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RESEARCH PAPER

Reversine, a 2,6-disubstituted Purine, as an Anti-cancer Agent in Differentiated and Undifferentiated Thyroid Cancer Cells

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ABSTRACT

Purpose A novel and effective treatment is urgently needed to deal with the current treatment dilemma in incurable differentiated thyroid cancer (DTC), poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC). Reversine, a small synthetic purine analogue (2,6-disubstituted purine), has been shown to be effective in tumor suppression.

Methods We performed *in vitro* evaluation of anti-tumor effects of reversine on proliferation, cell cycle, and apoptosis in human PDTC, ATC, and follicular thyroid cancer cell lines, respectively.

Results Treatment of these three lines with reversine inhibited proliferation in a time- and dose-dependent manner. G2/M accumulation was demonstrated in cell cycle analysis. Reversine induced apoptosis in PDTC cells with caspase-3 and caspase-8 activation, but not caspase-9. Use of a pan-caspase inhibitor before treatment with reversine attenuated cell death. Reversine also showed *in vivo* growth inhibitory effects on ATC cells in a xenograft nude mice model.

Conclusions Data demonstrated that reversine is effective in inhibiting the growth of thyroid cancer cells by cell cycle arrest or apoptosis, especially with the more aggressive ATC and PDTC. Apoptosis was induced by the mitochondria-independent pathway. Reversine is therefore worthy of further investigation in clinical therapeutics.

KEY WORDS reversine · thyroid cancer · cell cycle · apoptosis · xenograft nude mice

ABBREVIATIONS

ATC	anaplastic thyroid cancer
Aur-A	aurora kinase-A
Aur-B	aurora kinase-B
Aur-C	aurora kinase-C
BCRC	Bioresource Collection and Research Center
DTC	differentiated thyroid cancer
FTC	Follicular thyroid carcinoma
PDTC	poorly differentiated thyroid cancer
s.c.	subcutaneous injection

INTRODUCTION

Thyroid cancer is the most common cancer among endocrine malignancies, and has been classified as differentiated

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thyroid cancer (DTC), poorly differentiated thyroid cancer (PDTC), and anaplastic thyroid cancer (ATC). Both papillary and follicular carcinomas are classified as DTC, and patients with DTC have an excellent prognosis compared with other types of human carcinoma. More than 85 % of patients with follicular thyroid carcinoma (FTC) and DTC present with limited disease and become disease-free after initial treatment; however, 10–15 % of patients with thyroid cancer have recurrent disease (1,2). Radioactive iodide therapy is essential in current clinical treatment for thyroid cancer. Although ATC and PDTC are rare among human thyroid cancers, they account for a significant portion of the morbidity and mortality associated and fail to respond to all available chemotherapeutic agents and radiotherapy with these cancers (3). ATC is one of the most aggressive and lethal tumors in humans and the patients with ATC have a poor prognosis regardless of the types of treatment and almost die within a year of the diagnosis (4). Therefore, a novel and effective treatment modality is urgently needed for radio-resistant DTC, PDTC, and ATC.

A 2,6-disubstituted purine named reversine, originally identified by Ding et al. as a molecule, induces dedifferentiation of murine myoblasts into multipotent progenitor cells (5). Later, the role of reversine in regenerative medicine was well appreciated (6–10). Cell differentiation is also speculated to be relevant to cancer therapy. To our knowledge, only two studies on the anti-tumor capabilities of reversine have been published. First, reversine was reported to inhibit colony formation of acute myeloid leukemia cells and was as effective as VX-680, a potent Aurora kinases (Aur) inhibitor, but less toxic (11). In this cellular model, Aur inhibition was reported to be involved in tumor suppression (11). Recently, using a newly developed tumor cell-specific in vitro bioluminescence imaging (CS-BLI) anticancer drug screening assay, reversine was shown to inhibit a multiple myeloma cell line *in vitro* (12). In the cellular model of multiple myeloma cell lines, and in addition to the cell cycle regulator proteins Aurora kinase A (Aur-A) and Aurora kinase B (Aur-B), reversine was demonstrated to suppress enzymes involved in cell growth signals, such as JAK2 and SRC (12). Structurally, reversine is an ATP analogue and is suspected to be able to inhibit various kinds of cellular enzymatic activities, possibly not limited to Aur. The definite mechanisms of reversine's tumor suppression effect have not yet been clearly elucidated.

In the present study, we show that reversine is active against three thyroid cancer cell lines, including PDTC (SW579), ATC (ARO), and FTC (WRO). In the xenograft nude mice model, reversine significantly reduces growth of ARO. We also demonstrate that apoptosis is induced in SW579 cells with activation of a mitochondria-independent pathway. Our data suggests reversine is a potential anti-thyroid cancer drug candidate, and effective, regardless of the extent of differentiation.

MATERIALS AND METHODS

Thyroid Cancer Cell Lines and Cell Culture

Human ATC cell line (ARO) was kindly provided by Dr. Chih-Yuan Wang (13), human FTC cell line (WRO) was kindly provided by Prof. Jen-Der Lin (14), and human PDTC cell line (SW579) was purchased from Bioresource Collection and Research Center (BCRC), Taiwan. The ARO and WRO cells were maintained in RPMI1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 0.1 μg/ml streptomycin in a 5 % CO_2 humidified atmosphere at 37°C. The SW579 cells were maintained in 90 % Leibovitz's L-15 medium (Gibco BRL, Grand Island, NY) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 0.1 μg/ml streptomycin in a humidified atmosphere at 37°C without CO_2.

Cell Proliferation Assay (MTT Assay)

Reversine was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). 5×10^3 of ARO, WRO, and SW579 cells/100 μl were plated into 96-well tissue culture plates and grown in the above-mentioned medium. After an overnight attachment period, the cells were treated with medium only (containing 0.01 % DMSO) or medium containing reversine at 0.5, 1, 5, 10, 25, 50, and 100 μM. After incubation for 24, 48, and 72 h, the number of metabolically active cells was determined by MTT assay. The final results were analyzed by statistical methods in three independent studies.

Cell Cycle Analysis

ARO, WRO, and SW579 cells were incubated with either reversine at 1, 5, or 10 μM or DMSO 0.01 % for 24, 48, and 72 h. Cells were harvested and fixed in 70 % ethanol overnight. After double washing with PBS, cells were labeled with 500 μl PI staining buffer (Sigma, St. Louis, MO) and incubated at room temperature in the dark for 30 min. DNA content was analyzed using FACScan (Becton Dickinson, San Diego, CA) with Modfit. LT 3.3 software. In addition, the cell cycle markers of cyclin-A, cyclin-B1, cdc2 and cdc25c were determined by Western blot with the antibodies (Cell signaling, Danvers, US).

Cell Death Analysis

After treatment, Annexin-V staining (Sigma, St. Louis, MO) was performed to detect apoptotic cells. The cells were washed with PBS twice and centrifuged at $1500\times g$ for 10 min. The cell pellets were resuspended in 100 μl of

staining solution (2 μl Annexin-V-FITC and 2 μl PI in 100 μl binding buffer) and incubated for 15 min at room temperature in darkness. Annexin-V or PI fluorescent intensities were analyzed by FACScan (Becton Dickinson, San Diego, CA), and 10,000 cells were evaluated in each sample. Moreover, the mitochondria and cytochrome c were determined by immuno-fluorescence staining with anti-TOM20 (a subunit of mitochondrial import receptor) and anti-cytochrome c antibodies (all of the antibodies were purchased from Santa Cruz Biotechnology, Inc.).

Apoptotic Mechanisms Investigation

After data analysis of flow cytometry, apoptosis was concluded. Further apoptotic mechanisms were investigated by detecting the activation of caspase-3, caspase-8, or caspase-9 and PARP expression using Western blot (all of the antibodies were purchased from Santa Cruz Biotechnology, Inc.). We also used a pan-caspase inhibitor (Z-VAD-FMK; BioVision, Mountain View, CA) to confirm the mechanisms via a caspase-dependent pathway and observe whether it could reverse reversine's effects on cell death.

