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A novel synthetic oleanolic acid derivative with amino acid conjugate suppresses tumour growth by inducing cell cycle arrest

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Abstract

Oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid; OA) has a wide variety of bioactivities and is used for medicinal purposes in many Asian countries. Various derivatives of OA have been synthesized in attempts to improve the potency. Here we describe the anti-tumour activity of a novel OA derivative, N-[(3β)-3-(acetyloxy)-28-oxoolean-12-en-28-yl]-glycine methyl ester (AOA-GMe). AOA-GMe was a more potent inhibitor of the growth of B16 melanoma cells than its parent compound OA, both in-vitro and in-vivo. AOA-GMe also exhibited dose-dependent inhibition of human K562 leukaemia cells, but had almost no toxicity in normal human peripheral blood mononuclear cells. AOA-GMe induced cell cycle arrest in G0/G1 and blocked G1–S transition, which correlated well with marked decreases in levels of cyclin D, cyclin-dependent kinase CDK4 and phosphorylated retinoblastoma protein, and increases in the cyclin-dependent kinase inhibitor p15. OA did not show such activities. These results suggest that AOA-GMe may induce growth arrest in tumour cells through regulation of proteins involved in the cell cycle.

Introduction

Oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid; OA), one of the ubiquitous pentacyclic triterpenoids in the plant kingdom, has been reported to have numerous pharmacological activities (Liu 1995, 2005), including anti-inflammatory (Giner-Larza et al 2001), anti-HIV (Zhu et al 2001), anti-osteoporotic (Zhang et al 2005) and anti-complement activities (Assefa et al 1999) and hepatoprotective effects (Jeong 1999). In addition, OA and its derivatives have been shown to have anti-tumour activities such as inhibition of tumour growth in mouse skin (Oguro et al 1998), induction of differentiation (Huang et al 2006) and repression of angiogenesis (Sohn et al 1995). OA has also been found to be beneficial to the recovery of the haematopoietic system after irradiation (Hsu et al 1997). However, the efficacy of OA is relatively poor. To achieve a more potent effect, many researchers have synthesized various derivatives by adding new groups to OA (Suh et al 1999; Honda et al 2000; Ito et al 2000; Lapillonne et al 2003; Hail et al 2004), such as 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO). Cyano and carbonyl groups added to the structure of CDDO may contribute to the remarkable increase in the activity of this compound compared with OA (Honda et al 2000).

Increasing the solubility of a compound often improves its bioavailability. For example, conjugation of an amino acid to betulinic acid has been shown to improve its water solubility as well as its anti-melanoma activity (Jeong et al 1999). These findings prompted us to synthesize a series of novel derivatives of OA with amino acid conjugates (Zhang et al 2005) and to compare their activities.

Dysregulation of the cell cycle machinery is a fundamental hallmark of cancer progression. In recent years there has been growing interest in the crucial roles of proteins that regulate the cell cycle (Shapiro & Harper 1999). The key proteins in the regulation system for specific phase–phase progression are the cyclins and cyclin-dependent kinases (CDKs), whose activities are in turn constrained by CDK inhibitors (CDKIs). Retinoblastoma protein (Rb) is a key switch in the transition from G1 to S phase. During cell cycle progression,

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Rb phosphorylation is regulated by cyclin D–CDK4/6 complexes, as well as by CDKIs (such as p15, p16, p21, p27). Over-expression of CDKs and cyclin D, or loss of CDKIs may give tumour cells the potential for more aggressive growth (Shapiro & Harper 1999). These findings suggest that abnormalities in the proteins that regulate the cell cycle play important roles in carcinogenesis and may be targets for anti-tumour agents.

We have examined the anti-tumour activities of a series of OA derivatives. Among them, N-[(3β)-3-(acetyloxy)-28-oxoolean-12-en-28-yl]-glycine methyl ester (AOA-GMe) had the strongest anti-tumour activity. We also compared the anti-neoplastic potency of AOA-GMe with that of its parent compound OA in-vitro and in-vivo, and investigated its effect on the cell cycle.

