Acetyl-macrocalin B suppresses tumor growth in esophageal squamous cell carcinoma and exhibits synergistic anti-cancer effects with the Chk1/2 inhibitor AZD7762

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ABSTRACT

Natural products derived from herbal medicines have become a major focus of anti-cancer drug discovery studies. Acetyl-macrocalin B (A-macB) is an ent-diterpenoid isolated from Isodon silvatica. This study aimed to examine the effect and molecular action of A-macB in esophageal squamous cell carcinoma (ESCC) and explore possible drug synergistic modalities. A-macB induced cellular reactive oxygen species (ROS) generation, initiated the p38 mitogen-activated protein kinase (MAPK) signaling pathway, and triggered the caspase-9-dependent apoptosis cascade in ESCC cells. The ROS scavenger N-acetylcysteine (NAC) and the specific p38 inhibitor SB203580 reversed the effects of A-macB on the p38 network and thus rescued ESCC cells from apoptosis. The cellular ROS increase was at least partially due to the suppression of glutathione-S-transferase P1 (GSTP1) by A-macB. A-macB also upregulated the Chk1/Chk2-Cdc25C/Cdc2/Cyclin B1 axis to induce G2/M phase arrest. The cell growth inhibition induced by A-macB was further enhanced by AZD7762, a specific Chk1/Chk2 inhibitor, with a combination index (CI) of <1. Moreover, A-macB efficiently suppressed xenograft growth without inducing significant toxicity, and AZD7762 potentiated the effects of A-macB in the suppression of tumor growth in vivo. Taken together, A-macB is a promising lead compound for ESCC and exerts synergistic anti-cancer effects with AZD7762.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant types of cancer and has a high prevalence in Asia (Schweigert et al., 2013). Despite tremendous progress with respect to ESCC medical management, the outcome of the disease has remained unchanged during the last several decades. The five-year survival rate in patients with the disease ranges from 15% to 25% (Pennathur et al., 2013). A combination chemotherapy regimen comprising cisplatin (DDP) and 5-fluorouracil has been introduced worldwide as a treatment for ESCC (Nakajima and Kato, 2013). The overall response rate in patients with unresectable, metastatic, and recurrent esophageal cancer is 36% (Kato et al., 2011). The limited improvements in treatment outcomes and the relatively low response rate to conventional therapies have prompted a search for innovative strategies for treating ESCC (Baba et al., 2016).

Natural products derived from herbal medicines have tremendous potential and have been a major focus of drug discovery studies for decades (Shen et al., 2015). Isodon belongs to the Lamiaceae family and is widely used in traditional Chinese medicine (Zhan et al., 2013). The diterpenoids isolated from the Isodon species have been found to have many effects, including antibacterial, antiinflammatory and antitumor activities (Peters, 2010; Sun et al., 2006). Acetyl-macrocalin B (A-macB, Fig. 1A) is a major ent-diterpenoid isolated from Isodon silvatica, and we...
found that A-macB promoted apoptosis through an ROS-p38-caspase-9-dependent pathway and induced G2/M phase arrest via the Chk1/2-Cdc25c-Cdc2/Cyclin B axis in nonsmall cell lung cancer (Wang et al., 2018). However, the antitumor efficacy of A-macB against ESCC as well as the underlying mechanisms have not been investigated.

Previous studies have reported that ROS are associated with various cellular processes, including gene expression, cell proliferation, DNA damage and cell apoptosis (Park et al., 2017; Simon et al., 2000). The disruption of ROS homeostasis plays a critical role in the regulation of mitochondrial dysfunction and apoptotic events (Kim et al., 2011). Glutathione S-transferase P (GSTP) is one of the most prevalent cytosolic proteins in many tumors and is critical in facilitating and promoting glutathione (GSH)-dependent reactions under oxidative stress; the dominant member of the GSTP family is GSTP1 (Jones et al., 2016; Pasello et al., 2008). GSH provides a major source of thiol homeostasis, which is critical for the maintenance of a reduced cellular environment conducive to cell survival (Tew, 2007). GSTP1 catalyzes the conjugation of GSH with electrophilic compounds to regulate ROS generation and perform detoxification (Hang et al., 2018). Several anticancer chemicals extracted from traditional Chinese herbal medicines, such as cambogin (Shen et al., 2015), longikaurin A (Liao et al., 2014), and cudraflavone C (Lee et al., 2017), increase cellular ROS production to suppress cancer cell growth by mediating mitogen-activated protein kinase (MAPK) activation and inducing cell apoptosis. Conserved MAPKs include extracellular signal-regulated kinase (ERK), the p38 MAPKs and c-Jun NH2-terminal kinase (JNK) (Kim and Choi, 2015; Ono and Han, 2000). In particular, the p38 MAPKs are classified as “stress-activated” kinases and are associated with chemotherapeutic agent-induced apoptotic cell death (Grossi et al., 2014). Accumulating evidence shows that cytotoxic ROS signaling appears to trigger p38 MAPK activation and then induces mitochondrial membrane permeabilization and cell death (Hsieh et al., 2014; Xie et al., 2014; Xu et al., 2015).