In Vivo Anticancer Assay (Xenograft Nude Mice Model)

The 7-week-old male nude mice (BALB/cAnN.Cg-*Foxn1*^{nu}/CrlNarl) were purchased from the National Laboratory Animal Center. Mice were acclimatized at the Animal Facility of National Chung Cheng University. 2×10^6 cells of ARO, WRO or SW579 were separately injected subcutaneously (s.c.) into the right flank of the nude mice, but only the ARO xenograft nude mice model was successfully established. The experiment protocol was as follows: 8-week-old male nude mice were randomized into 3 groups (3 mice/group in each experiment); 2×10^6 cells of ARO were injected s.c. at Day 0. Control group mice were orally fed with DMSO, and the experimental group mice were orally fed with reversine 0.1 mg/kg or reversine 1.0 mg/kg at Day 1. The schedule of administration with reversine in our animal model was consulted with Dr. McMillin's report (12). Every 7 days, each mouse was fed with the drug dosage, and this was repeated 3 times (Day 8, Day 15, and Day 22). Tumor size was measured every 3 days. Tumor volume was estimated by the formula ($L\times S^2/2$, L as the longest diameter, S as the shortest diameter). Closing the experiment on Day 28, the nude mice were sacrificed and the tumor was harvested and weighed. Three independent experiments were done. All animal studies were conducted in accordance with the guidelines of the National Institutes of Health "Guide for the Care and Use of Animals".

Statistical Analysis

Data are presented as mean ± standard deviation for the indicated number of separate experiments. Using statistical software (SPSS 16.0), if the number was more than 30, Student's t test was performed for comparisons; if less than 30, the Mann-Whitney U test was used. Statistical significance was defined as a *P*-value less than 0.05 in all tests.

RESULTS

Reversine Inhibits the Cellular Viability of ARO, WRO, and SW579 Cell Lines

Three human thyroid cancer cell lines, including PDTC (SW579), ATC (ARO), and FTC (WRO), were used to evaluate the growth inhibition effect during reversine treatment. The ARO, WRO and SW570 cellular numbers were observed by microscopy after reversine treatment. The cellular numbers of ARO, WRO and SW579 were decreased significantly in a dosage- and time-dependent manner during reversine treatment (Fig. 1a, c and e). DMSO was used as a negative control. Moreover, using an MTT assay, cellular viability was quantified, and in a time- and dose-dependent manner after reversine treatment in all three thyroid cancer cells (Fig. 1b, d and f). However, in SW579 cells, treatment with reversine in low dose (<0.5 μM at 48 and 72 h) or short duration (<5 μM at 24 h) had no significant effect on cellular proliferation inhibition. The GI_{50} of reversine in SW579, ARO and WRO cells was 4, 5 and 25 μM after 48 h treatment, respectively (Fig. 1b, d and f). Of interest, SW579 and ARO cells, two of the more aggressive human thyroid cancers, showed more susceptibility than WRO cells during reversine treatment (Fig. 1). These data demonstrated that reversine could reduce cell viability in human PDTC, ATC and FTC cells.

Reversine Induced Tetrapolidization and Cell Cycle Arrest at the G_2/M Phase

Since reversine could reduce the numbers of ARO, SW579 and WRO cells, we further investigated whether cell cycle arrest was induced during reversine treatment. Flow cytometry was used to evaluate the cell cycle of ARO, SW579 and WRO cells after reversine treatment. DMSO was used as the negative control. Figure 2a shows that the ratio of G_0/G_1 (2 N) was decreased and the G_2/M (4 N; tetrapolidization) was increased in a dosage- and time-dependent manner in reversine-treated ARO cells. Moreover, this phenomenon was also found in reversine-treated WRO cells (Fig. 2a). In SW579 cells, G_2/M phase arrest was found in low-dosage treatment (1 μM) with reversine (Fig. 2c). In

addition, the sub-G1 phase was also found in these three cells, especially SW579 cells (Fig. 2). To further confirm the induction of G2/M phase arrest during reversine treatment, the cell cycle progression markers, cyclin A, cyclin B1, cdc2 and cdc25c were determined by immuno-blotting in reversine-treated ARO cells. Cyclin A levels decreased and cyclin B1, cdc2, and cdc25c accumulated after reversine treatment (Supplementary Material S1). These data demonstrated that reversine could induce cell cycle arrest at the G_2/M phase in ATC, FTC and PDTC cells. An important finding is that ARO cells were more susceptible than WRO and SW579 cells to reversine-induced cell cycle arrest (Fig. 2a and b). Moreover, apoptosis was found in reversine-treated ARO and WRO cells, and mostly in SW579 cells. Our data demonstrated that reversine could suppress human thyroid cancer growth by cell cycle arrest induction (mostly in ATC and FTC cells) and apoptosis (mostly in PTDC cells).

Reversine Induced Apoptosis in Human Thyroid Cancer Cells

Reversine treatment was able to induce cell cycle arrest; moreover, sub-G1 was also found in reversine-treated ARO, WRO and SW579 cells (Fig. 2). This suggested that apoptosis may occur in human ATC, FTC and PTDC cells during reversine treatment. Among these cell types, SW579 cells were more susceptible to reversine-mediated apoptosis induction. To confirm the apoptosis induction by reversine, all of the ARO, WRO and SW579 cells were treated with

