Mangrove dolabrane-type of diterpenes tagalsins suppresses tumor growth via ROS-mediated apoptosis and ATM/ATR–Chk1/Chk2-regulated cell cycle arrest

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Natural compounds are an important source for drug development. With an increasing cancer rate worldwide there is an urgent quest for new anti-cancer drugs. In this study, we show that a group of dolabrane-type of diterpenes, collectively named tagalsins, isolated from the Chinese mangrove genus Ceriops has potent cytotoxicity on a panel of hematologic cancer cells. Investigation of the molecular mechanisms by which tagalsins kill malignant cells revealed that it induces a ROS-mediated damage of DNA. This event leads to apoptosis induction and blockage of cell cycle progression at S-G2 phase via activation of the ATM/ATR–Chk1/Chk2 check point pathway. We further show that tagalsins suppress growth of human T-cell leukemia xenografts in vivo. Tagalsins show only minor toxicity on healthy cells and are well tolerated by mice. Our study shows a therapeutic potential of tagalsins for the treatment of hematologic malignancies and a new source of anticancer drugs.

Key words: apoptosis, cancer, ATR, ATM, Chk1, Chk2, Cdc25A

Additional Supporting Information may be found in the online version of this article.

With an increasing cancer rate worldwide and increasing acquired drug resistance during cancer therapy, there is an urgent quest for new anticancer drugs. Natural products are an important source of drugs in medicine. Marine mangroves, distributed widely in Africa, south pacific islands and south Asia including China, have long been used as a folk remedy for treatment of various diseases such as sores, hemorrhages, malaria and malignant ulcers.1,2 The mangrove genus Ceriops has recently attracted attention due to containing rich secondary metabolites with antibacterial and larvicidal activities.3,4 The main constituents of the secondary metabolites of Ceriops are diterpenoids and triterpenoids.5,6

In the course of searching for natural anticancer compounds, we have investigated 18 species of mangrove plants collected from South of China. Among them, the MeOH extract of the species Ceriops tagal showed significant cytotoxicity to a malignant cell line HL-60. Further investigation of the extract of C. tagal leads to identification of a group of dolabrane-type diterpenes and a nortriterpene, collectively named tagalsins compounds.7–9 So far, little is known about the biological activities of these compounds. Because some terpenoids have been reported to show cytotoxicity toward cancer cells,10–12 this information prompted us to investigate the therapeutic potential of the mangrove tagalsins for cancer treatment.

In this study, we show that 9 of 11 tagalsins are toxic to cancer cells. Investigation of the molecular mechanisms by which tagalsins exert their toxicities on cancer cells revealed that they block cell cycle progression at S-G2 phase and induce caspase-regulated apoptotic cell death in a ROS-dependent manner. The anticancer activity of tagalsins was further confirmed by a mouse model xenografted with human leukemic T cells. Our study suggests that diterpenes of mangroves may be a new source of anticancer compounds.

Material and Methods
Preparation of tagalsins
All tagalsins were isolated from stems and twigs of C. tagal as described previously.7–9 The structure characterizations of TA to TH were described in Ref. 7; T9 and T10 in Ref. 9, and T11 in Ref. 8. The yield of TC is about 25 mg kg−1.
What’s new?
Mangroves of genus *Ceriops*, widespread and highly utilized in China, are of growing interest in anticancer drug development due to their production of potentially cytotoxic diterpenoids and triterpenoids. Here, a group dolabrane-type diterpenes known as tagalsins isolated from the species *C. tagal* are shown to possess potent killing effects on cancer cells of hematologic origin. Cell death was associated with the production of reactive oxygen species and DNA damage. *In vivo*, tagalsins significantly delayed the development of human T-cell leukemia in a murine xenograft model.

Cells and cell cultures
The human malignant cell lines used in this study are the acute T cell leukemia lines Jurkat, SupT1, Molt-4 and CEM, the human myeloma cell lines U-266 and RPMI-8266, and the Hodgkin lymphoma cell lines L1236 and KM-H2. All cell lines were cultured in RPMI 1640 medium (GIBCO laboratories, Grand Island, NY) supplemented with 10% FCS, 50 μg ml⁻¹ gentamicin (GIBCO), 6 mM HEPES (GIBCO, 1 M solution), and 2 mM L-glutamine (GIBCO, 200 mM solution) at 37°C and 5% CO₂.

