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DNA intercalator korkormicin A preferentially kills tumor cells expressing wild type p53

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ABSTRACT

Korkormicin A belongs to a family of nature-produced cyclic depsipeptides. It has potent antitumor activity against both leukemia cell P388 and carcinoma cell M109. To further explore its potential as a cancer therapeutic, the mechanism of its antitumor activity was investigated. We found that korkormicin A can bind to DNA through intercalation. It also induces p53 phosphorylation, which leads to inhibition of p53 degradation and activation of p53-dependent transcription. Furthermore, korkormicin A preferentially induces apoptosis in transformed cells retaining wild type p53. As it has been shown that p53 usually induces apoptosis in transformed cells, but only growth arrest in untransformed cells, these results indicate that korkormicin A is a potential antitumor agent for cancers with wild type p53.

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1. Introduction

Despite tremendous progress in understanding the human genome, the signal transduction pathways, and the molecular mechanism of many cellular processes, treatment of cancer is still a major challenge as most of the drugs used in clinic displayed limited efficacy and/or significant toxicity. For the past decade, great attention has been paid to novel technologies including combinatorial chemical libraries and molecular targeting [1]. However, the small numbers of successful molecular targeting drugs and the decline of the rate of new chemotherapeutics approved by FDA lead to the argument that it is necessary to search for new anticancer agents on as many fronts as possible [2]. One of the proved methods is to screen cytotoxic agent from natural products. Korkormicin A ($C_{66}H_{84}N_{16}O_{22}$) is a cyclic depsipeptide antitumor antibiotic that was first isolated from a culture of *Micromonospora* sp. C39500 in the course of search for novel antitumor agents (Fig. 1A). It has potent antitumor activity against intraperitoneally implanted P388 lymphocytic leukemia cells and Madison 109 lung carcinoma cells [3]. The mechanism for this antitumor activity remains to be explored.

Korkormicin A is structurally similar to a number of cyclic depsipeptide antitumor antibiotics, including luzopeptins, sandramycin, echinomycin, thiocoraline, and quinoxapeptin [4,5]. Like korkormicin A, they were all identified from biological screenings and can inhibit the growth of tumor cells, such as leukemic P388 and melanoma B16, in xenographic animal tumor models [6–10]. It has been shown that at least some of these cyclic depsipeptides can bind to DNA through two planar intercalating moieties [5,11–14]. There was also evidence indicating that the binding of echinomycin to DNA affect the action of transcription factors such as HIF-1 [15]. The tumor suppressor p53 is a transcription factor that regulates cell cycle, apoptosis, and senescence [16]. In unstressed cells, p53 is maintained at very low levels mainly by ubiquitin-mediated proteasomal degradation. It has been shown that the RING finger ubiquitin ligase Hdm2 is essential for the regulation of p53. In response to diverse stresses such as DNA damage, p53 is phosphorylated at multiple residues, which activates p53 and prevents Hdm2-mediated ubiquitination, leading to the accumulation of transcriptionally active p53 in stressed cells [17]. It has also been shown that, while activated p53 often induces apoptosis in transformed cells, it only causes reversible growth inhibition in untransformed cells [16]. This led to the proposal that activation of p53 may be an effective therapeutic strategy to kill tumor cells harboring wild type p53. It has been shown that a number of small molecules that inhibit Hdm2-mediated p53 ubiquitination can selectively induce apoptosis in tumor cells, and that restoring p53 in animal models can effectively prevent tumor growth [18,19].

In the present study, we demonstrate that korkormicin A binds to DNA through intercalation. We also found that incubation of

Abbreviations: RPE, tert-immortalized human retinal pigment epithelial cells; PARP, poly (ADP-ribose) polymerase.