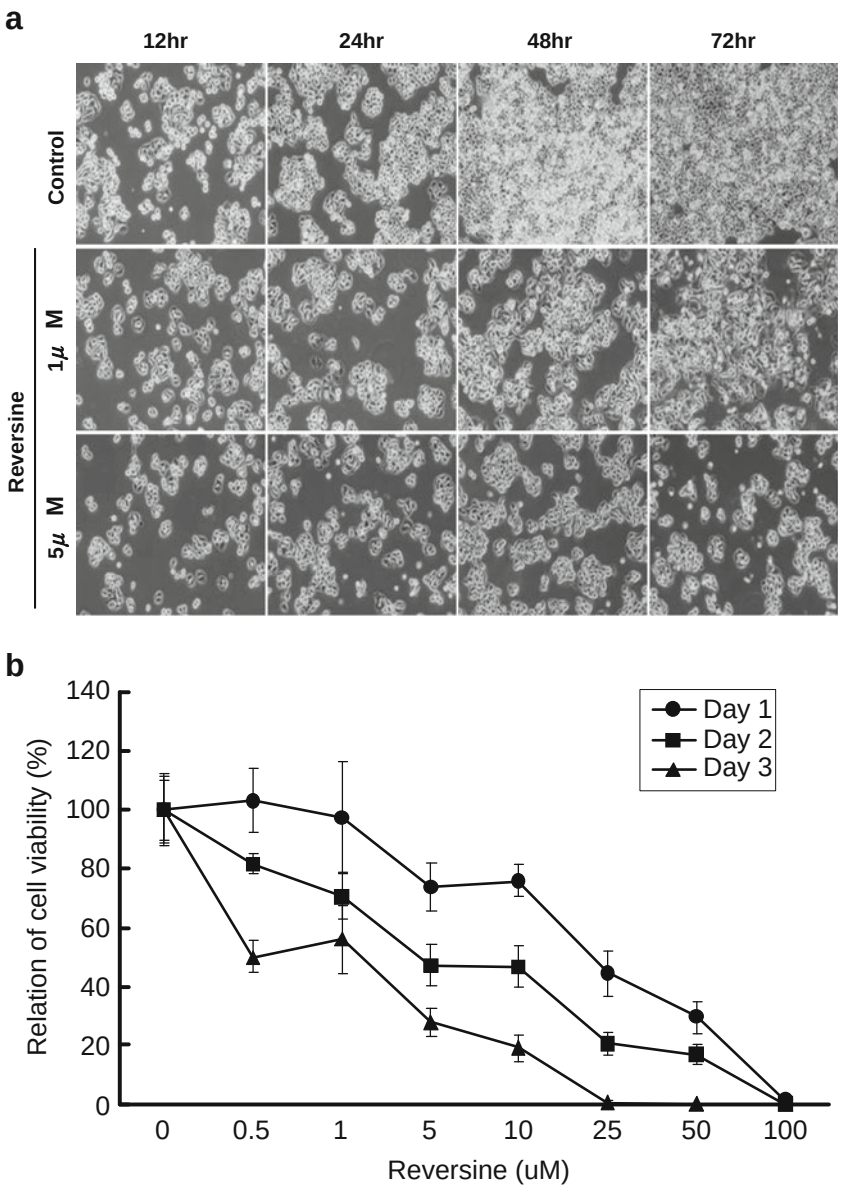
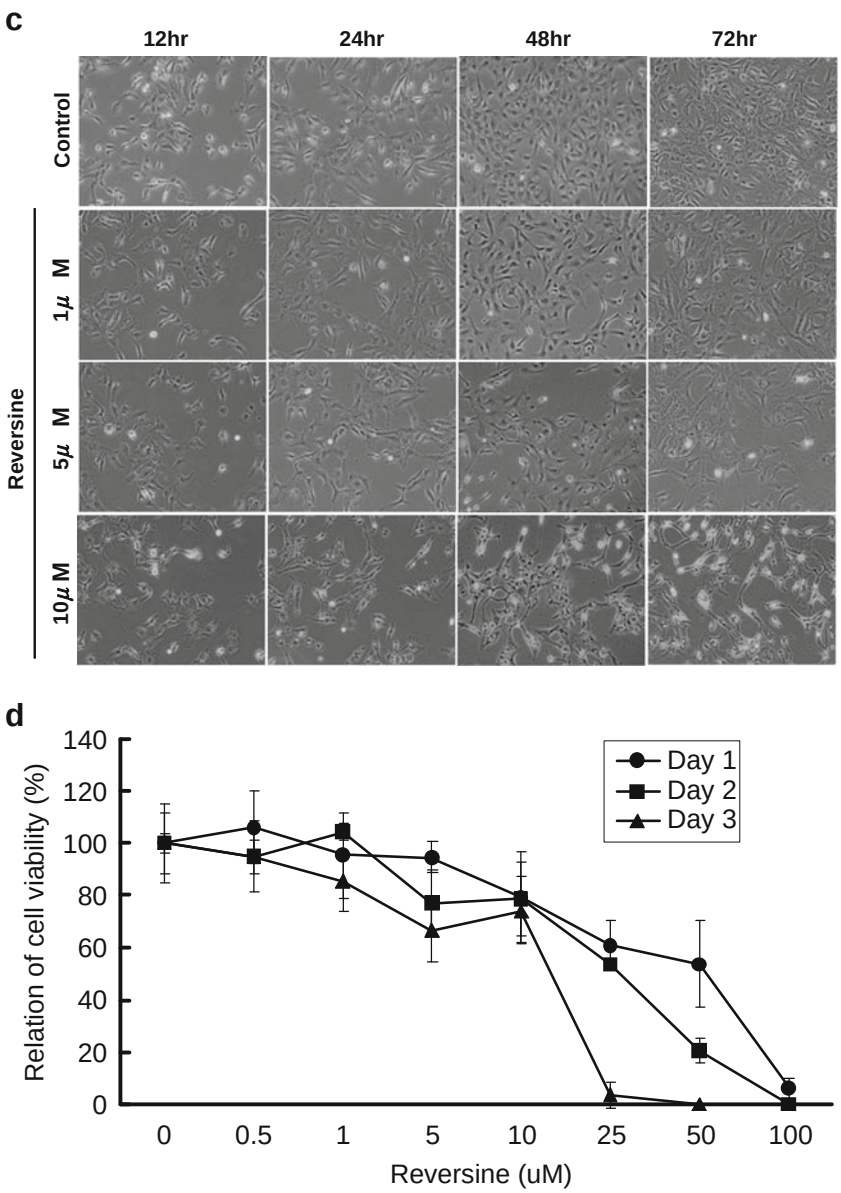


Fig. 1 Reversine inhibited the growth of human thyroid cancer cells. Cells were incubated with reversine for 24, 48 and 72 h, and the effects on the proliferation inhibition of (**a**, **b**) ARO, (**c**, **d**) WRO and (E and F) SW579 cells were determined by microscopy (**a**, **c**, **e**) and MTT assay (**b**, **d**, **f**). The results of MTT assay were expressed as mean ± SEM of three independent experiments.

Fig. 1 (continued)



reversine at various dosages and times. The apoptosis analysis was determined by flow cytometry with PI and Annexin-V double staining. Figure 3 showed that reversine could induce apoptosis in ARO, WRO and SW579 cells in a dosage- and time-dependent manner. The levels of apoptosis (both early and late apoptosis) in these three cell types with reversine (10 μM) treatment were SW579 > ARO> WRO, and were SW579 = ARO>WRO with 1 or 5 μM reversine treatment (Fig. 3). However, the apoptosis percentages in the WRO cells was very low (<10 % of WRO cells), even at high dosages (10 μM for WRO cells) and a long duration (72 h) of treatment. SW579 and ARO cells were more susceptible of the three cell types to reversine-induced apoptosis. This finding demonstrated that reversine could induce apoptosis in human thyroid cancer cells, especially in human PTDC and ATC cells.

Reversine Induced Apoptosis Through Activation of Mitochondria-Independent Pathway

Significant apoptosis was observed in reversine-treated SW579 cells (Fig. 3). To further investigate the mechanisms involved in reversine-mediated apoptosis induction, Western Blot was used and activated caspase-8 and -3 were determined during reversine treatment (Fig. 4a). This phenomenon was found to be significant after 10 μM reversine treatment. However, the active form of caspase-9 was not found after reversine treatment (Fig. 4a). To confirm

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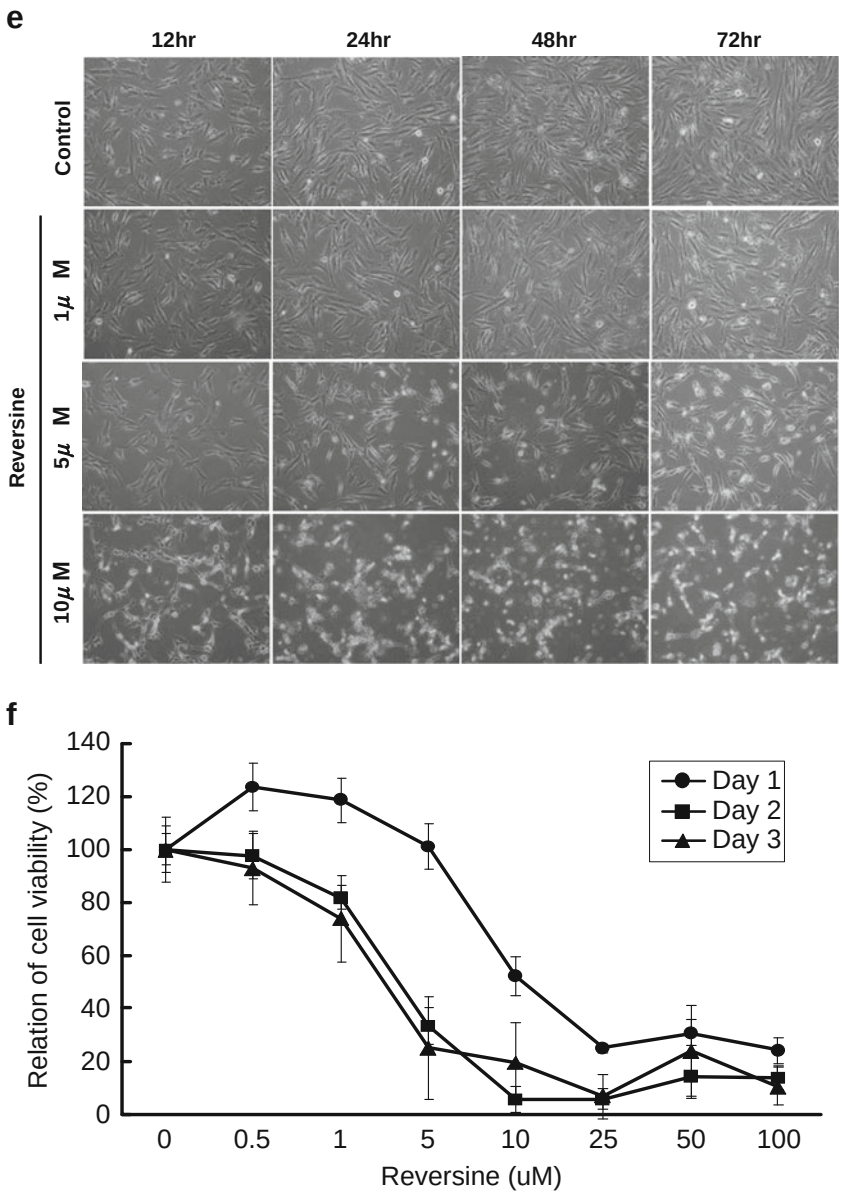