Preparation of human peripheral blood T cells
Human T cells (>90% CD3 positive) were isolated from peripheral blood of healthy donors as previously described.13 freshly isolated T cells were cultured as above at 2 × 10⁶ cells ml⁻¹ and activated with 1 μg ml⁻¹ PHA for 16 hrs. The activated T cells were then washed three times and further cultured for an additional 5 days (termed D6 T cells) in the presence of 25 U ml⁻¹ IL-2.

Preparation of leukemia cells from patients
Primary acute myeloid (AML) leukemia cells were obtained from patients (detailed information from the patients will be provided upon request) by Ficoll gradient and cultured in RPMI medium supplemented with 10% FCS, 2 mM glutamine, 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin at 37°C and 5% CO₂.

Cell cycle analysis
For cell cycle analysis, approximately 1 × 10⁶ cells were collected, lysed in 150 μl of Nicoletti-buffer (0.1% Na-citrate, 0.1% Triton X-100 and 50 μg ml⁻¹ propidium iodide) and stored at 4°C overnight in the dark. The propidium iodide stained DNA fragments were quantified by flow cytometry (FACSCanto II).

Determination of apoptosis
Cells were treated for the indicated periods of time at 37°C with solvent DMSO or different concentrations of tagalsins (>98% pure, assessed by HPLC) as indicated in the respective figures. Apoptotic cell death was determined by analysis of DNA fragmentation as previously described.13 Specific apoptosis was calculated as (percentage of experimental apoptosis – percentage of spontaneous apoptosis)/(100 – percentage of spontaneous apoptosis) × 100.

Western blot analysis
For each sample, 1 × 10⁷ cells were lysed as described previously.13 Equal amounts of proteins were separated on 7.5–13% SDS-PAGE depending on the molecular sizes of the proteins, blotted onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) and blocked with 5% non-fat dry milk in PBS/Tween (0.05% Tween-20 in PBS). The following antibodies were used: anti-ATM (D2E2), anti-phospho-ATM (10H11.E12), anti-ATR, anti-phospho-ATR, anti-Cdc25B, anti-Cdc25C (5H9), anti-Cdk4, anti-Cdk6, anti-phospho-Chk1 (Ser317), anti-phospho-Chk1 (133D3, Ser345), anti-Chk2, anti-phospho-Chk2 (Th68), anti-cyclin D3 and anti-cyclin E (HE12) from Cell Signaling Technology (Danvers, USA); anti-phospho-Cdc25A (Ser178) and anti-phospho-Cdc25A (Ser75) from Abgent (San Diego, CA, USA); anti-Cdc25A (Clone DCS-120) from NeoMarkers (Thermo Scientific, UK); anti-Chk1 (FL-475) from Santa Cruz Biotechnology (Heidelberg, Germany); anti-γH2AX (JBW301, Ser139) from Millipore (Millpore, MA); anti-γH2AX (Alexa-Fluor-488) from Biozol Diagnostics (Eching, Germany) and anti-tubulin from Sigma (St. Louis, USA).

Intracellular γH2AX staining analysis and comet assay
For intracellular FACS staining of γH2AX, 1 × 10⁶ cells were washed twice with PBS and fixed with 3% paraformaldehyde for 10 min at 37°C. Cells were then permeabilized with 90% methanol for a minimum of 30 min at 4°C or left overnight at −20°C. After fixation and permeabilisation cells were washed three times with 0.5% BSA in PBS and were blocked with 5% mouse serum (inactivated) in PBS and stained with 5 μl γH2AX Alexa Fluor 488 antibody for 1 hrs at RT in the dark. Cells were washed once again and co-stained with 25 μg ml⁻¹ propidium iodide containing 100 ng μl⁻¹ RNase A for 20 min at RT in the dark. Subsequent fluorescence intensities were examined by flow cytometry (FACSCanto II). A minimum of 1 × 10⁴ cells per sample were analysed. The comet assay was carried out with the OxiSelect™ Comet Staining Kit (Cell Signaling Technology, USA).

Stems and twigs. To obtain large amounts of TC for the *in vivo* mouse experiment, total 100 kg of stems and twigs of *C. Tagal* were used to obtain 2.5 g of TC by the same protocol. The purities of all compounds were controlled by HPLC and they were about 99% pure.
Assay Kit (Catalog Number STA-350, Cell Biolabs) (Biocat, Heidelberg, Germany).

**Redox measurement**
TC (2.5 and 5 μM) treated cells were stained for 30 min with 5 μM of the H$_2$O$_2$-sensitive fluorescent dye dichlorofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR) at 37°C in the dark, washed three times with PBS and subsequently assayed by FACScan (FL-1).