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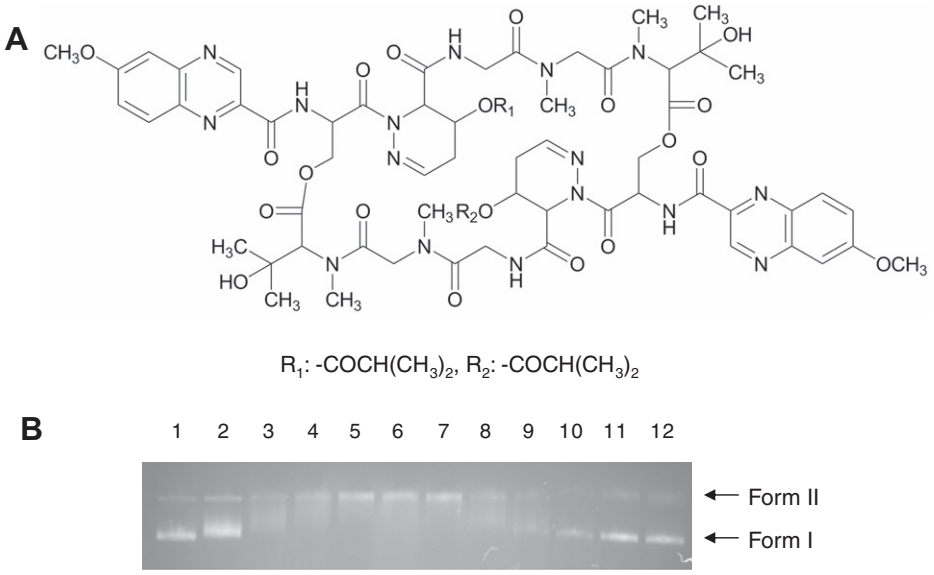

Fig. 1. Korkormicin A binds to DNA through intercalation. (A) Structure of korkormicin A. (B) pUC19 DNA was incubated with korkormicin A for 1 h and gel electrophoresis was conducted. Lane 1 is untreated supercoiled DNA. The concentration of korkormicin A is 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 1.0, 1.2, 1.5, 1.8, and 2.0 μM (lanes 2–12, respectively). Form I: supercoiled DNA, Form II: nicked or relaxed DNA.

cells with korkormicin A resulted in p53 phosphorylation and increased p53 level as well as p53-dependent transactivation. Furthermore, korkormicin A preferentially induces apoptosis in transformed cells expressing wild type p53, suggesting that it could be a potential therapeutic agent for certain types of cancers.

2. Materials and methods

2.1. Reagents, plasmids, antibodies and cell lines

Korkormicin A is a gift from Dr. J. Leet (Bristol-Myers Squibb, New York, NY). The proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) was purchased from Calbiochem (La Jolla, CA). Adriamycin and anti-β-Actin antibody were from Sigma (St. Louis, MO). Plasmid pUC19 was from New England Biolabs (Ipswich, MA). Antibodies recognizing p53 and poly (ADP-ribose) polymerase (PARP) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody recognizing phosphorylated-p53 (Ser15) was from Cell Signaling (Beverly, MA). Tert-immortalized human retinal pigment epithelial (RPE), RPE-E1A, U2OS-pG13, A9 and C8 cells were cultured as described previously [20].

2.2. DNA unwinding assay

DNA unwinding assay was performed as described previously [11,12]. Briefly, 0.18 μg of supercoiled pUC19 DNA was incubated with korkormicin A for 1 h at 25 °C. They were then separated by electrophoresis on a 0.8% agarose gel for 2 h at 90 V. The gel was stained with ethidium bromide and DNA in the gel was visualized using a UV transilluminator.

2.3. Immunoblotting

Immunoblotting was carried out as described previously [20]. Briefly, cells were lysed with the RIPA buffer supplemented with cocktails of protease inhibitors and phosphatase inhibitors. After centrifugation, resultant lysates were resolved by SDS–PAGE, transferred to PVDF membranes (GE, Kansas City, MO), incubated with specific antibodies and HRP-conjugated second antibodies, and visualized with chemiluminescence agents (Pierce, Rockford, IL) according to manufacturer's instructions.

