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Micheliolide inhibits gastric cancer growth *in vitro* and *in vivo via* blockade of the IL-6/STAT3 pathway

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Upregulation of pro-inflammatory cytokine interleukin (IL)-6 is observed in gastric cancer tissue, and high IL-6 serum levels predict a poor prognosis of gastric cancer patients. The IL-6/STAT3 pathway has been confirmed to play essential roles in the process of carcinogenesis, including gastric cancer. Thus, blockade of the IL-6/STAT3 pathway may be a potentially effective therapeutic option for gastric cancer. Micheliolide (MCL), a guaianolide sesquiterpene lactone, possesses anti-inflammatory properties and can attenuate the IL-6 level. In addition, MCL has been widely reported to possess anti-tumor activity. But the anti-cancer effect of MCL on gastric cancer is unclear. In this study, we detected the effects of MCL on gastric cancer cell proliferation and apoptosis by performing MTT, colony formation, TUNEL and western blot assays, and found that MCL inhibited gastric cancer cell proliferation and promoted apoptosis *in vitro.* We further investigated the molecular mechanism by which MCL played an efficient role against gastric cancer, and found that the IL-6/STAT3 pathway is involved in the anti-cancer effect of MCL on gastric cancer. *In vivo* experiments further confirmed this conclusion. Taken together, MCL inhibits gastric cancer growth *in vitro* and *in vivo* via blockade of IL-6/STAT3 pathway.

1. Introduction

Gastric cancer is the second most common cause of cancer-related deaths, and its incidence rate shows substantial variation internationally, with highest rates in East Asia, including Korea, Japan and China (Alberts et al. 2003; Larsson et al. 2006; Roder 2002). About 90% of gastric cancers are adenocarcinomas, and infection with *Helicobacter pylori* is identified as a key step of the carcinogenetic process (Correa et al. 2004; Fox and Wang 2007). The inflammatory response to *H. pylori* is very strong, and includes the release of pro-inflammatory cytokines such as interleukin (IL)-6, which has been reported to be enhanced in the gastric mucosa of *H. pylori*-infected subjects (Crabtree et al. 1991; Furukawa et al. 1998). In addition, upregulation of IL-6 is observed in gastric cancer tissue, and a high IL-6 serum level predicts a poor prognosis of gastric cancer patients). Several IL-6-related signaling pathways have been identified and associated with increased proliferation, epithelial-mesenchymal transition (EMT), invasion and migration, and decreased apoptosis of various tumor cells (Lou et al. 2000; Wei et al. 2001; Yadav et al. 2011). The IL-6/STAT3 pathway is among the most widely studied, and also has been confirmed to play a crucial role in the initiation and progression of gastric cancer (Mejías-Luque et al. 2008; To et al. 2004; Wang et al. 2013). Therefore, the blockade of IL-6/STAT3 pathway may be a potentially effective therapeutic option for gastric cancer.

Micheliolide (MCL), a guaianolide sesquiterpene lactone, can be isolated from *Michelia compressa* and *Michelia champaca* and semi-synthesized from parthenolide with 90% yield, which has been shown to attenuate the levels of multiple pro-inflammatory cytokines and mediators such as iNOS, COX-2, TNF-α, IL-6 and IL-1β, and inhibit various inflammatory responses (Sun et al. 2017; Zhai et al. 2012). In recent years, it has also been shown that MCL possesses anti-tumor activity, against acute myelogenous leukemia, colorectal cancer, malignant gliomas and breast cancer (An et al. 2015; Ji et al. 2016; Jia et al. 2015a; Jia et al. 2015b; Viennois et al. 2014; Zhang et al. 2012). However, it's role in gastric cancer is still unclear. In this study, we investigated the anti-cancer effect of MCL on gastric cancer and the potential mechanisms *in vitro* and *in vivo*.