In this study, we isolated A-macB from *Isodon silvatica* and evaluated its antitumor efficacy against ESCC in vitro and in vivo. We also elucidated the mechanisms underlying the effects of A-macB on ESCC. We discovered that A-macB was an effective lead compound that inhibited ESCC cell proliferation and induced cell apoptosis through the ROS-p38 MAPK signaling pathway. A-macB upregulated cellular ROS production partially through the inhibition of GSTP1. Furthermore, we found that A-macB induced G2/M arrest in ESCC cells through the Chk1/Chk2 checkpoint kinase-mediated pathway. The specific Chk1/Chk2 inhibitor AZD7762 sensitized ESCC cells to A-macB treatment both in vitro and in vivo. The results indicate that A-macB is effective against ESCC and warrants further evaluation.

Fig. 1. A-macB inhibits cell viability in ESCC cell lines. (A) The structure of A-macB. (B) IC50 curves for KYSE30 and KYSE450 cells treated with A-macB for 72 h. (C) Proliferation curves and inhibition rates for ESCC cells incubated with A-macB for different times and at different concentrations. (D) Clone formation by ESCC cells treated with A-macB. Data are expressed as means ± SD. (*p < .05, **p < .01, ***p < .001).
2. Materials and methods

2.1. Cell lines and animals

The ESCC cell lines KYSE30 and KYSE450 and the normal control cell lines Het-1a and HUVEC were authenticated by short tandem repeat (STR) analysis and matched to existing reference genotypes. KYSE30 and KYSE450 cells were cultivated in RPMI 1640 (Corning, Logan, UT, USA), and HUVECs were cultivated in DMEM (Corning, Logan, UT, USA) supplemented with 10% fetal bovine serum (Corning, Mediatech Inc., Manassas, VA, USA). Het-1a cells were maintained in BEGM (CC-3170, Lonza, Walkersville, MD, USA). Female BALB/c nude mice (3–4 weeks old, 13–17 g) were purchased from Huafukang Bioscience (Beijing, China). The mice were maintained under pathogen-free conditions and fed a standard commercial diet produced by the Experimental Animal Center of the Chinese Academy of Medical Sciences. Other main reagents and antibodies used in this study are described in the supplementary materials.

2.2. Cell viability assay

Cell viability was assessed by the CCK8 assay (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, cells were seeded in 96-well plates (2000/well) and treated with different doses of A-macB. The optical density (OD) was measured at 450 nm by a SpectraMax® 190 (Molecular Device, Sunnyvale, CA, USA). GraphPad Prism 5 (GraphPad Software Inc., CA, USA) was used to fit a dose-response curve to determine the IC50 value, where the X-axis values are logarithms of the A-macB concentration and the Y-axis values represent cell viability percentages upon treatment with A-macB. Then, the IC50
curve was fitted by nonlinear regression with the equation of “log(inhibitor) vs. response – Variable slope (four parameters)”.

For plate clone formation, cells were seeded in 6-well plates (500 cells/well), treated with A-macB or 0.1% DMSO for 24h and then cultured in cell culture medium supplemented with 10% fetal bovine serum containing neither drug nor DMSO for another 10 days. The colonies were then fixed and stained for visualization and counting.

2.3. Cell apoptosis analysis by flow cytometry

Cells were cultured in 6-cm plates overnight. Then, the cells were treated with different concentrations of A-macB. After treatment, the cells were collected, stained with Annexin V-FITC/propidium iodide (PI) (BD Biosciences, NJ, USA), and then immediately analyzed using a BD Flow Cytometer (Becton Dickinson FACSCanto II).