whether the mitochondria-dependent pathway was involved in reversine-mediated apoptosis, the expression and activation of Bcl-$_X$ and Bid were evaluated further. The expression levels of both Bcl-$_X$L and Bid showed no significant decrease in the reversine-treated groups (Fig. 4a). Furthermore, the apoptotic isoforms of Bcl-$_X$S and tBid were not detected after reversine treatment in SW579 cells. To further confirm this finding, the localization of cytochrome c and mitochondria were detected by immuno-fluorescence staining and determined by conforcal microscope. Figure 4b showed colocalization of mitochondria and cytochrome c both in control group and reversine treated group. However, the cytochrome c was detected evenly in the cytoplasm which was not colocalized with mitochondria in the etoposide treated group. In addition, the expression of PARP was also determined in human SW579 cells, the cleavage PARP was only found in the reversine treated group (Fig. 4c). These data suggested that reversine could induce apoptosis in SW579 cells through an extrinsic pathway.

In addition, to further evaluate whether the caspase-dependent apoptosis induced by reversine could suppress human thyroid cancer growth, the pan-caspase inhibitor, Z-VAD-FMK, was used to block the activation of caspase, and the activation of both caspase-8 and caspase-3 were determined after reversine treatment. Figure 4d shows that reversine-mediated activation of caspase-8 and -3 could be suppressed partially by Z-VAD-FMK pretreatment. Moreover, the cell number of SW579 cells was significantly restored and the apoptotic cells were also reduced (Fig. 4e). Altogether, we demonstrated that reversine could induce cellular apoptosis through an extrinsic pathway and further reduce human thyroid cancer growth.

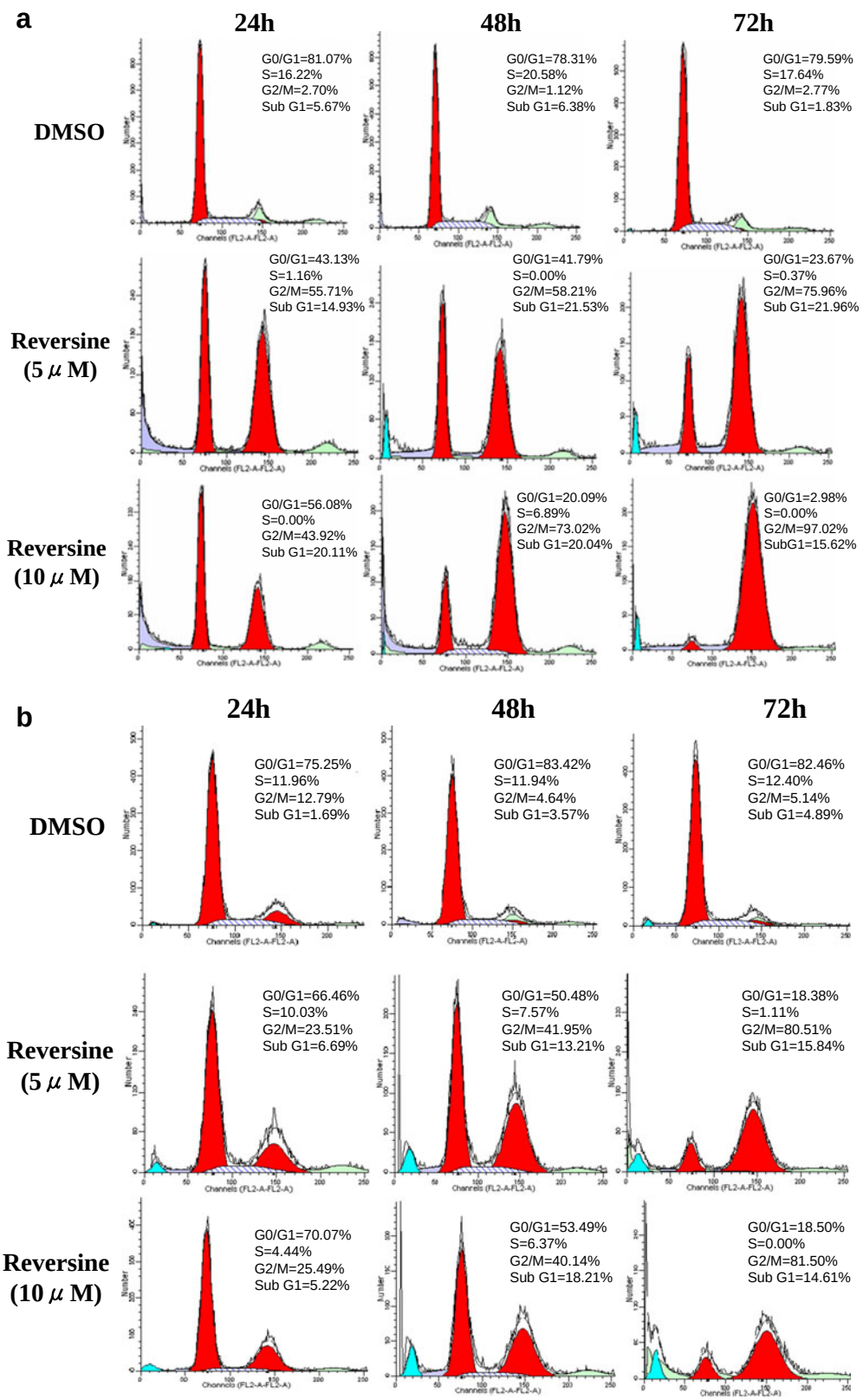
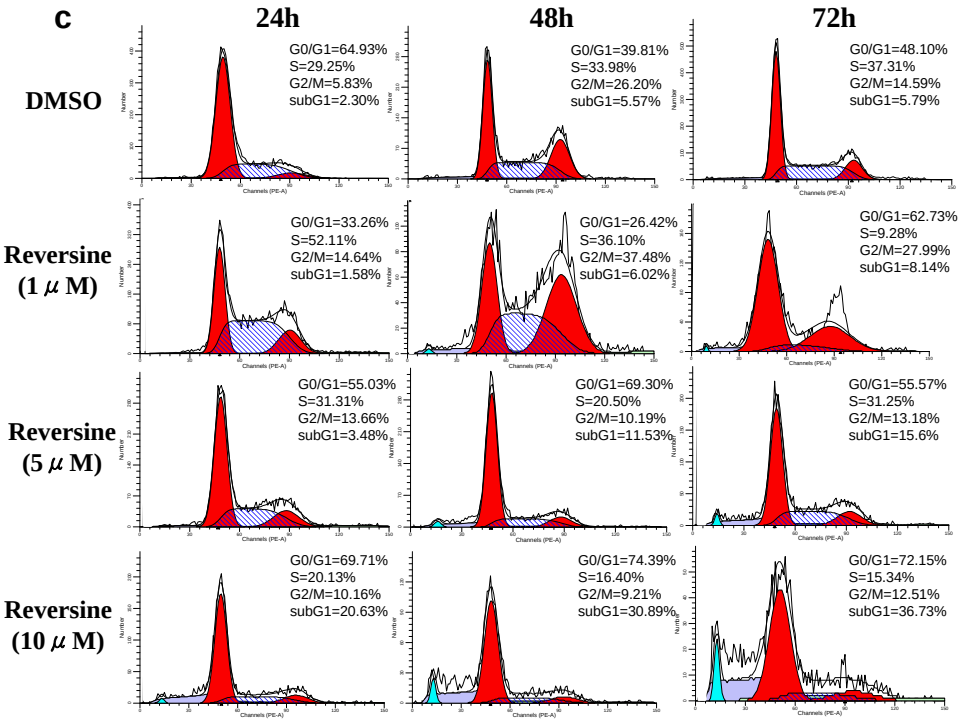


Fig. 2 Reversine induced cell cycle progression arrest at the G2/M phase and cell death in human thyroid cancer cells. Cells were incubated with reversine with various dosages and the DNA content of (**a**) ARO, (**b**) WRO and (**c**) SW579 cells were assessed by flow cytometry analysis with propidium iodide labeling at the indicated time period. The percentage of G0/G1, S, G2/M and sub-G1 phase distribution of reversine treated cells was measured by ModFit. LT 3.3 software. Three to four independent experiments were confirmed and one of them was shown.