**In vivo mouse studies**
Immunodeficient mice (Rag2$^{-/-}$/IIL2rg$^{-/-}$) were implanted subcutaneously in the dorsal flank region with CEM ($2 \times 10^7$ cells). Four days after xenografting, mice were separated into three groups. One group received no treatment and two groups were treated with either vehicle (DMSO) or TC 50 mg kg$^{-1}$ per day in the first week and followed by treatment three times per week. TC dissolved in DMSO was diluted in sunflower oil and were administered by intraperitoneal injection (i.p.). The tumor size was measured with a micrometer caliper and the tumor volume ($V$) was calculated by the formula $V = (\text{width}^2 \times \text{length})/2$. All protocols using and maintaining animals were approved by the German Animal Protection Authority (Office Regierungspräsidium, Karlsruhe). Treated and control animals were compared for differences in tumor weight after end of treatment using the non-parametric method in a one-sided statistical test (STEST) at the significance level of 0.05.

**Results**
**Tagalsins induce apoptotic cell death in cancer cells**
To investigate the effects of tagalsins on cancer cells, we first tested their cytotoxicities to the human acute T cell leukemia cell line Jurkat. Among 11 tagalsins tested (Fig. 1a), TC was shown to have highest toxicity to Jurkat T cells with a dose that kills 50% Jurkat T cells at ~1 μM (Fig. 1b). Tagalsins TA, TB, TE, TF, and T10 were 10 to 20 times less toxic than TC (EC$_{50}$ = ~10 to 20 μM) (Fig. 1c). At the concentrations of 40 μM, TD, TG and TW were also shown cytotoxicity to

![Chemical structures of tagalsins used in our study.](image1)

![Tagalsins induce apoptosis in Jurkat T cells.](image2)
Jurkat T cells. TH did not show detectable toxicity at the concentration of 40 μM (Fig. 1c).

We then examined the cytotoxicity of TC to various hematopoietic malignant cell lines including the human acute T cell leukemia Molt-4, CEM and Sup-T1, the human Hodgkin lymphoma L1236 and KM-H2 and the human myelomas U-266 and RPMI-8266. For all cell lines tested, TC was shown to kill the malignant cells with an EC50 concentration at about 1 μM (Figs. 2a and 2b). To investigate the potential use of tagalsins as anticancer drugs, primary AML cells freshly isolated from five patients were treated with TC. All AML samples were shown to undergo apoptotic cell death in a dose-dependent manner (Fig. 2c).

To further investigate the potential use of tagalsins for cancer treatment, we examined the toxicity of TC to normal cells. The human peripheral blood T cells were freshly isolated from four healthy donors. Because most anticancer drugs target proliferating cells, we generated proliferating T cells by pre-stimulation of the freshly isolated T cells with PHA overnight and then further culturing them for 5 days (D6 T cells). The proliferating T cells were treated with different doses of TC as indicated. After 48 hr treatment, apoptotic cells were determined by DNA fragmentation in triplicates. Results are average of duplicate assays.

To investigate the mechanisms by which tagalsins kill Jurkat T cells, we carried out a Western blot analysis to examine their effects on caspases. The analysis showed that TC (which showed the highest killing activity among the 12 tagalsins) and TF (which was 10 times less active than TC) activate caspase-9, -8, -3, and -2 and induce cleaved PARP in Jurkat T cells. TH did not show detectable toxicity at the concentration of 40 μM (Fig. 1c).

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Jurkat T cells (Fig. 3a). Consistent with its killing efficiency, TC showed stronger activation of caspases compared to TF (Fig. 3a). No caspase activity was induced by TG at the same concentration (20 μM) (Fig. 3a). TC-mediated activation of caspases and PARP cleavage were also observed in primary AML cells after TC treatment (Fig. 3b). To confirm the role of caspases in tagalsin-induced cell death, Jurkat T cells were treated with TC and TF in the absence or presence of the pan-caspase inhibitor zVAD-fmk. The experiment showed that TC- and TF-induced apoptotic cell death was almost completely blocked by zVAD-fmk (Figs. 3c and 3d). Thus, tagalsins kill tumor cells in a caspase-dependent manner.