2.4. Immunofluorescence

A total of $2 \times 10^5$ cells were seeded in chamber slides and cultured overnight. After treatment, the cells were fixed and stained directly with Phospho-Histone H2A.X mAb and Cleaved Caspase-3 mAb. The cytoskeleton was stained using Phalloidin-iFluor 488 (ab176753, Abcam, Cambridge, UK). Images were captured using the UltraVIEW® VoX (PerkinElmer Inc., Waltham, MA, USA) confocal system.

2.5. Detection of cell cycle distribution

Cells were seeded in 6-cm dishes overnight and then synchronized in serum-free medium for 12 h. After A-macB treatment, the cells were harvested and fixed with cold 70% ethanol overnight. Then, cells were subjected to RNase A digestion and PI solution staining (KGA512, KeygenTec., Jiangsu, China). Cell cycle distribution was determined by a BD Flow Cytometer.

2.6. Intracellular ROS production

Intracellular ROS production was monitored by the cell-permeable fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (S0033, Beyotime, Jiangsu, China). The cells were collected and stained with DCFH-DA for 30 min while protected from light. The stained cells were then assessed immediately at an excitation wavelength of 488 nm and an emission wavelength of 525 nm by a BD Flow Cytometer.

2.7. Western blot (WB) analyses

Cells lysates were separated by SDS-PAGE and electrotransferred onto PVDF membranes (Millipore, MA, USA). The membranes were incubated with specific primary antibodies overnight at 4°C and incubated with the appropriate secondary antibody. The bands were...
visualized by peroxidase reactions using an ECL detection system (Millipore) (Kumar et al., 2015; Yao et al., 2017).

2.8. In vivo tumor xenograft study

A total of \(1.2 \times 10^6\) KYSE30 cells were transplanted into the right flank of each mouse. The PDX model was established by our team and identified by two pathologists. At 9 days after transplantation, the mice were randomly divided into five groups and intraperitoneally injected with 1% Pluronic F68 (solvent, negative control), 3 mg/kg cisplatin (positive control), AZD7762 alone (25 mg/kg), A-macB (12 mg/kg), and A-macB (12 mg/kg) in combination with AZD7762 (25 mg/kg). Tumor volume and mouse weight were measured every two days. One month after treatment, the mice were sacrificed humanely, and the tumor xenografts were isolated, measured and fixed in formaldehyde solution. Tumor volume was calculated as \(V = \left(\frac{a^2 \times b}{2}\right)\), \((a, \text{ the small diameter}; b, \text{ the large diameter})\). The significance of the differences in tumor volume among the groups was analyzed by one-way ANOVA followed by Dunnett’s post hoc test, which was used to compare a set of treatments against a single control mean. Tumor growth inhibition \((\%\text{TGI})\) was calculated as \(\%\text{TGI} = \left[\frac{100 - 100 \times \text{RTV}(\text{Drug treated group})}{\text{RTV}(\text{Vehicle control group})}\right] \times 100\%\), where the relative tumor volume \((\text{RTV})\) = \(\sqrt{\frac{V_t}{V_1}}\), \(V_t\) (Tumor mean volume at a given time) \(V_1\) (Tumor mean volume at the start of treatment).

2.9. Immunohistochemistry (IHC)

Ki-67 and cleaved caspase-3 expression in tumor xenograft tissues were evaluated by IHC. Briefly, the tissue sections were deparaffinized, rehydrated, subjected to antigen retrieval and incubated with the appropriate primary antibodies overnight at 4°C. Then, the slides were incubated with the appropriate secondary antibody. The target proteins were visualized by DAB (Sun et al., 2017a).

2.10. Terminal deoxynucleotidetransferase-mediated dUTP end-labeling (TUNEL)

The TUNEL assay was performed to label apoptotic cells in xenograft tissues. After deparaffinization and rehydration, the tissues were fixed in paraformaldehyde and digested with Proteinase K solution. The samples were subsequently incubated with rTdT incubation buffer for 60 min at 37°C. The cell nuclei were stained by DAPI, and the apoptotic signals were analyzed by fluorescence microscopy (Nikon, Tokyo, Japan) (Kumar et al., 2015).

2.11. Statistical analysis

All in vitro experiments were repeated three times, and the data were expressed as means ± SD. GraphPad Prism 5 was used to analyze
the significance of the differences among the groups, and one-way ANOVA followed by Dunnett’s post hoc test was used to analyze multiple comparisons. Student’s t-test was performed to analyze comparisons between two independent groups. \( p < .05 \) was regarded as statistically significant.