2.4. Luciferase assay

U2OS-pG13 cells were lysed with reporter lysis buffer (Promega, Madison, WI). Luciferase activity in the supernatants was determined using the luciferase assay substrates according to manufacturer's instructions (Promega).

2.5. Cell viability

Cell viability was assessed under microscope through trypan blue staining. Equal volumes of culture medium-suspended cells and 0.4 w/v% trypan blue solution (WAKO, Osaka, Japan) were mixed for 1 min. Under a microscope, non-viable cells are stained with trypan blue, whereas viable cells excluded the dye.

2.6. WST-1 assay

The number of metabolic active cells was examined by using cell proliferation agent WST-1 according to the manufacturer's instructions (Roche, Basel, Switzerland). Briefly, cells were seeded into 96-well tissue culture cluster overnight and incubated with the compounds for indicated time in 200 μl of complete medium. Twenty micro liters of WST-1 reagent were then added to each well. Following incubation for 30 min, absorbance at 450 and 630 nm was measured using a microplate reader (Bio-Rad, Hercules, CA). The results are expressed as percentage of absorbance as compared to the solvent control wells.

3. Results

3.1. Korkormicin A binds to DNA through intercalation

Korkormicin A is structurally very similar to antitumor cyclic depsipeptide luzopeptins, sandramycin, and echinomycin, which are known to bind to DNA through intercalation [11–13]. We therefore examined whether korkormicin A could bind to DNA in a similar manner using a DNA unwinding assay. It is known that

DNA intercalators enhance the unwinding of negatively supercoiled DNA (form I), which leads to a decreased mobility in agarose gel during electrophoresis [11,12]. As shown in Fig. 1B, treatment with increasing amounts of korkormicin A gradually decreased the electrophoretic mobility of DNA (lanes 1–7), indicating that negatively supercoiled DNA turns into nicked or relaxed DNA (form II) due to the intercalation of korkormicin A. After reaching a minimal electrophoretic mobility, korkormicin A changed the DNA from form II DNA to positively supercoiled form I DNA, which led to an increased electrophoretic mobility (lanes 8–12). These data indicate that korkormicin A binds to DNA through intercalation.

3.2. Korkormicin A induces phosphorylation of p53 and increased p53 levels and p53-dependent transactivation

It has been shown that adriamycin, an effective chemotherapeutic agent and DNA intercalator, induces the phosphorylation of p53 in a variety of cells [21]. We asked whether korkormicin A also affects p53 regulation in target cells. As shown in Fig. 2A, similar to adriamycin induced p53 phosphorylation (Ser15) (lane 2), treatment with korkormicin A resulted in p53 phosphorylation in a dose-dependent manner (Fig. 2A, lane 3–8). Since the phosphorylation of p53 is a critical event in regulating its level and activity [16], we also examined the effect of korkormicin A on p53 level and activities in cells. As shown in Fig. 2A, korkormicin A increased p53 levels in a dose-dependent manner (lanes 3–8). The increase of p53 level is comparable to that was seen with adriamycin (Fig. 2A, lane 2). To assess whether korkormicin A increases transactivation of p53, we analyzed the cellular levels of Hdm2 and p21, whose transcriptions are activated by target p53. In associated with the increase of p53, both Hdm2 and p21 levels in cells were induced by korkormicin A (Fig. 2B, lane 3–6). To further evaluate the ability of korkormicin A to increase p53-dependent transactivation, we used a cell line that contains wild type p53 gene and stably transfected p53-responsive luciferase reporter pG13 (U2OS-pG13 cell). Like adriamycin, korkormicin A effectively induced luciferase activity in a dose-dependent manner (Fig. 2C). These data indicate that korkormicin A is an activator of p53 function in cells.

