *ent*-kaurane diterpenoids (eKDs) from *Croton tonkinensis* induce apoptosis of colorectal cancer cell lines, a study in which we examined 10 eKDs with slightly different structures [10].

In addition to Tan IIA, we have also isolated tanshinone I and cryptotanshinone. Since these 3 tanshinones possess slightly different structures one from another (Figure 4), a comparative study of their anti-growth activity could be useful to understand the molecular basis of their action towards NSCLC cells. Especially, it is interesting to compare the effect of these 3 tanshinones on the growth of PC-14 cells, whose driver mutation genes leading to gefitinib resistance are not known.

CONCLUSION

We studied the anti-growth activity of Tan IIA against 6 NSCLC cell lines, which included gefitinib-sensitive PC-9 cells and gefitinib-resistant PC-14, AY-01, H358, H1650, and A549 cells. Growth of all the cells, except PC-14 cells, was inhibited by Tan IIA. Therefore, Tan IIA will be of help to develop new anti-cancer drugs for the treatment of a variety of gefitinib-sensitive and -resistant NSCLC.

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