3. Results

3.1. A-macB suppresses cell growth and colony formation in ESCC cell lines

The extraction and isolation of A-macB is described in the supplementary methods, and the spectroscopic A-macB data are shown in the supplementary results and Fig. S1–S4. The effects of A-macB on the cell viability of KYSE30 and KYSE450 cells were evaluated by CCK8 assays. The results showed that A-macB significantly inhibited KYSE30 and KYSE450 cell proliferation (Fig. 1B–C) and colony formation (Fig. 1D) in a dose-dependent manner. The corresponding IC50 values were 1.42 \( \mu M \) and 1.43 \( \mu M \), respectively, when A-macB was administered for 72 h. In addition, the results showed that the normal cell lines Het-1a and HUVEC were more tolerant of A-macB treatment than the ESCC cell lines (Fig. S5).

3.2. A-macB induces ESCC cell apoptosis via the p38 MAPK-mediated intrinsic apoptotic pathway

The impact of A-macB on ESCC cell apoptosis was evaluated by flow cytometry analysis. As shown in Fig. 2A and Fig. S6, the apoptosis rates in KYSE30 and KYSE450 cells increased in a dose-dependent manner upon treatment with A-macB. After incubation with A-macB, shrinkage, blebbing, and floating cells were observed (Fig. 2B). Phalloidin labeling of the cytoskeleton also revealed changes in cell morphology (Fig. 2C). Immunofluorescence staining of phosphorylated histone H2AX (γH2AX) and cleaved caspase-3 expression indicated that DNA damage and apoptosis were induced by A-macB (Fig. 2C).

To gain insight into how A-macB induces ESCC cell apoptosis, we utilized stress and apoptosis signaling antibody arrays with Fluorescent
Readout analysis (Supplementary methods). The results showed that A-macB activated the p38 MAPK signaling pathway in a dose-dependent manner (Fig. S7), consistent with the results of the flow cytometry analyses of apoptosis. p38 MAPK activation was confirmed by WB analysis. As shown in Fig. 2D, treatment with increasing doses of A-macB resulted in gradual increases in the levels of p-p38 MAPK (Thr180/Tyr182) and its downstream target p-HSP27 (Ser82) but did not change the levels of total p38. The levels of intrinsic apoptosis-inducing proteins, such as cleaved caspase-9, cleaved caspase-3 and cleaved PARP, were upregulated in conjunction with p38 MAPK signaling, while those of the extrinsic initiator cleaved caspase-8 were not increased. These results indicated that A-macB treatment significantly
involved cell cycle arrest, we examined the cell cycle distributions by flow cytometry. (B) Dose-effect curves for AZD7762, A-macB and combination treatment with A-macB and AZD7762. (C) The combination index (CI) values of AZD7762 and A-macB against KYS30 and KYSE450 cells were calculated using the Chou-Talalay method. CI values < 1 indicated synergistic interactions between AZD7762 and A-macB. (D) WB analyses showed that AZD7762 inhibits A-macB-induced Chk1/2 activity and enhances cell apoptosis. Chk1/2 activity is suppressed by AZD7762, leading to Cdc25C hyperactivity and decreased Cdc2/Cyclin B dephosphorylation. This process enables cells to enter mitosis with damaged DNA, resulting in mitotic catastrophe, a phenomenon reflected by the expression of γH2AX, p-h3 and cleaved caspase-3. (E, F) Knockdown of Chk1/2 abrogated A-macB-induced G2/M arrest. Cells were transfected with siRNA targeting Chk1, Chk2 or Chk1 + 2 (E) and incubated with 1 μM A-macB for 24 h, and the cell cycle distributions were analyzed by flow cytometry (F). Data quantification is shown in (G). (H) Depletion of Chk1/2 stabilized Cdc25A expression, led to Cdc25C hyperactivity and decreased Cdc2/Cyclin B activity. (I, J) A-macB-induced cell cycle arrest could not be abolished by AZD7762 when the Cdc2/cyclin B inhibitor NU6102 was administered. KYS30 and KYSE450 cells were pretreated with 100 nM AZD7762 with or without 1 μM NU6102 for 2 h, and 1 μM A-macB was then administered for another 24 h; cell cycle distributions were analyzed by flow cytometry. (K) NU6102 inhibited Cdc2/Cyclin B1 activation, which was originally activated by the cotreatment of A-macB and AZD7762, forcing the arrest of cells in G2/M phase. Data are presented as means ± SD. (*p < .05, **p < .01, ***p < .001).