**Tagalsins induce apoptosis via ROS-mediated DNA damage**

Two diterpenoids, clerocidin and salvicine, have been shown to exert their cytotoxicity by inducing DNA double-strand breaks (DSBs). Therefore, we asked whether tagalsins also induce cell death via DNA damage. In general, when DNA is damaged, DSBs trigger recruitment of ataxiatelangctasia mutated kinase (ATM) to the damage site which in turn phosphorylates histone H2AX (yielding γH2AX) resulting in foci formation at the damage site. Thus, we used γH2AX as readout to follow the status of foci formation in TC-treated Jurkat T cells. Consistent with the cytotoxic concentrations shown in Figure 1, at the dose of 5 μM we observed strong induction of γH2AX by TC but not by the other tagalsins (Fig. 4a).

We further investigated foci formation by FACS following tagalsin treatment. For a positive control, we used doxorubicin, a well-known DNA damage inducing anticancer drug, in parallel. The experiments showed that similar to the effect of doxorubicin, treatment with 5 μM TC resulted in γH2AX foci formation in 6.1% cells within 4 hrs and in more than 60% cells after 8 hrs (Fig. 4b). An approximate 20% of γH2AX foci formation could also be observed by treatment with 20 μM TF at 8 hrs of treatment (Fig. 4b). Consistent with the cytotoxicities (Figs. 1c and 2b), no increase in γH2AX foci-positive cells was detected by treatment with 20 μM TG (Fig. 4b). To further investigate the effect of TC on DNA, we carried out a single cell gel electrophoresis assay (or comet assay) to measure DNA damage in individual cells. As shown in Figure 4c, treatment with 5 μM TC for 12 hrs yielded a classic “comet tail” observed under the microscope. These data demonstrate that tagalsins exert their cytotoxicity by inducing DNA damage.

A number of currently used anticancer drugs are DNA cross-linking reagents. Reactive oxygen species (ROS) may also cause DNA damage. To investigate the mechanisms of tagalsins-induced DNA damage, we compared tagalsins with doxorubicin (trade name adriamycin) which damages DNA.
by d(CGTACG) intercalation. Jurkat T cells were treated with TC, TF or doxorubicin in the absence or presence of the anti-oxidant N-acetyl-cysteine (NAC). The experiment showed that NAC completely inhibited TC- and TF-induced γH2AX foci formation (Fig. 4d). In contrast, NAC only partially (35%) blocked doxorubicin-induced γH2AX foci...
formation (Fig. 4d). This experiment demonstrates that tagalsin-mediated DNA damage is mainly induced by ROS.

To further verify the role of ROS in tagalsin-induced apoptosis, we examined the TC-induced cell death in the absence or presence of NAC. Consistent with the effect of NAC on γH2AX foci formation, NAC largely (about 80%) blocked TC-induced apoptotic cell death (Fig. 4e). Western blot analysis showed that NAC inhibits TC-induced caspase activities (Fig. 1f). This data demonstrate that tagalsins-induced cell death is largely regulated by a ROS-mediated DNA damage mechanism.

A study reported that salvicine, a diterpenoid quinone compound, triggers DSBs and apoptosis by generation of hydrogen peroxide (H2O2).14 Therefore, the redox status of Jurkat T cells treated with TC was monitored by the oxidation-sensitive fluorescent dyes, DCFDA (for detecting H2O2). However, no H2O2 could be detected in Jurkat T cells treated with TC (Supporting Information Fig. S1). Therefore, TC acts differently than salvicine.

**Tagalsins activate ATM and ATR and block cell cycle at S-G2 phase**

ATM and ataxia-telangiectasia and Rad3-related (ATR) kinases are generally activated in response to DNA damage.21,22 Indeed, we observed that TC induces phosphorylation of ATM and ATR at the Ser1981 and Ser428 site, respectively (Fig. 5a). Chk1 and Chk2 are the cell cycle checkpoint kinases downstream of ATM and ATR.21,23 Chk1 and Chk2 were shown to be phosphorylated at the Ser317 and Thr68 site, respectively (Fig. 5a). Chk1 and Chk2 are known to regulate the stability of the cell division cycle phosphatases 25A (Cdc25A) and Cdc25B.21,23 TC treatment was shown to induce phosphorylation of Cdc25A at the Ser178 site, and, consequently, degradation (Fig. 5a). Degradation of Cdc25B was also seen after 4 hrs treatment with TC (Fig. 5a).