Materials and Methods

Materials and reagents

AOA-GMe and its analogues were synthesized by Dr Jianxin Li (Nanjing University, Nanjing, China). OA was a kind gift from Dr Yihua Zhang (China Pharmaceutical University, Nanjing, China). The structures of OA, AOA-GMe and their analogues are given in Figure 1. Stock solutions in DMSO were stored at −20°C and freshly diluted to the desired concentration for use in in-vitro assays. Appropriate amounts of DMSO were used as controls. Doxorubicin was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd (Taizhou, China). All compounds used for in-vivo assays were dissolved in phosphate-buffered saline (PBS) containing 2% Tween 80. The primary antibody against α-tubulin (sc-8035) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against cyclin D (#2926), CDK4/6 (#2906 and #3136), p15 (#4822), Rb phosphorylated at Ser 807/811 (#9308) and the secondary antibodies of anti-mouse and rabbit IgG horseradish peroxidase (HRP) conjugate (#7076 and #7074) were purchased from Cell Signaling Technology (Beverly, MA, USA).

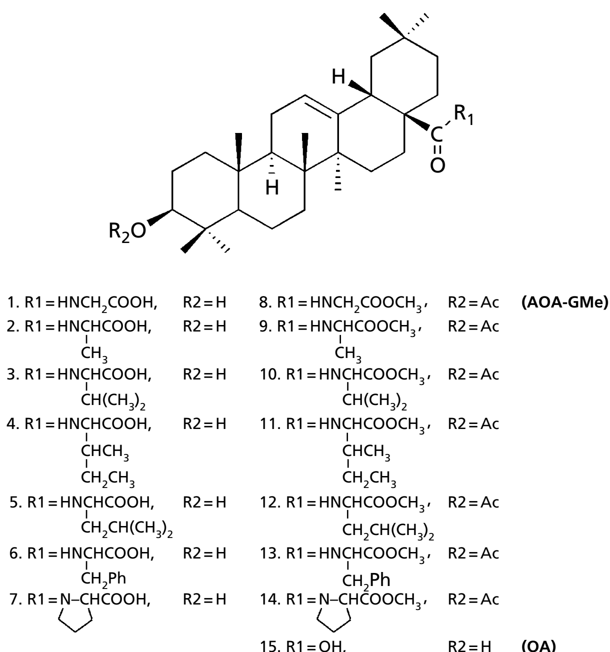

Figure 1 Chemical structures of OA and its derivatives.

Cell culture

Murine B16 melanoma and human chronic myelogenous K562 leukaemia cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Grand Island, NY, USA) and Roswell Park Memorial Institute medium (RPMI 1640; Gibco, Scotland, UK), respectively, supplemented with penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}) and 10% fetal bovine serum (FBS; Life Technologies) at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized whole blood donated by healthy volunteers who gave informed consent. Separation of blood cells was performed using density gradient centrifugation (LTS1077, Hao Yang Biological Manufacture Co. Ltd, Tianjin, China). The buffy coat layer containing PBMC at the interface was carefully removed, washed twice with PBS and centrifuged at 1000 rpm for 5 min. The cells were suspended in RPMI 1640 supplemented with 10% FBS and antibiotics (as above), and incubated in the presence of 5% CO_2 in air at 37°C.

Animals

Female C57BL/6 mice (7–8 weeks old) were obtained from Shanghai Laboratory Animal Center (Shanghai, China) and were maintained with free access to pellet food and water at 21 ± 2°C in a 12-h light–dark cycle. Animal welfare and experimental procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council of USA) and the related ethical regulations of the Ministry of Science and Technology of China (2006). All efforts were made to minimize suffering and the number of animals used. This study was approved ethically by our university.