3.3. A-macB increases ROS generation partially via GSTP1 inhibition and promotes ROS-mediated p38 MAPK activation

ROS generation plays an important role in the proapoptotic activity of various anticancer agents (Hsieh et al., 2014; Kuo et al., 2007). Consequently, we determined the ROS status of the above cell lines by examining DCFH-DA fluorescence intensity. We observed that A-macB treatment resulted in a > 2-fold increase in cellular ROS levels (Fig. 3A, B) and an increase in the number of Annexin V (+) cells in the ESCC cell population (Fig. 3C and Fig. S9A). The antioxidant NAC effectively prevented A-macB-induced ROS production (Fig. 3A, B) and thus largely protected ESCC cells from apoptosis (Fig. 3C and Fig. S9A, B). Western blot analysis demonstrated that NAC pretreatment significantly suppressed HSP27 phosphorylation and the intrinsic apoptotic signaling pathway (Fig. 2F). These results confirmed that p38 MAPK pathway inhibition prevented ESCC cell apoptosis.

3.4. A-macB induces G2/M phase arrest through a Chk1/2-mediated pathway

As AZD7762 is a specific Chk1/2 inhibitor, we sought to determine whether the drug influences cell cycle progression and whether its inhibitory effects would sensitize ESCC cells to the cytotoxic effects of A-macB. To prevent A-macB-induced apoptotic cell death, we treated ESCC cells with nontoxic concentrations of A-macB (1 μM) (Morgan et al., 2010). The results (Fig. 3D, E) showed that combination treatment with AZD7762 and A-macB significantly abrogated A-macB-induced G2/M arrest, while AZD7762 alone had no effect on the cell cycle distribution. Furthermore, the combination treatment induced significant cell apoptosis, while single incubation with any of the agents did not cause notable cell death (Fig. 3A and supplementary Fig. S10). In addition, as shown in Fig. 6B and C, cell growth inhibition by A-macB was further enhanced when combined with AZD7762, and the combination index (CI) values were < 1, indicating a synergistic effect between A-macB and AZD7762 (Chou, 2010). WB analysis confirmed that AZD7762 effectively suppressed A-macB-induced Chk1/2 pathway activation (Fig. 6D). p-Chk1 (Ser345) and p-Chk2 (Thr68) expression levels increased significantly after A-macB exposure. These results indicated that A-macB caused cell DNA injury and triggered the Chk1/2-Cdc25C-Cdc2/Cyclin B axis to arrest the cell cycle at G2/M phase.

3.5. The Chk1/2 inhibitor AZD7762 abrogates A-macB-induced G2/M arrest and enhances cell cytotoxicity


activated the p38 MAPK signaling pathway and induced intrinsic cell apoptosis. Pretreatment with the specific p38 MAPK inhibitor SB203580 significantly reversed A-macB-induced cell apoptosis, as shown in the flow cytometry results and morphology investigation (Fig. 2E and Fig. S8). Western blot analysis illustrated that SB203580 pretreatment significantly suppressed HSP27 phosphorylation and the intrinsic apoptotic signaling pathway (Fig. 2F). These results confirmed that A-macB-induced Chk1/2 pathway activation prevents ESCC cell apoptosis.

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As GSH is one of the most abundant multifunctional antioxidants and GSTP1 participates in GSH regulation and ROS production (Hang et al., 2018; Zhang et al., 2018), we investigated whether GSTP1 and GSH were influenced by A-macB. The results indicated that both the level and activity of GSTP1 were significantly decreased by A-macB (Fig. 4A). As a consequence, GSH levels and the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) significantly decreased, and cellular ROS levels increased accordingly (Fig. 4B–D). Another important antioxidant enzyme, SOD, was not affected by A-macB (Fig. 4E). Furthermore, GSTP1 overexpression markedly depressed A-macB-induced cellular ROS generation; GSTP1 knockdown enhanced ROS levels, but the effect of GSTP1 knockdown was milder than that of A-macB treatment (Fig. 4F–I). These results indicated that A-macB upregulated cellular ROS production partially through inhibition of GSTP1.