2. Investigations and results

2.1. MCL inhibits gastric cancer cell proliferation in vitro

The effects of MCL on the viability of AGS and N87 cells were determined by MTT assay. As shown in Fig. 1A and B, the viability of AGS and N87 cells decreased with MCL concentration increasing from 2.5 to 20 μM, with no difference at the concentration of 20 and 40 μM. We also detected the proliferation markers Ki67 and PCNA expression in AGS and N87 cells treated with 20 μM of MCL or not by western blot assay. The results showed that MCL treatment significantly decreased Ki67 and PCNA expression (Fig. 1C and D). To further characterize the effects of MCL on gastric cancer cell proliferation, colony formation assay was performed. The results showed that the number of colonies was markedly reduced in response to 20 μM of MCL treatment compared with untreated controls (Fig. 1E and F). Taken together, these data indicated that MCL inhibits gastric cancer cell growth *in vitro.*

2.2. MCL promotes gastric cancer cell apoptosis in vitro

The effects of MCL on the apoptosis of AGS and N87 cells were determined by TUNEL assay. As shown in Fig. 2A and B, exposure of AGS and N87 cells to 20 μM of MCL caused a significant increase in the number of TUNEL-positive cells compared with the control group. In addition, pro-apoptotic protein Bax, and anti-apoptotic protein XIAP were detected by western blot assay. The results showed that MCL treatment significantly increased Bax expression, and decreased XIAP expression (Fig. 2C and D). These data indicated that MCL promotes gastric cancer cell apoptosis *in vitro.*

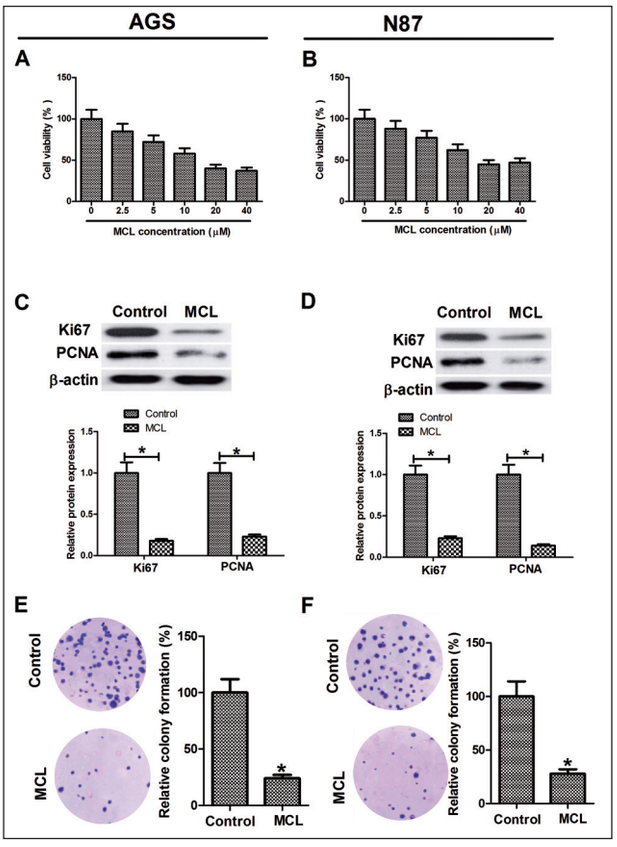

Fig. 1: Effects of MCL on proliferation in AGS and N87 cells. (A and B) After treatment with serial concentrations (0, 2.5, 5, 10, 20 and 40 μM) of MCL for 48 h, the viability of AGS and N87 cells was determined by MTT assay. (C and D) After treatment with 20 μM of MCL for 48 h, Ki67 and PCNA expression in AGS and N87 cells were detected by western blot assay. (E and F) The number of colonies in AGS and N87 cells was markedly reduced in response to 20 μM of MCL treatment compared with untreated controls. $^*P < 0.05$.

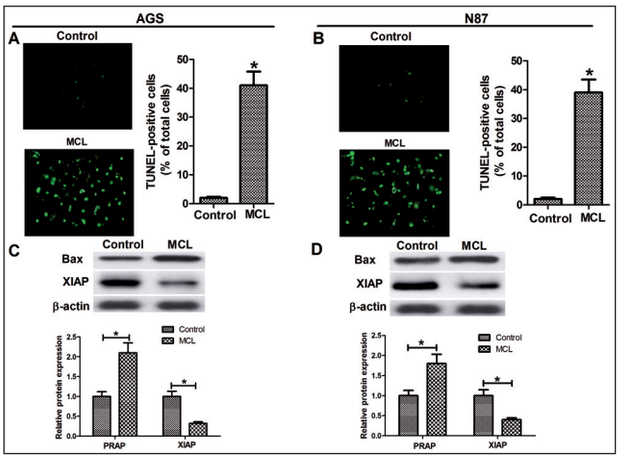

Fig. 2: Effects of MCL on apoptosis in AGS and N87 cells. (A and B) After MCL treatment (20 μM) for 48 h, cellular apoptosis in AGS and N87 cells was examined by TUNEL assay. The stained cells were counted, and the percentage of positive cells was calculated. (C and D) AGS and N87 cells were treated with MCL (20 μM) for 48 h, and Bax and XIAP protein expression were determined by western blot assay. $^*P < 0.05$.