Measurement of cell viability by MTT assay

Tumour cells were cultured in 96-well plates at a density of 1×10^4 cells mL^{-1} in medium for 72 h in the presence or absence of AOA-GMe or OA. Isolated PBMC were plated at a density of 3×10^6 cells mL^{-1}, with or without 5 μg mL^{-1} phytohaemagglutinin (PHA; Sigma, St Louis, MO, USA) to stimulate proliferation, and incubated with or without AOA-GMe for 72 h. Cell viability was evaluated using a modified 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sargent & Taylor 1989). Briefly, MTT (20 μL 4 mg mL^{-1} in medium; Sigma) was added to cells, which were then incubated for a further 4 h. After removing the supernatant, 200 μL DMSO was added to dissolve the formazan crystals. The absorbance was read on an

ELISA reader (Tecan, Austria) at 540 nm. The relative survival rate was determined in relation to that of untreated cells, which was set as 100%. Data were analysed using SigmaPlot 10.0 software (Jandel Scientific, San Rafael, CA, USA).

Cell count using trypan blue dye exclusion

Starting inoculums of 2×10^4 B16 cells per 2 mL were added to each well of 24-well plates in triplicate and were incubated in the presence or absence of AOA-GMe or OA (0, 1, 5, 10 and 50×10^{-6} M). Cells were then harvested on the days noted in the figure legends. After trypsinization, viable cells that excluded trypan blue were counted.

B16 tumour growth in mice

Mice were shaved before receiving s.c. injection of B16 cells (3×10^5 in 100 μl PBS) into the flank. After injection, mice were randomly divided into five groups (n = 12) treated with AOA-GMe (5 and 10 mg kg^{-1}), OA (10 mg kg^{-1}) or doxorubicin (10 mg kg^{-1}) or the vehicle (2% Tween 80 in PBS), injected i.p. in a volume of 10 mL kg^{-1} every 2 days for 20 days from 24 h after tumour inoculation. Tumour diameter was measured with vernier calipers every 2 days and tumour volume was calculated using the formula: $volume = 0.5\times L_1\times(L_2)^2$, where L_1 is the long axis and L_2 is the short axis of the tumour. Animal survival was monitored daily.

Cell cycle analysis by flow cytometry

After treatment with OA, AOA-GMe or vehicle control for 24 h or 48 h in 12-well plates, cells were harvested, washed with PBS and fixed in 75% ethanol at 4 °C for 2 h. They were then stained in PBS containing propidium iodide (PI; 50 μg mL^{-1}; Sigma), RNase A (300 μg mL^{-1}) and 1% Triton X-100 at room temperature for 30 min. Cell cycle phase distribution was analysed by cell cytometry using a FACScalibur (Becton-Dickinson, San Jose, CA, USA).

Western blot analysis

Western blot assay was performed as described previously (Zhang et al 2000). Briefly, after treatment with 0, 5, 10 or 50×10^{-6} M AOA-GMe or OA, cells were collected, lysed in lysis buffer and centrifuged at 12 000 *g* for 5 min at 4°C. The supernatant was collected and 30–50 μg protein was electrophoresed on a 7.5–10% SDS-PAGE, followed by immunoblotting onto polyvinylidene difluoride membrane. The membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature. Proteins were visualized using the enhanced chemiluminescence detection system (Cell Signaling Technology, Beverly, MA, USA) after incubation with primary antibodies overnight and secondary antibodies for 1 h. The primary antibodies included anti-α-tubulin, cyclin D, CDK4/6, p15 and p-Rb (Ser 807/811).

Statistical analysis

Data were expressed as mean ± s.d. One-way analysis of variance for multiple comparisons was used to determine differences between the different treatments. Once significant differences had been detected ($P < 0.05$), Student's *t*-test was used to evaluate the differences between two groups.

Results

Effect of OA derivatives

B16 cells were treated with OA and a series of OA derivatives (all at 1×10^{-5} M for 72 h). As shown in Table 1, compounds 6, 8, 9 and 11–14 had significant inhibitory effect against B16 tumour cells; compounds 1–5, 7, and 10 displayed a tendency towards inhibition. AOA-GMe had the greatest efficacy.