Fig. 2 (continued)



In Vivo Anticancer Assay (Xenograft Nude Mice Model)

In this study, we demonstrated that reversine is an anti-cancer agent that can induce cell cycle arrest and apoptosis *in vitro*. To evaluate the anti-cancer effect of reversine *in vivo*, we established a xenograft nude mice model by s.c. injection of WRO or SW579 or ARO cells into the right flank of nude mice. However, only ARO cells were tumorigenesis in the xenograft nude mice model; the WRO and SW579 cells failed to grow into tumor *in vivo* (data not shown). Therefore, the ARO xenograft nude mice model was used to evaluate the anti-cancer effect of reversine *in vivo*. After s.c. injection with ARO cells (2 x 10^6 cells/mouse), the mice were fed orally with or without reversine as described in the method. After 28 days of treatment, the tumor weight in the control group was 0.581± 0.09 g, and in the groups treated with reversine 0.1 mg/kg and 1.0 mg/kg, tumor weight was 0.41±0.13 g (compared with the control group, p=0.0068) and 0.38±0.12 g (compared with the control group, p=0.0011), respectively (Fig. 5a). However, there was no significant difference between the reversine 0.1 mg/kg and 1.0 mg/kg groups (p= 0.5774) (Fig. 5a). In addition, there were no significant differences in body weight between the control group (22.25± 0.90 g/mouse) and the reversine-treated groups (21.99± 0.98 g/mouse in 0.1 mg/kg reversine treated group, and 22.76±1.35 g/mouse in 1 mg/kg reversine treated group). Furthermore, no infiltration or liver pathology was found in the reversine treated group and control group (Supplementary Mineral S2). Tumor volume in the control group and the reversine-treated groups was also measured from Day 7 to Day 28. At Day 19, differences began to be seen—tumor volume in the control group was 322.54±67.33 mm^3, and that in the reversine 0.1 mg/kg group was 193.81± 91.10 mm^3 (compared with the control group, p=0.0036) and in the reversine 1.0 mg/kg group was 225.40± 110.98 mm^3 (compared with the control group, p=0.0393) (Fig. 5b). Moreover, tumor growth suppression with reversine treatment was more significantly different at Day 28—tumor volume in the control group was 855.08±318.53 mm^3, and that of the reversine 0.1 mg/kg group was 546.86± 187.50 mm^3 (compared with the control group, p=0.024) and of the reversine 1.0 mg/kg group was 490.60± 253.39 mm^3 (compared with the control group, p=0.0162) (Fig. 5b). Moreover, there was no significant tumor volume difference between the reversine 0.1 mg/kg group and the 1.0 mg/kg group throughout the entire experiment course (Fig. 5b). These data demonstrated that reversine not only inhibited human thyroid cancer cell growth through cell cycle arrest and apoptosis *in vitro*, but also suppressed tumorigenesis *in vivo*. Of most importance, reversine was safe for the nude mice, even with 1.0 mg/kg treatment.

DISCUSSION

In the present study, reversine, a 2,6-disubstituted purine, exhibited significant antitumor actions against human follicular thyroid cancer (WRO), ATC (ARO) and PDTC (SW579) cells. Cell proliferation and viability were significantly reduced by cell cycle arrest and/or apoptosis induction (Figs. 1, 2 and 3). Moreover, reversine-mediated apoptosis was

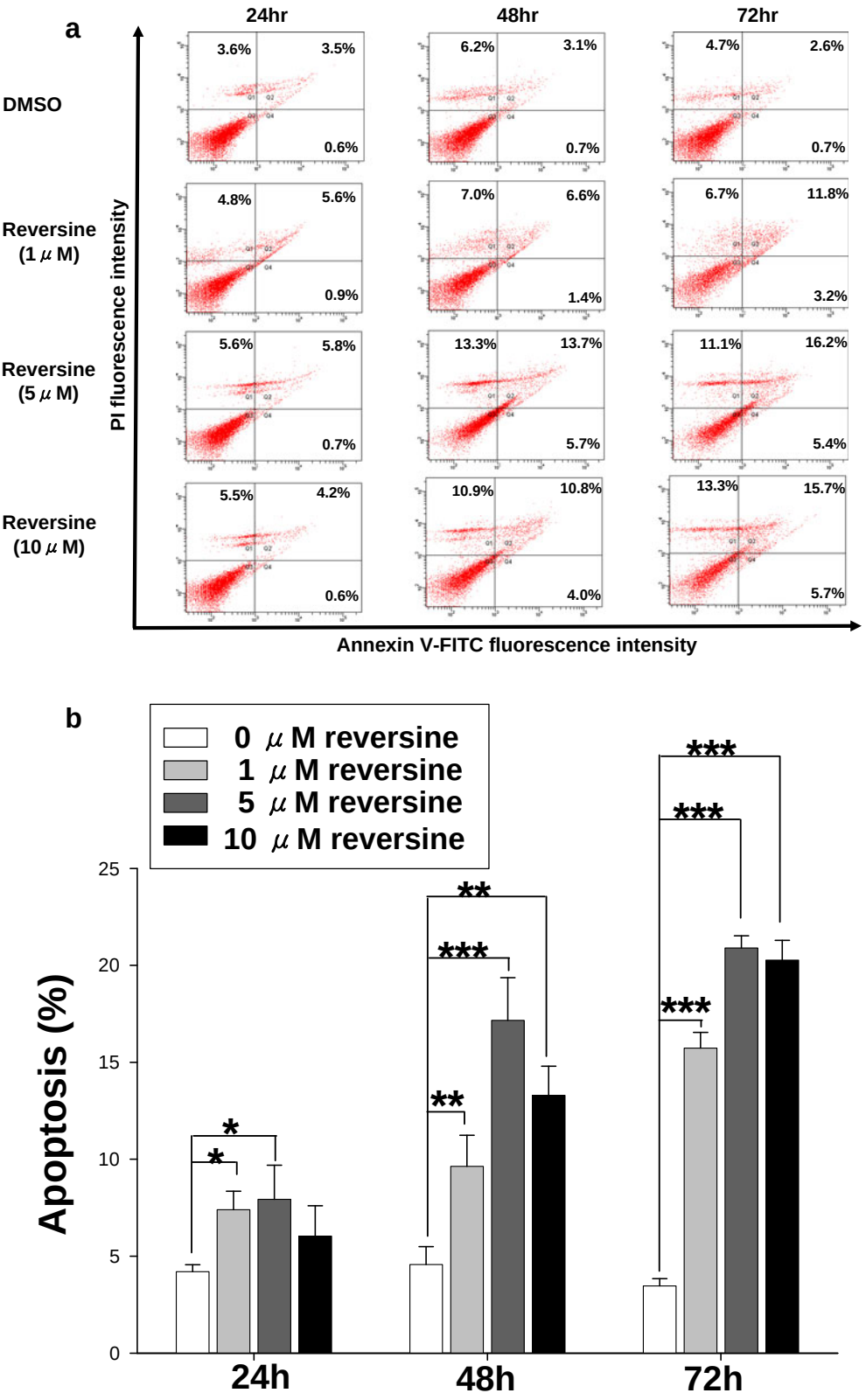
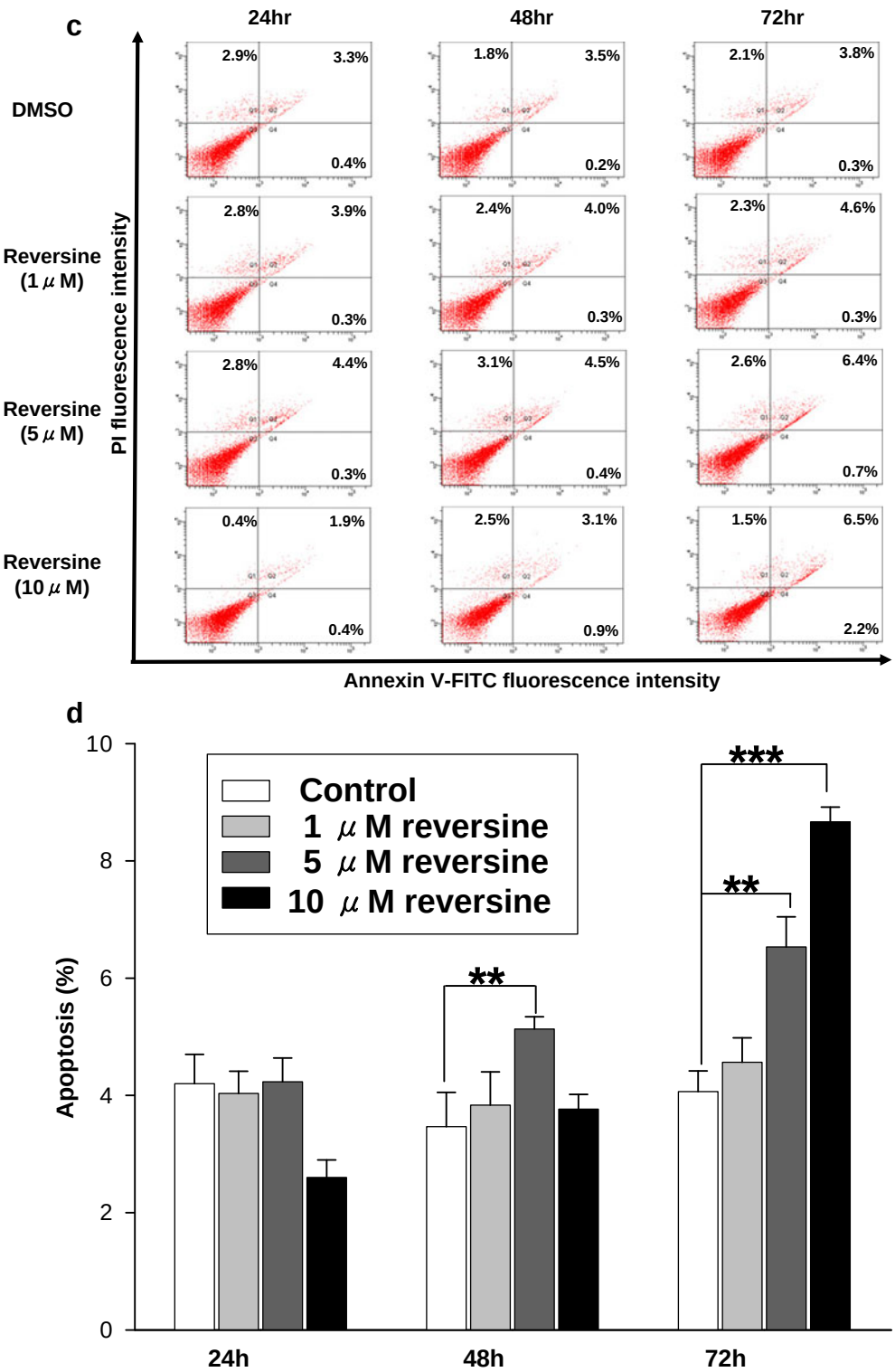