Cdc25A and Cdc25B are known to play an important role in promoting the G1-S and S-G2 transition during the cell cycle by activation of CDK2-Cyclin E and CDK2-Cyclin A.21,23 Consistent with the degradation of Cdc25A and Cdc25B (Fig. 5a), cell cycle was arrested at the S-G2 phase after TC treatment (Fig. 5b). To investigate the role of ROS in TC-induced cell cycle arrest, we examined the status of TC treatment in the absence or presence of NAC. NAC was shown to completely prevent TC-induced S-G2 arrest. In contrast, doxorubicin-induced S-G2 arrest could not be completely rescued by NAC (Fig. 5b). These data further demonstrate that TC inhibits cell cycle progression and induces...
apoptosis in cancer cells by a ROS-mediated DNA damage mechanism (Fig. 5c).

**TC suppresses growth of xenografted human tumor in vivo**

To further confirm the anti-cancer activity of TC, we carried out an in vivo study in Rag2<sup>-/-</sup>II2rg<sup>-/-</sup> immunodeficient mice xenografted with the human CEM leukemic T cells. Four days after xenografting, mice were randomly separated into three groups (seven mice in each group). One group received no treatment and two groups were treated with either vehicle (DMSO) or TC (i.p.). Two weeks after xenografting, the tumor sizes were measured each second or third day. The experiment showed that all control mice (DMSO and untreated) developed tumors in 2 weeks after xenografting. In contrast, in TC-treated group no tumor developing was seen in two mice and significantly delayed tumor growth in the rest of the mice (Fig. 6a). At the end of the experiment, mice were sacrificed and the tumors were weighted. Treatment with TC showed significant reduction in tumor weight ($p$ values $= 0.017$) (Figs. 6b and 6c). TC treatment was well tolerated with only little loss of body weight (Fig. 6d). These data demonstrate that TC has an anti-cancer effect in vivo. Because the tolerant single dose of TC in mice is 1.85 g kg<sup>-1</sup>, higher doses may be tested in future studies.

**Discussion**

In this study, we show that tagalsins induce apoptotic cell death in a panel of hematologic cancer cell lines and in primary AML cells in vitro and suppress growth of xenografted human leukemic T cells in vivo in mice. Importantly, tagalsins show very low toxicity on normal peripheral blood lymphocytes.

Induction of apoptosis in cancer cells is one of the strategies of anti-cancer therapy. We show that tagalsins induce cancer cells to undergo apoptotic cell death in a dose- and caspase-dependent manner (Figs. 1 and 2). Investigation of the molecular mechanism of tagalsins-induced cell death revealed that ROS plays an essential role because the death can be completely blocked by the anti-oxidant NAC (Fig. 4c). Interestingly, tagalsins seem to preferentially kill malignant cells rather than normal cells (Fig. 3). The selectivity of tagalsins may be explained by the fact that tumors have an increased metabolism and often show a metabolic switch to aerobic glycolysis (Warburg effect) known to influence the metabolism of cancer cells.

Figure 6. Effect of tagalsin on tumor growth in vivo. (a) TC suppresses tumor growth in vivo. H-2<sup>q</sup>Rag<sup>-/-</sup>II2rg<sup>-/-</sup> mice were xenografted with $2 \times 10^7$ CEM cells per mouse. Four days after grafting, mice were randomly separated into three groups (seven mice in each group). One group was treated with TC. The other two groups were either not treated or treated with DMSO, respectively. After 2 weeks of grafting, tumor size was measured on days indicated. (b) Tumor weight was determined at the end of the experiment. (c) Photographs of the tumors. (d) The body weight at the end of the experiment. $p$ values are indicated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
redox status. Thus, tumors, particularly in advanced stage, produce elevated levels of ROS.\textsuperscript{24–26} Cancer cells with increased oxidative stress are likely more vulnerable by further ROS insults induced by exogenous agents. Recent studies suggest that this biochemical property of cancer cells can be exploited to achieve therapeutic activity and selectivity.\textsuperscript{24,26}

Although a number of natural compounds has been reported to kill tumor cells by generation of ROS, their mechanisms how ROS is induced are still largely unknown. The terpenoids artesunate and artemisunic have been shown to kill tumor cells through a ROS-dependent mechanism.\textsuperscript{27–28} The mechanism underlying pharmacological activity of Artemisinin in both malaria and cancer is thought to involve the heme-mediated decomposition of the endoperoxide bridges of the compound leading to production of carbon-centred free radicals.\textsuperscript{29} Glutathione (GSH), the major small-molecule antioxidant in cells, has been implicated in the regulation of cell proliferation and apoptosis. Salvicine, a diterpenoid quinone compound, was shown to directly react with GSH leading to depletion of GSH and generation of H\textsubscript{2}O\textsubscript{2}.\textsuperscript{14} However, we did not detect generation of H\textsubscript{2}O\textsubscript{2} by TC treatment (Supporting Information Fig. S1). ROS may also be generated via DNA damage. However, we saw that tagalsin-mediated DNA damage could be completely blocked by NAC, whereas, doxorubicin-mediated DNA damage could not (Fig. 4d). Thus, DNA damage is unlikely the cause of ROS in the tagalsin case.