Effect of AOA-GMe and OA on cell growth

To compare the effect of AOA-GMe with that of its parent compound OA, tumour cells were exposed to different concentrations of the compounds for 24, 48 and 72 h. Both AOA-GMe and OA inhibited the viability of B16 cells in a concentration- and time-dependent manner (Figure 2). AOA-GMe had an IC50 of 5.68×10^{-6} M against B16 cells (assessed by MTT assay), which is lower than the IC50 of OA (1.24×10^{-4} M (Figure 2A). We also counted cells using the trypan blue exclusion method. AOA-GMe showed significant inhibition at concentrations of 5×10^{-6} M and above (Figure 2B) whereas OA inhibited cell growth only at the highest concentration of 5×10^{-5} M (Figure 2C).

Table 1 Comparison of anti-tumour activities of oleanolic acid (OA) derivatives in B16 cells. Cells were treated with OA and a series of derivatives (1×10^{-5} M for 72 h) and the survival rate was determined by MTT assay. Chemical structures of OA and the derivatives 1–14 are shown in Figure 1

Compound	Cell viability (% of control)
Vehicle control (DMSO)	100
1	87.2 ± 3.1
2	82.6 ± 6.1
3	80.9 ± 5.2
4	81.0 ± 7.6
5	81.4 ± 6.8
6	76.4 ± 5.4**
7	84.6 ± 3.3
8 (AOA-GMe)	39.6 ± 2.5**
9	67.8 ± 0.4**
10	80.5 ± 1.5
11	77.5 ± 0.3**
12	73.0 ± 1.1**
13	57.8 ± 1.0**
14	72.3 ± 3.4**
15 (OA)	99.7 ± 2.8

Data are mean ± s.d. of three experiments, each performed in triplicate wells. **$P < 0.01$ vs vehicle control.

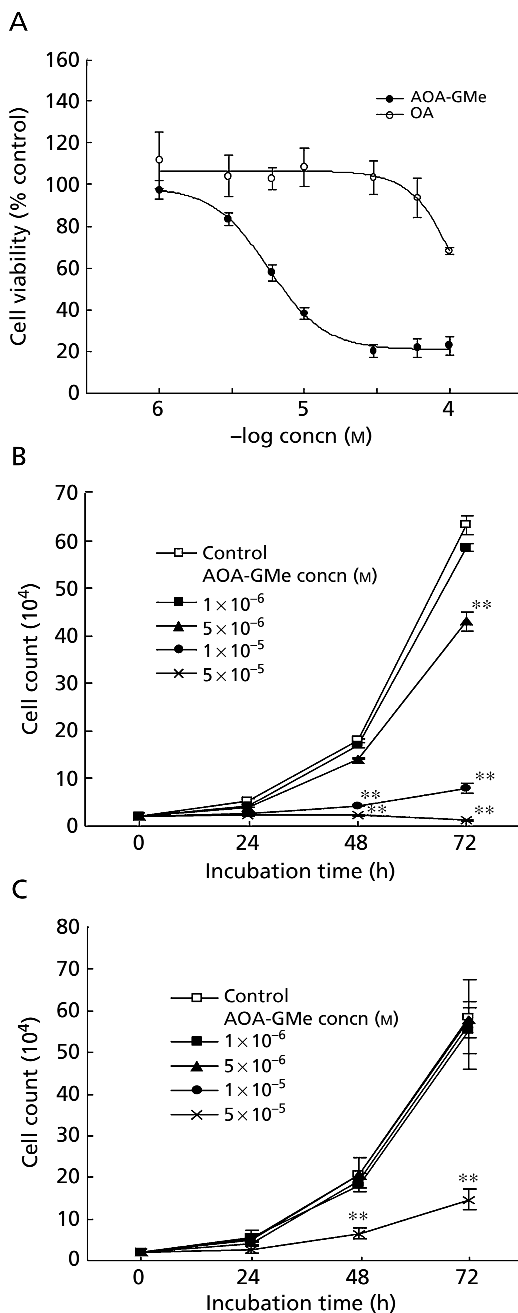

Figure 2 Inhibitory effect of AOA-GMe and OA in B16 cells in-vitro. A. Survival of B16 cells determined by MTT assay. Cells were exposed to AOA-GMe or OA for 72 h. The relative survival rate was determined in relation to that of untreated control cells, which was set as 100%. Data are mean ± s.d. of three triplicate experiments. B and C. Growth curves of B16 cells. Cells were incubated with different concentrations of AOA-GMe or OA for 24, 48 and 72 h, harvested, stained with trypan blue and counted. $^{**}P < 0.01$ vs vehicle control.