3.3. Korkormicin A inhibits p53 ubiquitination

In addition to affect its activity, phosphorylation of p53 can also reduce its ubiquitination through inhibiting its binding to Hdm2, which lead to decreased ubiquitin-mediated degradation and accumulation of p53 protein [22]. Therefore, we examined the effect of korkormicin A on p53 ubiquitination. Treatment of cells with

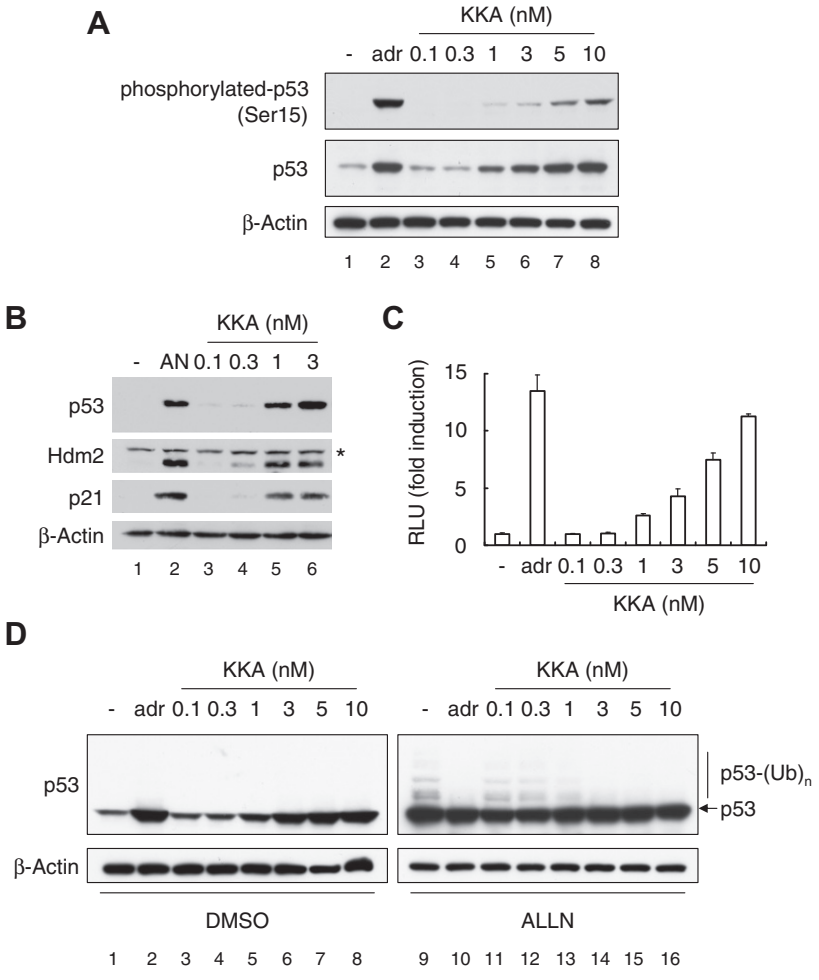

Fig. 2. Korkormicin A increases p53 phosphorylation, cellular p53 protein levels, and p53-dependent transactivation. (A) RPE cells were treated with 1 μg/ml adriamycin (adr) or 0.1–10 nM korkormicin A (KKA) for 8 h. Phosphorylated-p53 (Ser15), p53, and β-Actin levels in cells were assessed by immunoblotting. (B) RPE cells were treated with 50 μM ALLN (AN), or 0.1–3 nM korkormicin A for 8 h. Cellular p53, Hdm2, p21 and β-Actin levels were assessed by immunoblotting. The asterisk represents a nonspecific band. (C) U2OS-pG13 cells were incubated with 1 μg/ml adriamycin or 0.1–10 nM korkormicin A for 20 h and p53-dependent luciferase activity was assessed. Data represents an average and standard deviation of three independent experiments. (D) RPE cells were treated with 1 μg/ml adriamycin or 0.1–10 nM korkormicin A for 1 h prior to incubation with either DMSO (1–8) or 50 μM ALLN (9–16) for 7 h. p53 and β-Actin levels in cells were assessed by immunoblotting.

proteasome inhibitor ALLN alone prevented the degradation of ubiquitinated p53, which resulted in an increase of p53 and the appearance of ubiquitinated p53 species migrated above the native p53 band (Fig. 2D, compare to lane 1 and lane 9). When cells were exposed to ALLN in the presence of korkormicin A, the ALLN-induced accumulation of ubiquitinated p53 species was reduced in a dose-dependent manner, similar to the treatment with adriamycin (Fig. 2D, lane 10–16). These results indicated that korkormicin A induces phosphorylation of p53 and inhibits p53 ubiquitination, leading to a stabilization of cellular p53. This is also consistent with our finding that korkormicin A had no effect on p53 mRNA levels assessed by semi-quantitative RT-PCR (data not shown).