3.4. A-macB induces G2/M phase arrest through a Chk1/2-mediated pathway

To examine whether the cell growth inhibition induced by A-macB involved cell cycle arrest, we examined the cell cycle distributions by flow cytometry. As shown in Fig. 5A and B, 2 μM A-macB treatment significantly arrested cells in G2/M phase, while 4 μM A-macB exposure induced cell apoptosis at a notable level. WB (Fig. 5C) showed that A-macB treatment significantly increased the expression of γH2AX, an indicator of DNA damage. The expression of p-Chk1 (Ser345) and p-Chk2 (Thr68) subsequently increased in response to the DNA damage to delay the cell cycle at G2/M phase. The activated Cdc2/Cyclin B1 complex acts as the G2/M entry engine (Lee et al., 2016; Rhind and Russell, 2012). Cdc2 can be inactivated by phosphorylation by Cdc25C (Shaltiel et al., 2015; Zhang et al., 2016), which is the downstream target of Chk1/2 (Sun et al., 2017b). In the present study, the expression levels of the positive regulatory molecules, namely Cdc25C, Cdc2 and Cyclin B1, were suppressed by A-macB treatment (Fig. 5C). By contrast, p-Cdc2 (Tyr15) levels gradually increased after A-macB exposure. These results indicated that A-macB caused cell DNA injury and triggered the Chk1/2-Cdc25C-Cdc2/Cyclin B axis to arrest the cell cycle at G2/M phase.
Knockdown of Chk1 and Chk2 markedly abrogated A-macB-induced G2/M arrest; moreover, Chk1 and Chk2 double knockdown not only abolished G2/M arrest but also induced considerable cell apoptosis (Fig. 6E–G). WB assessment indicated that Chk1/2 knockdown stabilized Cdc25A expression, restored the expression of Cdc25C and Cyclin B1, and abrogated Cdc2 dephosphorylation (Fig. 6H). These results indicated that the Chk1/2-Cdc25C-Cdc2/Cyclin B axis plays a crucial role in A-macB-induced cell cycle arrest. In a complementary study, using the Cdc2/Cyclin B inhibitor NU6102 (Johnson et al., 2010), A-macB-induced cell cycle arrest could not be abolished by AZD7762 (Fig. 6I, J). WB showed that AZD7762 restored the activation of Cdc2/Cyclin B1, which was suppressed by A-macB, but NU6102 inhibited Cdc2/Cyclin B1 activation to arrest cells in G2/M phase (Fig. 6K). Collectively, these results demonstrated that AZD7762 could abrogate A-macB-induced G2/M arrest and enhance A-macB-induced cell cytotoxicity.

Fig. 7. A-macB treatment suppresses tumor growth in vivo. (A) A-macB and DDP significantly inhibited xenograft tumor tissue growth both in KYSE30 cells and in an ESCC PDX model. Furthermore, the administration of AZD7762 enhanced the antitumor activity of A-macB. The mice were randomly divided into five groups (n = 6). (Control: 1% Pluronic F68 treated group, AZD7762: 25 mg/kg AZD7762 treated group, DDP: 3 mg/kg cisplatin treated group, A-macB: 12 mg/kg A-macB treated group, A-macB + AZD7762: 12 mg/kg A-macB in combination with 25 mg/kg AZD7762 treated group). (B) Growth curves of the different treatment groups. The significance indicated the differences of tumor volume compared with controls, which was analyzed by one-way ANOVA followed by Dunnett’s post hoc test. (*p < .05, **p < .01, ***p < .001). (C) Representative IHC images of Ki67 and cleaved caspase-3 staining in ESCC xenografts.

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3.6. A-macB inhibits tumor growth in vivo

To detect the antitumor activity of A-macB in vivo, we established KYSE30 xenografts and a patient-derived xenograft (PDX) model and treated the model with different modalities as described in the methods section. The results showed that the inhibition rates of both A-macB and DDP exceeded 50%. In addition, the combination group exhibited an inhibition rate > 90% (Fig. 7A, B).

The inhibitory effects of A-macB and the combination treatment were verified in tumor tissues using IHC. As shown in Fig. 7C, in the vehicle control-treated samples, strong nuclear Ki67 staining was observed in the absence of cleaved caspase-3 expression. However, a lack of Ki-67 staining and considerable cleaved caspase-3 expression were observed in the A-macB- and DDP-treated samples, especially in the A-macB+AZD7762 combination treatment group. The levels of apoptosis induced by each treatment were assessed via TUNEL assay. As anticipated, we detected significant apoptotic signals in the A-macB, DDP and A-macB+AZD7762 combination groups but detected rare apoptotic signals in the negative control-treated group (Fig. 8A, B). In addition, hematoxylin and eosin (H&E) staining of tumor tissues showed apparent histopathological disorganization in the A-macB- and DDP-treated samples, especially in the combination treatment group (Fig. S11A).