Similar results were obtained in K562 leukaemia cells: AOA-GMe was a more potent inhibitor than OA (IC50 7.31×10^{-6} M and 5.55×10^{-5} M respectively; Figure 3).

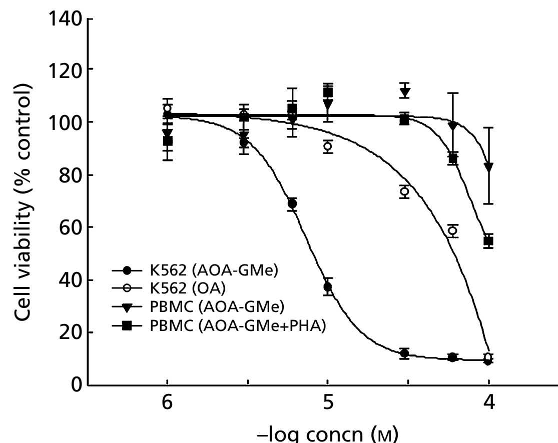

Figure 3 Effect of AOA-GMe and OA on the viability of K562 leukaemia cells and normal human peripheral blood mononuclear cells (PBMC). K562 cells and human PBMC were exposed to AOA-GMe or OA for 72 h, and cell viability was determined by MTT assay. In the case of normal human PBMC, the incubation was performed in the presence or absence of phytohaemagglutinin (5 μg mL^{-1}), and the relative survival rate was determined in relation to that of AOA-GMe-untreated control cells, which was set as 100%. Data are the mean ± s.d. of three experiments, each performed in triplicate wells.

We also examined the effects of OA and AOA-GMe on normal human PBMC, unstimulated or stimulated with PHA. In both cases PBMC were almost insensitive to AOA-GMe, except for slight inhibition at the highest concentration of 1×10^{-4} M (Figure 3).

Suppression of tumour growth in mice

AOA-GMe significantly inhibited tumour growth in a dose-dependent manner and prolonged the life span of mice bearing B16-cell tumours (Figure 4). By contrast, OA showed only a slight inhibition of tumour growth. AOA-GMe did not induce weight loss in the mice, unlike doxorubicin.

Cell cycle arrest

AOA-GMe induced G0/G1 cell cycle arrest in a dose-dependent manner (Figure 5). At 24 h after exposure to AOA-GMe at $5–50\times10^{-6}$ M, the proportions of cells in G0/G1 phase increased from 65.28% to 78.81%, while those in S phase and G2/M phase declined from 19.42% to 12.24% and from 11.91% to 9.24%, respectively. The changes at 48 h were similar to those at 24 h (data not shown). OA did not cause cell cycle arrest, except for a slight tendency at 5×10^{-5} M (Figure 5).

Expression of cell cycle regulatory proteins

Cells were exposed to AOA-GMe or OA (0, 5, 10 and 50×10^{-6} M for 24 h). AOA-GMe markedly reduced the expression of cyclin D, CDK4 and phosphorylated Rb, and