Fig. 3 Detection of reversine mediated apoptosis in human thyroid cancer cells. Cells were incubated with DMSO or with reversine in various dosages, and the apoptotic cells of (A and B) ARO, (C and D) WRO and (E and F) SW579 cells were evaluated by flow cytometry analysis with PI/Annexin-V double staining. Three independent experiments of (**a**, **c**, **e**) were confirmed, and the results were expressed as mean ± SEM and the apoptosis (early + late) was shown as (**b**, **d**, **f**). * means $P < 0.05$; ** means $P < 0.01$; *** means $P < 0.001$.

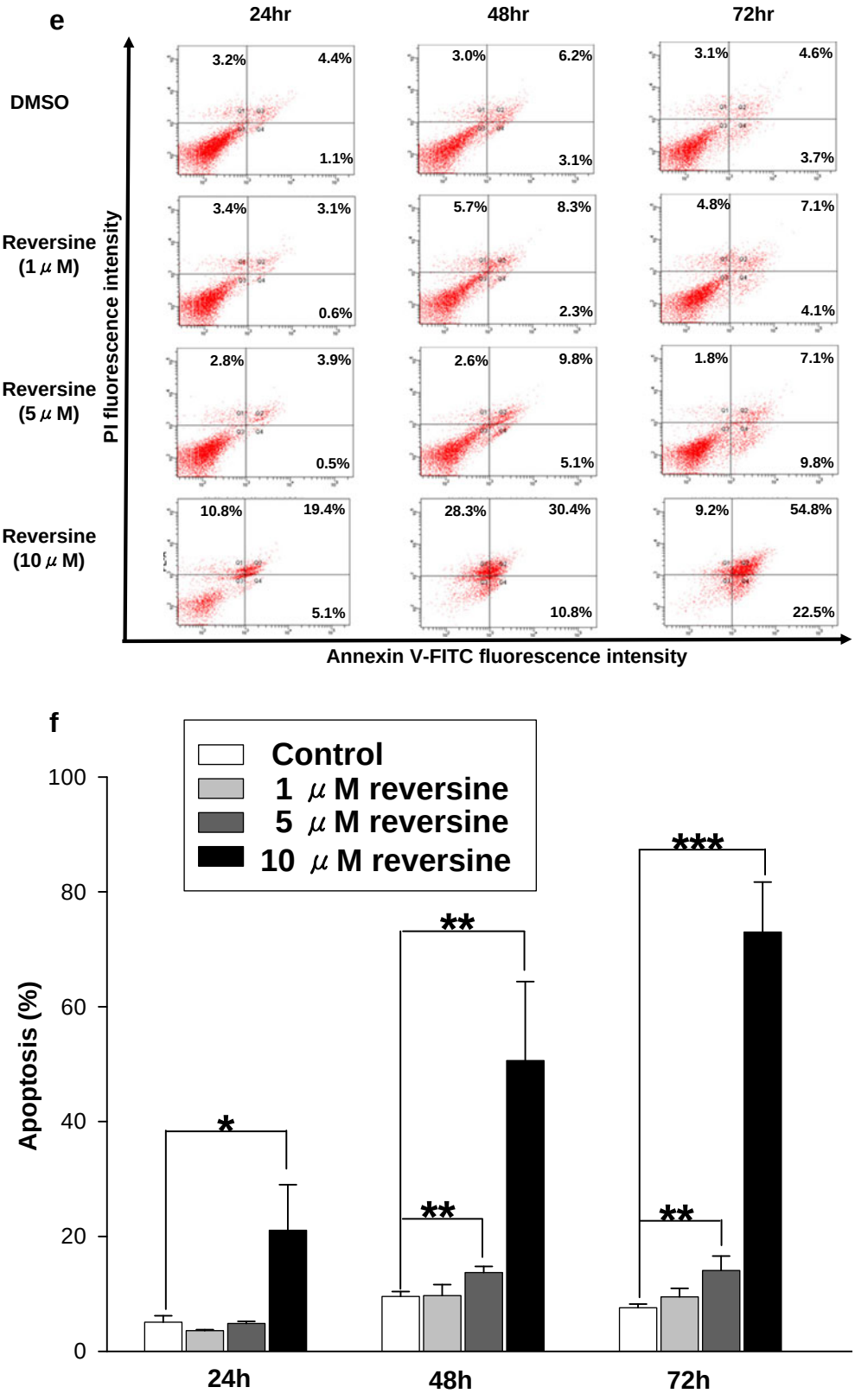
Fig. 3 (continued)



induced through a mitochondria-independent pathway (Fig. 4).

Cell cycle progression is subject to regulation by several different Cdk regulatory mechanisms (15). The cyclin B1/cdc2 kinase complex is inactive before mitosis, but cyclin B1/cdc2 kinase is activated by cdc25c phosphatase, and this complex promotes cell cycle progression from the G2 phase to the M phase. Both mitosis and cytokinesis occur during the M phase. In this report, reversine was observed to induce significant cell cycle arrest at the G2/M phase (Fig. 2 and Supplementary Material S1). Although cdc25c kinase was not down-regulated during reversine treatment,

Fig. 3 (continued)



cyclin B1 and cdc2 expression was observed to be accumulated (Supplementary Material S1). This observation is consistent with previous reports (11).