2.3. MCL inhibits IL-6/STAT3 signaling in gastric cancer cells

Since MCL can attenuate IL-6 level, and IL-6-induced STAT3 pathway plays a crucial role in the initiation and progression of gastric cancer, we hypothesized that MCL may function *via*

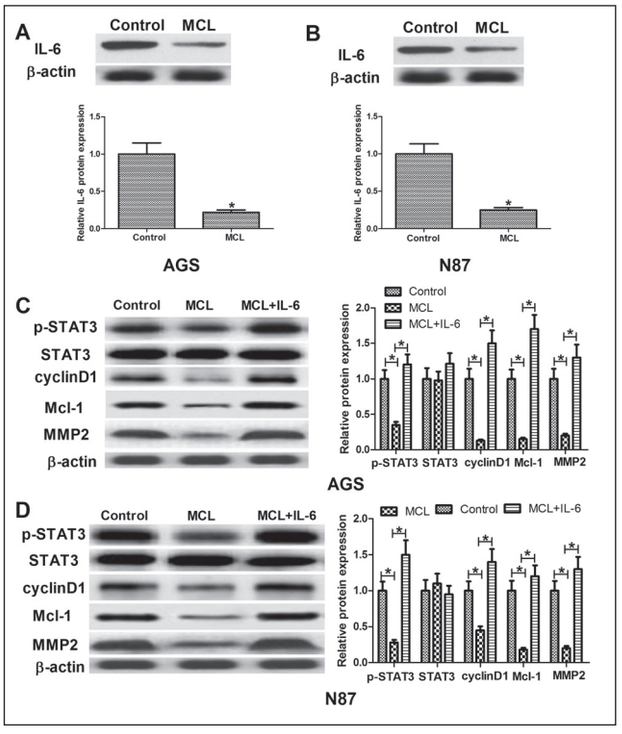

Fig. 3: MCL inhibited IL-6/STAT3 signaling in gastric cancer cells. (A and B) After treatment of AGS and N87 cells with MCL (20 μM) for 48 h, IL-6 expression was detected by western blot assay. (C and D) After treatment of AGS and N87 cells with MCL (20 μM) and/or human recombinant IL-6 (50 ng/mL) for 48 h, phosphorylated STAT3 (p-STAT3), STAT3, and STAT3 target genes cyclinD1, Mcl-1 and MMP2 expression were detected by western blot assay. $^*P < 0.05$.

blockade of IL-6-induced STAT3 pathway in gastric cancer cells. We first examined the expression of IL-6 protein in MCL-treated AGS and N87 cells by western blot assay. As shown in Fig. 3A and B, IL-6 expression was obviously reduced by the treatment of 20 μM MCL. In addition, we also determined the effects of MCL on phosphorylated STAT3 (p-STAT3), STAT3, and STAT3 target genes cyclinD1, Mcl-1 and MMP2 expression. The results showed that the treatment of cells with 20 μM MCL significantly reduced p-STAT3, cyclinD1, Mcl-1 and MMP2 expression, but had no effect on total STAT3 protein expression (Fig. 3C and D). These data indicated that MCL treatment inhibits IL-6 expression and STAT3 pathway. We further examined whether MCL suppressed the STAT3 pathway *via* inhibition of IL-6 expression. We cultured AGS and N87 cells in a medium with human recombinant IL-6 (50 ng/mL) and MCL (20 μM) for 48 h, and then detected p-STAT3, STAT3, cyclinD1, Mcl-1 and MMP2 expression. The results showed that combination treatment of IL-6 and MCL abolished the effects of MCL treatment on p-STAT3, cyclinD1, Mcl-1 and MMP2 expression, which suggested that exogenous IL-6 could restore the STAT3 pathway. Collectively, these data indicated that MCL inhibits STAT3 pathway via suppression of IL-6 expression in gastric cancer cells.