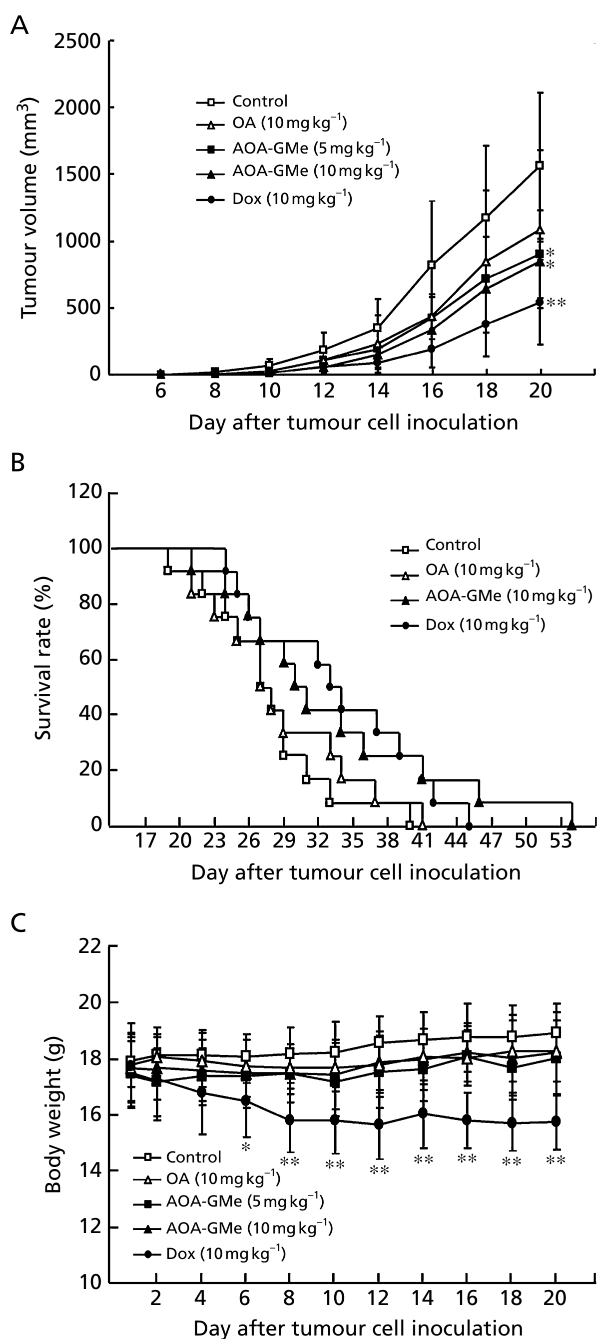

Figure 4 Anti-tumour activities of AOA-GMe and OA against B16 cells grown as tumours in mice: changes in tumour volume (A), survival (B) and changes in body weights (C) in mice inoculated with B16 cells. B16 cells (3×10^6 cells mL^{-1} in 100 μL phosphate-buffered saline (PBS)) were injected subcutaneously into flanks of C57BL/6 mice. Mice were treated with AOA-GMe, OA, doxorubicin (Dox) or vehicle (2% Tween 80 in PBS) injected i.p. every 2 days for 20 days, starting 24 h after tumour inoculation. Data are mean ± s.d of 12 mice in each group. *$P < 0.05$ and **$P < 0.01$ compared with vehicle control.

increased the amount of p15, but did not affect CDK6. OA had no effect on these cell cycle regulators except for a slight decrease in cyclin D at 5×10^{-5} M (Figure 6).

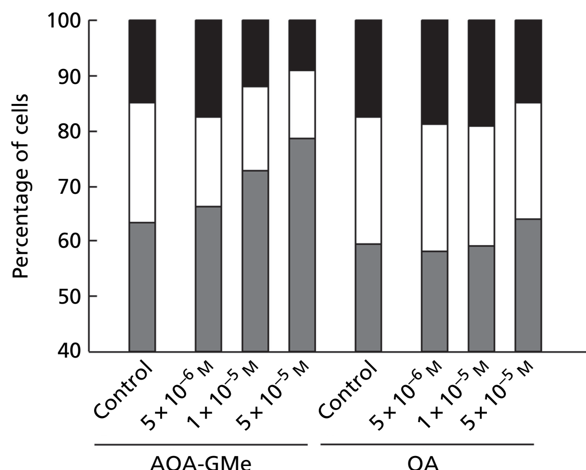

Figure 5 Effect of AOA-GMe and OA on cell cycle distribution in B16 cells. Cells were incubated with various concentrations of AOA-GMe or OA for 24 h, harvested and stained with propidium iodide. Cell cycle stages were determined by flow cytometry. Grey bars correspond to cells at G0/G1, white bars to S phase and black bars to G2/M.