In addition, reversine has been reported to be an Aurora kinase inhibitor (11). Therefore, the impact of reversine on cell cycle arrest, with the resulting G2/M accumulation and

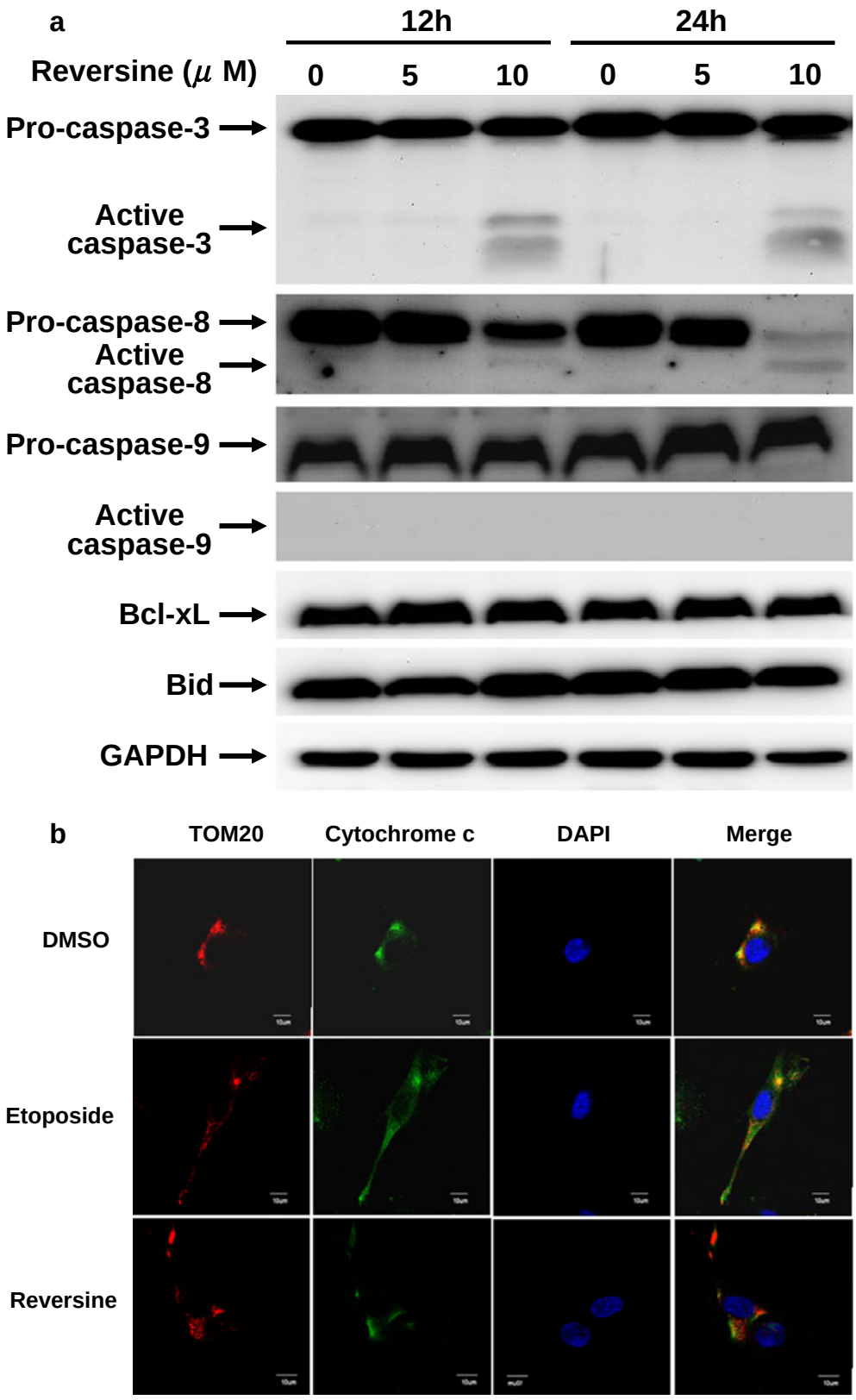
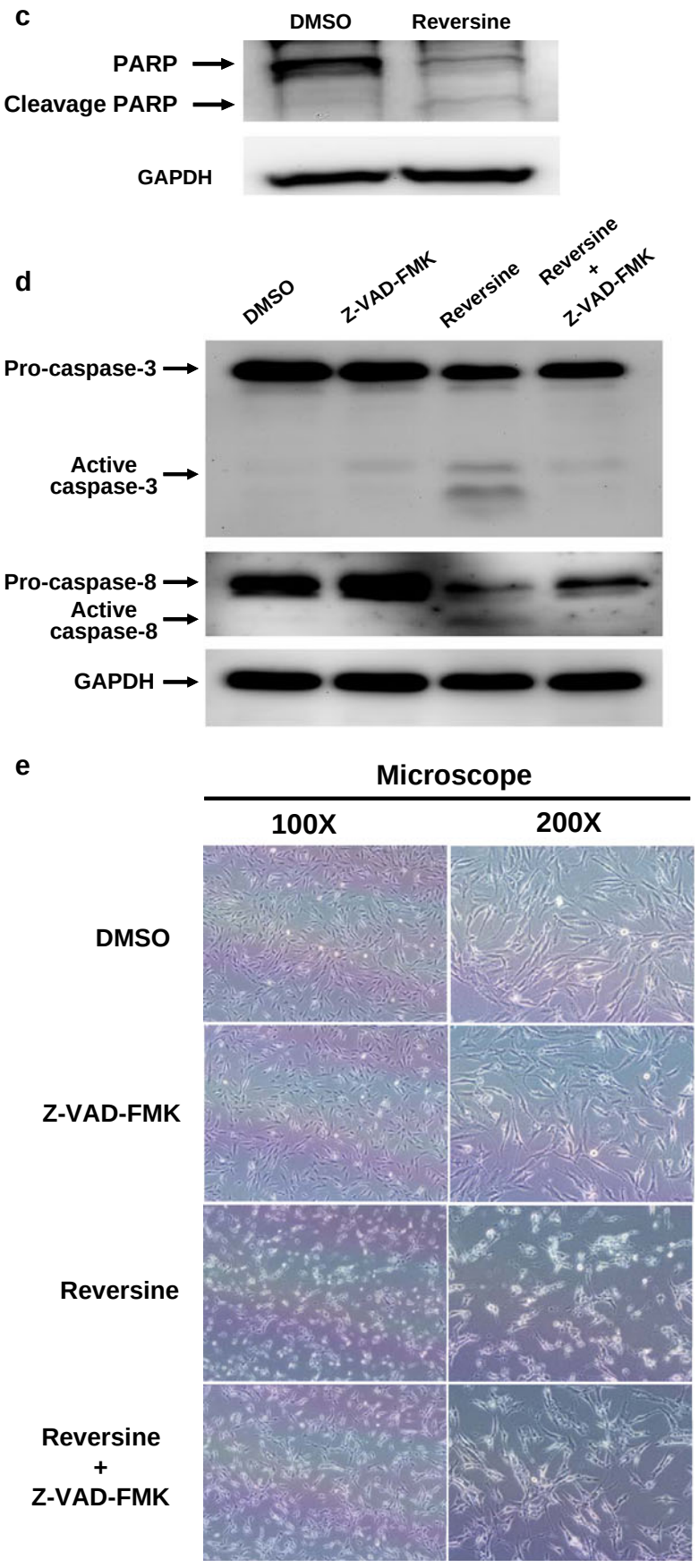