3.4. Korkormicin A preferentially induces apoptosis in transformed cells

It has been found that, compared to their untransformed parental cells, transformed cells become more sensitive to p53-induced apoptosis [16]. To examine whether p53 activator korkormicin A induces preferential cell death in transformed cells, we compared RPE cells and RPE cells transformed with adenovirus E1A (RPE-E1A), which interacts with the retinoblastoma tumor suppressor gene product but not p53 [23,24]. As shown in Fig. 3A, korkormicin A preferentially killed RPE-E1A cells but had no effect on parental RPE cells as assessed by trypan blue exclusion. To determine whether korkormicin A preferentially kills RPE-E1A cells by apoptosis, we assessed the cleavage of caspase substrate PARP in korkormicin A-treated cells. Korkormicin A induced dose-dependently PARP cleavage in RPE-E1A cells but not in RPE cells (Fig. 3B). These results indicate that korkormicin A treatment preferentially causes apoptosis of transformed cells.

3.5. Tumor cells retaining wild type p53 are more sensitive to korkormicin A

The ability of korkormicin A to activate p53 and preferentially kill transformed cells led us to ask whether it could selectively kill tumor cells retaining wild type p53. Use was made of adenovirus E1A and activated Ha-ras transformed mouse embryonic fibroblasts (MEFs) from $p53^{+/+}$ (C8) and these from $p53^{-/-}$ mouse (A9) [25]. As shown in Fig. 4A, while korkormicin A increased cell death in C8 cells in a dose-dependent manner, A9 cells were relative resistant to korkormicin A-induced cell death as assessed by WST-1 assay. Furthermore, korkormicin A induced the cleavage of PARP in C8 cells (Fig. 4B), indicating that it preferentially activates apoptotic program in tumor cells with wild type p53.

4. Discussion

Since the isolation of echinomycin in 1950s, more than 20 natural produced cyclic depsipeptides have been found from a variety of microorganisms [5]. The potent antitumor, antiviral, and antibiotic activities of this family of compounds have attracted continued interests in understanding their mechanisms of action and in identifying potential leads for clinical applications [1]. It also led to studies to decipher how these unique peptide compounds are produced and their physiological roles [26]. While korkormicins were identified as an antitumor agent, its structurally most closely related compound in the family, quinoxapeptins were initially discovered as novel inhibitors of HIV transcriptase [10]. It is generally believed that the antitumor activities of the cyclic depsipeptides depend on their ability to bind to DNA as intercalators [5]. The structural variations among the family members affect their sequence selection and induced changes in cellular processes, which include blocking the action of transcription factor HIF-1, inhibiting DNA topoisomerase II, and activating DNA repair machinery [15,27]. In the present study, we first showed that korkormicin A is indeed a DNA bisintercalator, and then demonstrated that it activates p53 system in cells. The importance of p53 activation in the antitumor activity was further indicated by the findings that korkormicin A preferentially kills transformed cells and tumor cells expressing wild type p53.