Although DNA damage causes genomic instability and, ultimately, may lead to cancer, induction of DNA damage, such as DNA double-strand breaks (DSB), has been shown to be an effective treatment of cancer.\textsuperscript{18} In fact, most currently used anti-cancer drugs, e.g., Cisplatin, Camptothecin, Etoposide, Doxorubicin, etc., act by inflicting DSB.\textsuperscript{18} We show that tagalsin-induced cell death involves DNA damage. This is confirmed by detection of increased levels of phosphorylated H\textsubscript{2}AX (γH\textsubscript{2}AX) by Western blot and formation of a comet tail in individual cells by comet assays (Fig. 4). γH\textsubscript{2}AX is considered to be a sensitive marker for DSB and is essential for caspase-activated DNase-mediated nucleosomal DNA fragmentation.\textsuperscript{30} In contrast to doxorubicin, we observed that TC- and TF-induced γH\textsubscript{2}AX formation can be completely blocked by NAC (Fig. 4d). Therefore, we conclude that tagalins induce DNA damage mainly via oxidative DNA damage. Several terpenoids, e.g., artemisunic, clerodin and salvicine, have been shown to induce DSBs.\textsuperscript{14–16,31} Oxidative DNA damage was implicated to be the reason for the anticancer effect of artesunate.\textsuperscript{31} Clerodin and salvicine were shown to induce DNA DSBs by topoisomerase inhibition.\textsuperscript{14–16} It was shown that clerodin alkylates non-paired guanines of DNA resulting in irreversible stimulation of DNA cleavage.\textsuperscript{15} Thus, terpenoids may induce DNA damage through different mechanisms.

Analysis of relationships between the structures and activities shows that (i) the vinyl unit at the C ring is important for the activity of the compound since replacement of this unit by hydroxycetyl (T9, IC\textsubscript{50} > 50 μM and T10, IC\textsubscript{50} = 8.75 μM) dramatically reduces the effect of the compound (TC, IC\textsubscript{50} = 1.35 μM); (ii) A ring is critical for maintaining the activity of the compounds since breaking the A ring (TH, no detectable activity, NT) results in complete loss of the activity; (iii) in the A ring, the exovinyl unit at C-4 is more active comparing to those with an epoxy group (TA, IC\textsubscript{50} = 11.25 and TB, IC\textsubscript{50} = 7.5μM); (iv) the 1,2-enol unit in the A ring is necessary for the activity of the compounds since TE (IC\textsubscript{50} = 15.31 μM) is more active than TW (IC\textsubscript{50} = 45.0 μM) which does not have this unit; (v) also, when the 1,2-enol (TC) migrates to 3,4-enol (TG, IC\textsubscript{50} > 50 μM), the activity is dramatically reduced; and (vi), hydroxylation at C-18 might be able to further enhance the activity of the compounds as shown that TF (IC\textsubscript{50} = 9.4 μM) which is hydroxylated has higher activity than TE (IC\textsubscript{50} = 15.31 μM). Taken together, 1,2-enol and 4,13-divinyl apparently favor the function of tagalins.

Deregulation of cell cycle progression is one of the hallmarks of cancer.\textsuperscript{32} Hence, targeting the regulatory components of the cell cycle machinery has been considered to be an important strategy for cancer treatment. Cdc25A regulates both G1-S and G2-M transitions and Cdc25B is involved in G2-M regulation.\textsuperscript{23,33} We show that TC down-regulates Cdc25A and Cdc25B protein expression and, consequently, arrests cell cycle at the S-G2 phase (Figs. 5a and 5b). The stability of Cdc25A protein is largely regulated by the DNA damage response kinases ATM/ATR-Chk1/Chk2.\textsuperscript{34} We show that TC induces a rapid activation of ATM/ATR-Chk1/Chk2 kinases (Fig. 5a). Current evidence suggests that Cdc25A is a rate-limiting oncogene and is therefore an important therapeutic target.\textsuperscript{35} Thus, compounds which down-regulate Cdc25A expression have potential use for cancer treatment.

Taken together, we show that tagalins inhibit tumor growth by two mechanisms: induction of apoptosis and inhibition of cell cycle progression.

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