Discussion

We have studied the anti-tumour activities of a series of novel OA derivatives. Compounds 1–7, in which amino acids were introduced, inhibited growth of B16 cells; their derivatives with the corresponding amino acid methyl ester and acetoxy groups (compounds 8–14) were more potent in this respect. Among them, AOA-GMe had the highest efficacy (Table 1). Glycin methyl ester and acetoxy groups jointly led to an increased activity. These findings suggest that the increased anti-B16 tumour activity of these OA derivatives may result not only from increased water solubility but also from optimization of hydrophilic–lipophilic balance, which is potentially important for anti-tumour activity (McKeage et al 2002; Seiler 2005).

AOA-GMe had quite different properties to its parent compound OA. AOA-GMe was a potent inhibitor of the proliferation of B16 cells in-vitro in a concentration- and time-dependent manner, showing a much higher activity than OA (Figure 2). This activity was consistent with the inhibitory effect of AOA-GMe against tumour growth in mice, where it significantly reduced tumour size and prolonged the life span of tumour-bearing mice, without inducing significant weight loss (Figure 4). AOA-GMe was also a more potent inhibitor than OA of the proliferation of K562 cells, suggesting that AOA-GMe has this activity against various kinds of tumour cells. Furthermore, AOA-GMe exhibited almost no toxicity in normal human PBMC, except for a slight inhibition at a high concentration (1×10^{-4} M; Figure 3). These findings together suggest that AOA-GMe may have some selectivity for tumour cells over normal cells, which may be beneficial in the treatment of cancer.

To investigate the mechanism underlying the anti-proliferation effect of AOA-GMe on tumour cells, we focused on the

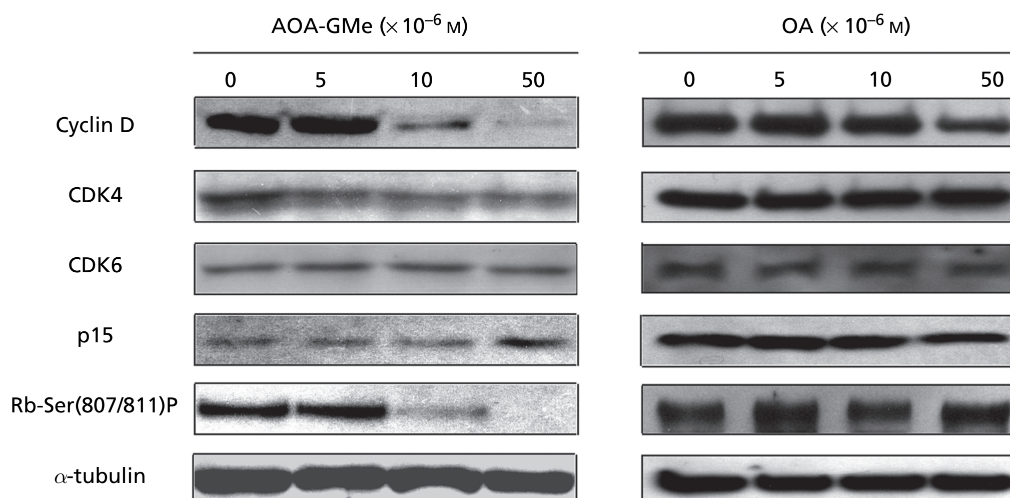

Figure 6 Changes in the expression of cell cycle regulatory proteins induced by AOA-GMe and OA. Lysates were prepared from the cells after treatment with 0, 5, 10 and 50×10^{-6} M AOA-GMe or OA for 24 h. Cells were then subjected to immunoblot analysis with anti-cyclin D, anti-CDK4/6, anti-p15 and anti-Rb phosphorylated at Ser 807/811 antibodies. Equal protein loading was confirmed by probing the Western blots with an anti-α-tubulin antibody.

cell cycle. In normal cells the cell cycle is a highly orderly process that results in the duplication and transmission of genetic information from one cell generation to the next. Abnormal cells are no longer subject to normal internal control and continue to proliferate even in the absence of external stimuli. Regulation of the cell cycle therefore offers an approach to controlling the growth of tumour cells. In the present study, AOA-GMe had a concentration-dependent effect on the cell cycle, increased the percentage of B16 cells in G0/G1 phase but decreasing the proportion in S phase (Figure 5). By contrast, OA caused negligible change in the ratio of the cells in these phases, except for a slight tendency to increase the G0/G1 populations at high concentrations (5×10^{-5} M; Figure 5). These results show that AOA-GMe is more potent than OA, and probably induces cell cycle arrest at G0/G1 and blocks the entry of cells into DNA synthesis phase.