2.4. MCL inhibits gastric cancer growth in vivo

To further confirm that the anti-cancer effect of MCL on gastric cancer, we established subcutaneous tumor xenografts by injection of AGS cells into a single side of the posterior flank of each mouse. Thirteen days after injection, the tumors were measurable (about 2 mm), and MCL treatment began. Mice received the prodrug form of MCL DMAMCL (dimethylamino Michael adduct of MCL, 50 mg/kg p.o.) that slowly releases MCL *in vivo* every day. Tumor size was monitored once every three days. Eighteen days after treatment, all mice were sacrificed, and the tumors were excised, weighed, imaged, and used for western blot detection. The results showed that the treatment group had less tumor volume and

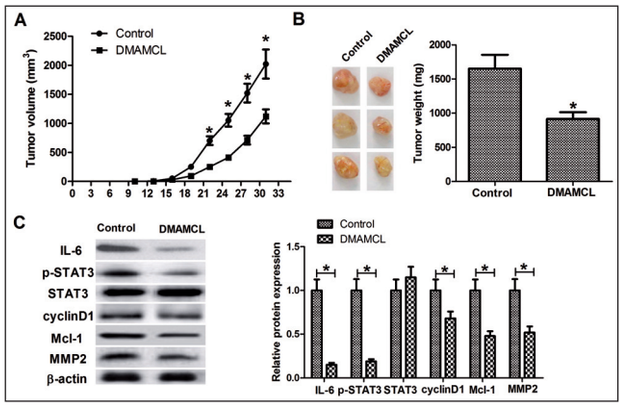

Fig. 4: MCL inhibited gastric cancer growth *in vivo*. Subcutaneous tumor xenografts were established by injection of AGS cells into a single side of the posterior flank of each mouse. When the tumors were measurable (13 days after injection), MCL treatment began. Mice received oral administration of the prodrug form of MCL DMAMCL (50 mg/kg) every day. Tumor size was monitored once every three days. Eighteen days after treatment, all mice were sacrificed, and the tumors were isolated, weighed, imaged, and used for western blot detection. (A and B) The DMAMCL treatment group had significantly less tumor volume and tumor weight than the control group. (C) IL-6, p-STAT3, cyclinD1, Mcl-1 and MMP-2 expression were significantly decreased in the xenografts of the DMAMCL treatment group compared with the xenografts of the control group. $*P < 0.05$.

tumor weight than the control group (Fig. 4A and B). In addition, compared with the control group, the xenografts in the treatment group showed significant decreases in IL-6, p-STAT3, cyclinD1, Mcl-1 and MMP-2 expression (Fig. 4C). Taken together, these data indicated that MCL inhibits gastric cancer growth *in vitro* and *in vivo* via blockade of IL-6/STAT3 pathway.

3. Discussion

Gastric cancer is one of the most common malignancies worldwide, and nearly 1 million new cases are diagnosed each year (Ezzati et al. 2005), and more efficacious anti-cancer agents are needed. Many natural compounds and their derivatives have been reported to inhibit the pathogenesis and development of gastric cancer. For example, flavonoids, a group of polyphenolic compounds, induce G2/M phase arrest and apoptosis in human gastric cancer AGS cells; hesperidin, a bioflavonoid, induces apoptosis in human gastric cancer SNU-668 cells; sulforaphene, a structural analog of sulforaphane, promotes Bax/Bcl2, MAPK-dependent human gastric cancer AGS cell apoptosis and inhibits migration via EGFR, p-ERK1/2 down-regulation; and parthenolide, a natural sesquiterpene lactone, suppresses tumor growth and enhances response to chemotherapy in gastric cancer (Lee et al. 2012; Mondal et al. 2016; Park et al. 2007; Sohma et al. 2011). Micheliolide (MCL), a novel guaianolide sesquiterpene lactone, can be synthesized from parthenolide, and has higher stability, less toxicity, and more sustainable release from prodrug than parthenolide (Zhang et al. 2012). In recent years, many studies have shown that MCL has high anti-cancer activity in different cancers. For example, Ji et al. (2016) and Zhang et al. (2012) reported that MCL inhibits cell proliferation and selectively induces apoptosis in leukemic stem/progenitor cells. Viennois et al. (2014) reported that MCL inhibits the activation of pro-inflammatory pathways and attenuates carcinogenesis in AOM/DSS-induced colorectal cancer. Jia et al. (2015a, b) reported that MCL induces breast cancer cell death *via* regulation of dynamin-related protein 1 expression, and overcomes KlF4-mediated cisplatin resistance by downregulating glutathione. In addition, DMAMCL, a pro-drug form of MCL, has also been reported to significantly prolong the lifespan of a mouse model of human acute myelogenous leukemia and inhibit glioma cell growth *in vitro* and *in vivo* (An et al. 2015; Ji et al. 2016). But the anti-cancer effect of MCL on gastric cancer is unclear. In this study, we detected the effects of MCL on gastric cancer cell proliferation and apoptosis by performing MTT, colony formation, TUNEL and western blot assays, and the results showed that MCL treatment inhibited gastric cancer cell proliferation and promoted apoptosis *in vitro*. In addition, nude mice xenograft studies also showed that MCL inhibited gastric cancer cell growth *in vivo*. These results indicated that MCL possesses anti-cancer effect on gastric cancer and may serve as a potentially novel anti-cancer agent for gastric cancer treatment. Then we further investigated the molecular mechanism by which MCL played an efficient role against gastric cancer.