Fig. 4 Reversine promoted apoptosis through the activation of caspases in SW579 cells. (**a**) Cells were incubated with reversine for various dosages and the caspase-3, -8 and -9 were detected by western blot. Moreover, the Bid and Bcl-xL were further detected to demonstrate the extrinsic apoptosis pathways of type I was existence. (**b**) The localization of cytochrome c and mitochondria was detected by immunofluorescence staining after reversine (10 μM) treatment. The etoposide (50 μM) was used as positive control. (**c**) Reversine mediated apoptosis was evaluated by the cleavage of PARP. Cells were treated with or without reversine (10 μM), and the expression of PARP was determined by Western blot after 24 h treatment. (**d**) To evaluate whether the growth inhibition was mediated by reversine (10 μM) induced apoptosis, the pan-caspase inhibitor (Z-VAD-FMK; 20 μM) was used to block the activation of caspases, and the caspase-8 and -3 were determined after 24 h treatment. (**e**) After 2 h pre-treatment with Z-VAD-FMK (20 μM) and incubating with reversine (10 μM), the cellular phenomena was determined by microscope and the cell number were recovered in Z-VAD-FMK pre-treatment group. The GAPDH in the Western blot was used as the internal control, and the DMSO was a negative control.

tetrapolidization formation, also may contribute to Aurora kinase inhibition. Aurora kinases have three family members, including Aurora kinase A (Aur-A), Aurora kinase B (Aur-B), and Aurora kinase C (Aur-C), that play a role in the regulation of multiple aspects of chromosome segregation and cytokinesis (16–20). Aur-B, a chromosomal passenger

Fig. 4 (continued)



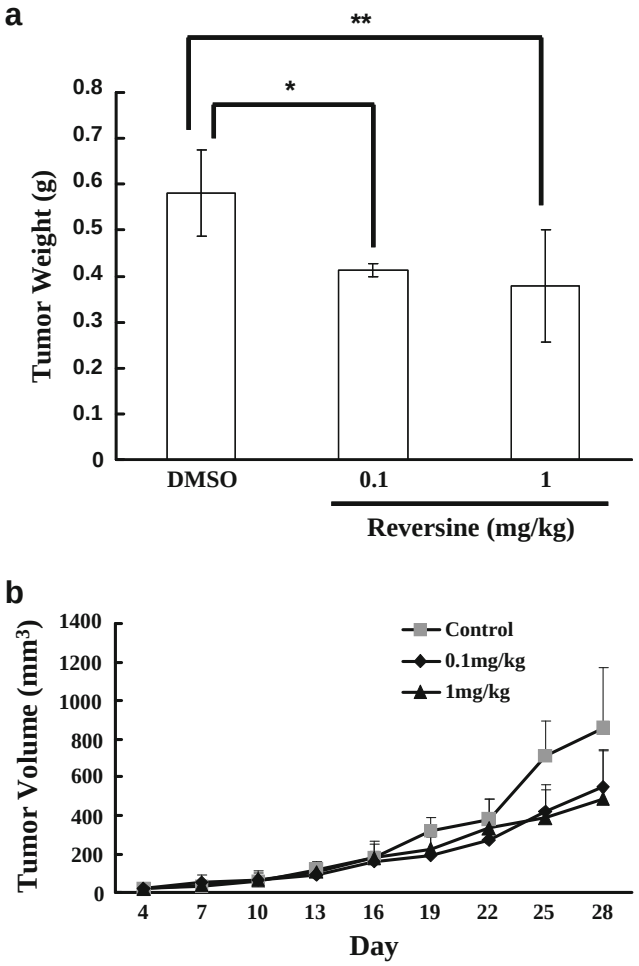


Fig. 5 Reversine inhibited tumorigenesis of ARO cells in nude mice. Details of the methods and the treatments of reversine were described in the Materials and Methods. (**a**) The mice were injected subcutaneously with ARO cells, and treated without or with reversine, the tumor weight were determined after 28 days treatment. (**b**) The tumor volume in different treatment groups were detected of days as described. Three independent experiments were done, and total nine mice were analyzed in each group. * means $P < 0.05$; ** means $P < 0.01$.

protein, associates with chromatin at the beginning of mitosis and forms a complex with proteins as inner centromere protein (INCENP), survivin, and borealin, inducing the phosphorylation of histone H3 (17,18,21). During the transition from anaphase to telophase, Aur-B also plays a role in the mitotic spindle dynamics and cleavage furrow, and can be observed in the midbody of cytokinetic cells. Reversine has been shown to bind to the active site of Aur-B by X-ray diffraction crystallography and inhibit Aur-B downstream target histone H3 phosphorylation (11). Previous studies have shown that although cells treated with reversine enter and exit the mitotic phase normally, however it fails in cytokinesis in human acute myeloid leukemia and prostate cancer cells. The cell cycle returns to the G_1-S phase to synthesize DNA, resulting in polyploidy cells—so-called "endoreplication" (11,22). This phenomenon is similar to the other Aur inhibitor ADZ1152 (23,24). Using an *in vitro* kinase assay, reversine has been reported to inhibit Aur-A, Aur-B, and Aur-C and other factors (11,12). On the other hand, Aur-A or Aur-B over-expression in human thyroid cancer-derived cell lines and tissues have also been reported in previous studies (25,26). Moreover, inhibition of Aur-B by siRNA suppresses ATC tumor growth *in vivo* (26). Another study showed VX-680, a potent Aur inhibitor, inhibits ATC cell lines and induces apoptosis (27). Therefore, whether reversine can suppress human thyroid cancer cell growth through inhibition of Aur kinases and/or other factors should be further investigated.

In our data, reversine showed potent anti-tumor activities (SW579 ≧ ARO >WRO cells) in thyroid cancer cells (Fig. 1). Apoptosis was induced significantly in PDTC cell lines, but partially in ATC or FTC (Fig. 4). Reversine was also active against ATC *in vivo* (Fig. 5). The significant tetrapolidy observed in these three thyroid cancer cell lines is consequent to cell cycle regulator protein inhibition by reversine, and Aur is most likely involved. In our study, ARO and WRO cells treated with reversine had obvious tetrapolidization but less apoptosis, compared to SW579 cells. The possible explanation is that reversine-mediated cell death in ARO and WRO cells was through different mechanisms than in SW579 cells. The definite mechanisms need to be further explored. However, we cannot rule out the involvement of other types of cell death mechanisms in the reversine-mediated anti-thyroid cancer effect, especially with ATC and FTC.

Reversine, a 2,6-disubstituted purine, is an ATP analogue and suspected to be able to inhibit various kinds of cellular enzymatic activities, not only limited to Aur (12). According to the literature, in the cellular model of human acute myeloid leukemia, concentrations of reversine at 1.0 μM, in addition to Aur, could suppress other enzymes involved in cell cycle regulation, such as CKD2/cycin E and CKD3/cyclin E, and could also suppress enzymes involved in cell growth signaling, such as MEK1, PDK1, and PKA (12). In another cellular model of multiple myeloma cell lines and mouse embryonic fibroblast-adipose-like cells, in addition to Aur-B and Aur-C, reversine was shown to suppress enzymes involved in cell growth signaling, such as in the JAK2, SRC and Akt pathways (6,12). Therefore, further investigation of the impact of reversine on the kinase profiles in ARO, WRO, and SW579 cell lines would be worthwhile. This would answer questions about the different cellular response to reversine in cell cycle changes and cell death. Further more detailed exploration of kinase profile differences between various types of thyroid cancer tissues and normal thyroid tissues is needed.

In the ARO xenograft tumor model, 28-day reversine treatment reduced tumor growth significantly compared to

the control group. However, there was no significant difference between oral administration of 0.1 mg/kg and 1.0 mg/kg once weekly. This suggested that higher-dosage reversine does not increase its efficacy. The limitations of this study are the few dosage titration comparisons, short treatment duration, and inadequate dosage administration. Moreover, whether drug delivery by other routes could elevate the anti-tumorigenic efficacy should be further investigated. However, at the end of the experiment, all mice were alive and active. The safety of reversine can be appreciated.

CONCLUSION

In this study, we prove the anti-tumor effects *in vitro* and *in vivo* of reversine on human thyroid cancer cells through cell cycle arrest or cell apoptosis. ATC is more susceptible to the anti-cancer effects of reversine than PDTC or FTC. In addition, we are the first to demonstrate that apoptosis is induced by reversine through a mitochondria-independent pathway. Altogether, reversine is a potential drug candidate against incurable thyroid cancer and is worthy of further clinical investigation.

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