The cell cycle is driven by the CDK family of proteins. These kinases are positively regulated by cyclins and negatively regulated by CDKIs. During the G1 phase, cyclin D associates with CDK4 and/or CDK6 to form cyclin–CDK complexes, which result in the activation of CDKs (Hartwell & Kastan 1994; Shapiro & Harper 1999; Israels & Israels 2001). To address the mechanisms of cell cycle arrest in G0/G1 phase induced by AOA-GMe in B16 cells, the levels of several regulatory proteins were examined by Western blot analysis. Levels of CDK4 and cyclin D proteins were decreased by AOA-GMe, whereas levels of p15, a specific inhibitor of CDK4 and CDK6, were increased after exposure to AOA-GMe (Figure 6). OA had only a slight inhibitory effect on cyclin D at high concentrations (5×10^{-5} M) and had no effect on other proteins (Figure 6). These results suggest that AOA-GMe may regulate proteins involved in regulating the cell cycle, differing from its parent compound OA. Investigations on the G1 phase have clearly demonstrated that CDKs are significant in the diagnosis and prognosis of cancer. For example, remarkably elevated CDK2 activity is evident in gastrointestinal carcinoma tissues, and the direct measurement of CDK activities and expression (CDK profile) may produce clinically relevant values for diagnosis (Ishihara et al 2005). CDK4, but not CDK6, is strongly involved in the development of melanoma (Zuo et al 1996), while CDK6 activity is remarkably elevated in squamous cell carcinomas, without alteration of CDK4 activity (Timmermann et al 1997). These findings suggest that the CDK profile differs between tumour types. The changes in cyclin D, p15 and CDK4 but not CDK6 induced by AOA-GMe may therefore be linked to the particular tumour cells studied, and regulation of these cell cycle proteins may contribute to the inhibitory efficacy of AOA-GMe against melanoma cells in-vivo. These mechanisms need to be studied in more detail in further research.

It is well known that the activated CDKs can phosphorylate Rb protein, which disrupts the binding of Rb to E2F, allowing activation of E2F and the transcription of genes necessary for G1–S transition and progression (Hartwell & Kastan 1994; Shapiro & Harper 1999; Israels & Israels 2001). In this study, levels of phosphorylated Rb were reduced by AOA-GMe (Figure 6), suggesting that AOA-GMe may block G1–S transition in these cells. In fact, AOA-GMe dose-dependently decreased the proportion of the cell population in S phase (Figure 5).

Cell cycle regulatory proteins have received increasing attention in recent years and they have been confirmed as targets for some novel anti-tumour drugs (Lapillonne et al 2003; Dispenzieri et al 2006; Radhakrishnan & Gartel 2006; Shapiro 2006). The pattern of activity of AOA-GMe in this respect, regulating CDK4, cyclin D and p15, and inducing G0/G1 arrest, as well as blocking G1–S transition, is different from that of other analogues of OA reported previously, such

as CDDO, which showed a strong anti-tumour efficacy (Suh et al 1999). The underlying mechanisms of CDDO were consistent with targeting of cell cycle regulatory genes, including cyclin D and p21, inducing cell cycle arrest in G1/S and G2/M, and apoptosis (Ito et al 2000; Lapillonne et al 2003). The different activities of AOA-GMe and CDDO may reflect the different functional groups introduced into OA.

Conclusions

AOA-GMe, a novel derivative of OA, showed potent anti-tumour efficacy but with almost no toxicity to normal human PBMCs. This effect of AOA-GMe is consistent with its effects on the cell cycle, blocking G1–S phase transition and inducing G0/G1 arrest.

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