IL-6 is a pleiotropic cytokine primarily involved in the regulation of inflammation and aberrant immunity, and can be attenuated by MCL (Sun et al. 2017). In recent years, many studies have shown that IL-6 contributes to malignant progression *via* activation of multiple pathways and regulation of various biological processes, such as proliferation, apoptosis, EMT, invasion, and migration. IL-6 acts through different classic protein kinase pathways depending upon the cell type. For example, IL-6 induces prostate cancer cell growth accompanied by activation of STAT3 signaling pathway; IL-6 promotes head and neck tumor metastasis by inducing EMT via the JAK-STAT3-SNAIL signaling pathway; and IL-6 inhibits human cervical cancer cell apoptosis by upregulation of Mcl-1 through a PI3-K/Akt pathway (Lou et al. 2000; Wei et al. 2001; Yadav et al. 2011). Among these pathways, IL-6/STAT3 pathway is the most widely studied, and plays essential roles in the process of carcinogenesis. Briefly, IL-6 binds to the IL-6 receptor (IL-6R) and triggers the dimerization of the signal transducer receptor gp130, leading the activation of STAT3 *via* the Janus-associated kinase. Upon activation, STAT3 rapidly translocates into the nucleus, and subsequently activates target genes such as cyclinD1, Mcl-1, MMP2, and Bcl-2 *via* binding to recognition sequence in the promoter of target genes. All these target genes are involved in the major cellular responses, including cell proliferation, apoptosis, invasion and migration. In fact, IL-6/STAT3 pathway has been confirmed to promote gastric cancer progression. For example, Wang et al. (2013) reported that increased IL-6-induced activation of STAT3 is observed in neoplastic gastric tissue, which positively correlates with tumor progression; To et al. (2004) found that the IL-6-mediated STAT3 pathway is constitutionally activated through hypermethylation of SOCS-1 in gastric cancer cells; and Mejías-Luque et al. (2008) reported that IL-6 activates the STAT3 pathway and induces MUC4 expression, thereby promoting gastric cancer progression. Based on these findings, we performed cell experiments and further investigated whether IL-6/STAT3 pathway was involved in the anti-cancer effect of MCL on gastric cancer. The results showed that MCL treatment inhibited IL-6 expression and the STAT3 pathway, and exogenous IL-6 could restore the STAT3 pathway, which suggested that MCL plays anti-cancer effect via inhibition of the STAT3 pathway by down-regulation of IL-6 expression. *In vivo* experiments further confirmed this conclusion.

In conclusion, we have demonstrated for the first time that MCL inhibits gastric cancer growth *in vitro* and *in vivo* via blockade of the IL-6/STAT3 pathway. These findings suggest that MCL might be a potentially powerful anti-cancer agent for various IL-6/STAT3-dependent cancers, including gastric cancer.

4. Experimental

4.1. Cell culture

Gastric cancer cell lines AGS and N87 were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and 100 U/mL penicillin (Invitrogen). Cell cultures were maintained in a humidified incubator at 37 °C with 5% CO_2.

4.2. MTT assay

Cell viability was determined using an MTT assay. Briefly, exponentially growing AGS and N87 cells were seeded into 96-well plates at a density of 5×10^4 cells/well, and then treated with serial concentrations (0, 2.5, 5, 10, 20 and 40 µM) of MCL (Accendatech Co., Ltd., Tianjin, China) for 48 h after adhesion. After medium removal, 1 mg/mL MTT-containing medium was added for further incubation at 37 °C for 4 h, followed by addition of 100 µl dimethylsulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA), and shaking for 15 min in the dark. The absorbance at 570 nm was measured using a microplate reader (UVM 340, ASYS Hitech GmbH, Eugendorf, Austria).