We also evaluated markers of liver, kidney, heart and bone marrow injury to evaluate drug safety. As shown in Fig. S11B, no significant histological structural or pathological alterations were found in the mouse organs, including the heart, liver, and kidney, in all groups, while DDP treatment led to significant injury to liver and marrow function as indicated by increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and decreased white blood cells (WBC) (Fig. 9A, B). No mouse body weight loss was observed in any of the treatment modalities (Fig. S12A, B).

4. Discussion

Natural products are rich sources of drugs and have garnered increasing attention as anti-cancer agents (Ma et al., 2013). Our team discovered that A-macB from Isodon silvatica exhibited potent cytotoxicity against ESCC while displaying a good safety profile both in vitro and in vivo. We investigated the mechanisms underlying the effects of A-macB against ESCC and discovered that the compound induced G2/M phase arrest and promoted cell apoptosis. We also determined that A-macB induced G2/M phase arrest via the Chk1/2-Cdc25C-Cdc2/Cyclin B axis and induced apoptosis through the ROS/p38/caspase-9-dependent apoptotic pathway in KYSE30 and KYSE450 cells. Moreover, Chk1/2 inhibition by AZD7762 sensitized ESCC cells to the chemotherapeutic effects of A-macB.

Oxidative stress and ROS hyperactivity play critical roles in the determination of cell fate (Son et al., 2011; Son et al., 2013). Several chemotherapeutic agents, including many natural products, exert their antitumor effects by inducing ROS overproduction and eventually apoptosis (Hao et al., 2015; Liao et al., 2014; Ma et al., 2015). In the
In the present study, A-macB significantly increased ROS generation, leading to severe oxidative damage and, consequently, cell apoptosis in ESCC cells. Pretreatment with NAC, an ROS scavenger, suppressed the expression of the DNA damage marker γH2AX and prevented cell apoptosis. GSH is one of the most abundant and multifunctional intracellular antioxidants in the defense against oxidative stress (Lu, 2013; Sentellas et al., 2014). Glutathione disulfide (GSSG) is the oxidized form of GSH. GSH/GSSG is often used as a measure of cellular oxidative stress, with a higher ratio signifying less oxidative stress (Lu, 2013). GSTP1 is a putative catalyst of protein S-glutathionylation reactions and plays an important role in GSH homeostasis (Hang et al., 2018; Jones et al., 2016; Khan et al., 2018). In our research, we demonstrated that A-macB treatment downregulated GSTP1 expression and activity, resulting in the inhibition of cellular GSH, thus increasing ROS generation. GSTP1 overexpression mitigated ROS accumulation, while depletion of GSTP1 with siRNA increased ROS. These results indicated that, at least partially, A-macB upregulated cellular ROS production through the inhibition of GSTP1.

Accumulating evidence has demonstrated that chemotherapeutic agent-induced p38 MAPK activation and oxidative stress are interdependently correlated (Hao et al., 2015; Olson and Hallahan, 2004; Park et al., 2011; Park et al., 2017). In the present study, we showed that A-macB increased ROS generation and promoted ROS-mediated p38 MAPK activation. Consequently, activated p38 signaling initiated caspase-9-dependent intrinsic cell apoptosis. This finding was confirmed by the results of experiments involving the antioxidant NAC and the p38-specific inhibitor SB203580. Both NAC and SB203580 decreased the levels of apoptosis induced by A-macB by inhibiting p38 MAPK signaling. Pretreatment with NAC, which prevented A-macB-induced ROS accumulation, significantly suppressed p38 MAPK phosphorylation and inhibited p38 MAPK enzyme activity. Co-administration of SB203580 with A-macB significantly inhibited the p38 signaling pathway without influencing p38 phosphorylation; this inhibitory function was reflected by a reduction of the phosphorylation of HSP27, which is the downstream target of p38 MAPK. Previous studies have reported that SB203580 exerts its inhibitory effect by preventing ATP binding without affecting p38 phosphorylation (Han and Sun, 2007; Xu et al., 2015), consistent with our result.

Many anticancer agents cause DNA damage, resulting in the activation of cell cycle checkpoints and proliferation arrest (Montano et al., 2012). In the present study, A-macB treatment caused obvious DNA damage as indicated by the increased expression of γH2AX. In response to DNA injury, the critical kinases Chk1/Chk2 were activated. As a result, Cdc25C, Cdc2 and Cyclin B1 were suppressed to arrest cells in G2/M phase. AZD7762 is a specific Chk1/2 inhibitor and is reported to sensitize tumor cells to some chemotherapeutic agents (Isono et al., 2015).