4.3. Colony formation assay

AGS and N87 cells were seeded into 6-well plates at a density of 300 cells per well, and cultured in medium containing 20 μM of MCL or not to allow colony formation. The medium was replaced every 3 days. After 2 weeks, colonies were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet (Sigma Chemical Co.) for 1 h. The stained colonies were photographed, and manually counted.

4.4. TdT-mediated dUTP nick-end labeling (TUNEL)

Cell apoptosis was determined using the One Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Briefly, AGS and N87 cells were seeded into 6-well plates, and maintained in media containing 20 μM of MCL or not for 48 h. Then cells were collected and washed with PBS, followed by fixation using 4 % paraformaldehyde phosphate for 30 min, incubation in cold PBS containing 0.1 % Triton X-100 for 2 min in a dark condition, and incubation in a TUNEL reaction buffer for 1 h at 37 °C. Fluorescence images were obtained using a fluorescent microscopy (Olympus, Japan), and TUNEL-positive cells (apoptotic cells) were counted.

4.5. MCL and IL-6 treatment of gastric cancer cells

AGS and N87 cells were cultured in the medium with or without human recombinant IL-6 (50 ng/mL, R&D systems, Minneapolis, MN, USA) and MCL (20 μM) for 48 h. Then protein expression was detected by western blot assay as described below.

4.6. Western blot assay

Total proteins were extracted from tissues or cultured cells with RIPA lysis buffer containing 1 % protease inhibitor. Protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) according to the instructions of the manufacturer. About 30 μg of total proteins was mixed with an equal volume of 2 × sample buffer, boiled at 95 °C for 5 min, subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred onto a 0.22 μm PVDF membrane (BD Biosciences, San Diego, CA, USA) using a semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). After blocking with 5% nonfat dried milk for 1 h, the membranes were probed with specific primary antibodies overnight at 4 °C. After washing in Tris-buffered saline with 0.1 % Tween 20 (TBST), the membranes were probed with horseradish-peroxidase (HRP)-conjugated secondary antibodies (Beyotime, China) for 2 h at room temperature. The bands were developed by using ECL Western blotting detection kit (Amersham Biosciences, Uppsala, Sweden), and quantified using Quantity One Software (Bio-Rad). The following primary antibodies were employed in this study: anti-Ki67 (Abcam, Cambridge, UK), anti-PCNA (Abcam), anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-XIAP (Abcam), anti-IL-6 (R&D system), anti-p-STAT3 (Cell Signaling Technology, Beverly, MA, USA), anti-STAT3 (Cell Signaling Technology), anti-cyclinD1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Mcl-1 (Chemicon, Temecula, CA, USA), anti-MMP2 (Chemicon), and anti-β-actin (Santa Cruz Biotechnology) that was used as housekeeping gene control.

4.7. Nude mice xenograft studies

All animal procedures were in agreement with the Guide to the Care and Use of Experimental Animals formulated by the Canadian Council of Animal Care (1993, CCAC, 2nd ed.) and approved by the Ethics Committee of Tiantai Branch of Zhejiang Provincial People's Hospital. Six-week old BALB/c athymic nude mice were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and housed in sterile laminar flow cabinets under appropriate pathogen-free conditions. Subcutaneous tumor xenografts were established by injection of 100 μl PBS containing 1×10^7 AGS cells into a single side of the posterior flank of each mouse. Tumor size was measured once every three days using calipers, and tumor volume was calculated using the formula: (length × width^2)/2. When the tumors were measurable, mice were randomly divided into two groups, control group and DMAMCL (dimethylamino Michael adduct of MCL) group. Mice in the DMAMCL group were treated orally with DMAMCL (50 mg/kg, Accendatech Co., Ltd.) every day. Eighteen days after DMAMCL treatment, all mice were sacrificed, and the tumors were excised, weighed, imaged, and used for western blot detection.

4.8. Statistical analyses

All statistical tests were performed on SPSS 17.0 statistical software (IBM, Chicago, IL, USA). All data were expressed as mean ± standard deviation (SD) of at least three independent experiments, and analyzed by Student's *t*-test or ANOVA test. A probability value of $P < 0.05$ was used as the criterion for statistical significance.

Conflicts of interest: The authors have stated that they have no conflicts of interest.

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