Fig. 9. Measurement of serum biochemical parameters of the xenograft mice. (A and B) Measurement of serum biochemical parameters in xenograft mice, including serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), urine nitrogen (UN), creatinine (CREA), creatine kinase (CK), white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb) and platelets (PLT). P values were determined by one-way ANOVA followed by Dunnett’s post hoc test. (**p < .01, ***p < .001). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2017; Itamochi et al., 2014; Zabludoff et al., 2008). In our study, coadministration with AZD7762 abrogated A-macB-induced G2/M phase arrest and caused more severe DNA damage, leading cells to premature mitosis. The enhanced phosphorylation of Chk1/2 may be a consequence of more pronounced DNA injury induced by the combination drug treatment, consistent with previous studies (Barker et al., 2016; Morgan et al., 2010; Zabludoff et al., 2008). Activating Chk1/2 usually induces Cdc25A protosomal degradation (Furusawa et al., 2017; Visconti et al., 2016); thus, the high Cdc25A expression noted in our study indicated the inhibitory effect of AZD7762 on Chk1/2. Loss of Chk1/2 function facilitated Cdc25C activity and prevented the inhibitory phosphorylation of Cdc2, resulting in Cdc2/Cyclin B hyperactivity. Ultimately, cells containing severely damaged DNA were forced into mitosis, leading to mitotic catastrophe and cell death. In addition, coadministration of A-macB and AZD7762 resulted in enhanced inhibitory efficacy with CI values of $< 1$, indicating a synergistic effect between A-macB and AZD7762 (Chou, 2010). In complementary studies, using si-Chk1/2 or the Cdc2/cyclin B inhibitor NU6102 confirmed that the Chk1/2-Cdc25C-Cdc2/cyclin B axis is involved in A-macB-induced cell cycle regulation.

The inhibitory effects of A-macB were confirmed in vivo. In the present study, A-macB showed significant antitumor activity both in KYSE30-derived xenografts and an ESCC PDX model. Previous studies have reported that AZD7762 shows chemosensitizing activity in xenograft models with a variety of DNA-damaging agents, including gemcitabine, irinotecan and DDP (Goteti et al., 2010; Itamochi et al., 2014; Xu et al., 2011; Zabludoff et al., 2008). Given that A-macB functioned as a DNA-damaging agent and AZD7762 enhanced the inhibitory response of A-macB in ESCC cell lines, the synergistic effect of AZD7762 and A-macB was evaluated in mouse xenograft. The results showed that the in vivo inhibition rate of A-macB exceeded 50%, and the combination group exhibited an inhibition rate > 90%. Furthermore, no obvious adverse effects have been detected in A-macB monotherapy and AZD7762 + A-macB co-administration, while DDP treatment led to significant liver and marrow function injury as indicated by increased ALT and AST levels and decreased WBC. Taken together, the in vivo studies demonstrated that AZD7762 potentiated A-macB in the suppression of tumor growth without potentially harmful side effects.

In summary, the results of this study showed that the novel di-terpenoid A-macB is a promising lead compound that may be a useful and relatively safe chemotherapy for ESCC. In addition, combination of A-macB with AZD7762 may have a chemosensitizing effect. Further evaluation of the clinical efficacy and combination modalities of A-macB or its derivatives with possible sensitizers may provide clinicians with a strategy for fighting ESCC.

Author contributions

J.H., P.T.P. and N.S. perceived, designed and supervised the study. J.N.W. performed the experiments and wrote the paper; Y.C., Z.Y.Y., Z.R.Z., and R.D.L. helped with experimental design and exertion. Y.C., Z.Y.Y., Z.L.L., and Y.L. analyzed the data; J.H., P.T.P., N.S. and N.L. provided the funding acquisition; J.W. and H.D.S. provided necessary and support in the animal experiments. The clinical ESCC samples used for PDX establishment were histopathologically and clinically diagnosed at the Cancer Institute and Hospital of the Chinese Academy of Medical Science with written consent and approval from the institutional research ethics committee. The animal experiments were approved by the Cancer Institute and Hospital of the Chinese Academy of Medical Sciences Institutional Animal Care and Use Committee (IACUC; permission number: NCC2015A095).

Declaration of competing interests

The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2019.01.005.

References