Nuclear DNA is an important target of chemotherapeutic agents, including platinum-containing compounds and adriamycin, which are DNA intercalators and are center elements of many cancer treatment regimens [28,29]. The therapeutic effects of additional DNA intercalators, such as pixantrone, are being investigated [30]. It has been shown that korkormicin A is a more effective DNA intercalator compared with pixantrone [31]. Meanwhile, several cyclic depsipeptides have also been tried in clinic for various can-

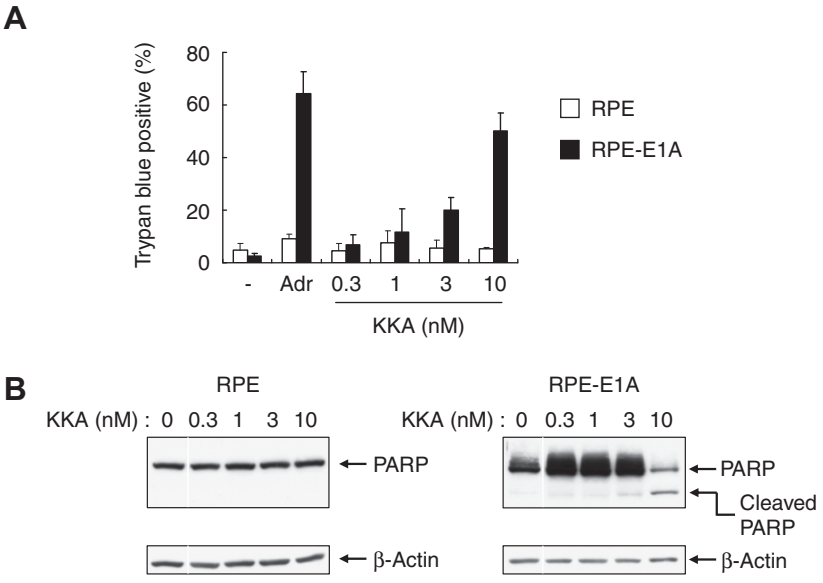

Fig. 3. Korkormicin A preferentially kills transformed cells. (A) RPE and RPE-E1A cells were treated with 1 μg/ml adriamycin or 0.3–10 nM korkormicin A for 20 h. Cell death was determined by trypan blue exclusion. Data represents an average and standard deviation of three independent experiments. (B) RPE and RPE-E1A cells were incubated with 0.3–10 nM korkormicin A for 10 h. Cells were then lysed and immunoblotted by using anti-PARP or anti-β-Actin specific antibodies.

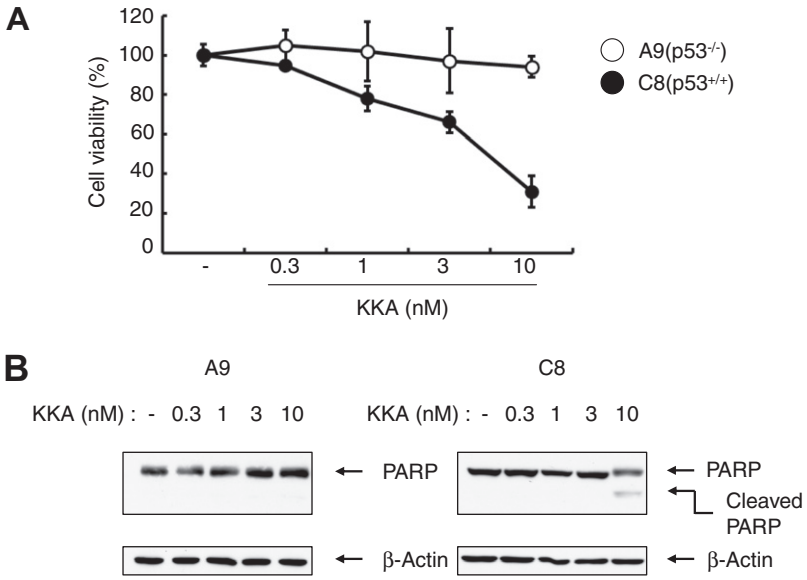

Fig. 4. Korkormicin A preferentially kills cells expressing wild type p53. (A) A9 and C8 were incubated with 0.3–10 nM korkormicin A for 11 h. Cell death was assessed by WST-1 assay. Data represents an average and standard error of three independent experiments. (B) A9 and C8 cells were exposed to 0.3–10 nM korkormicin A for 10 h. Cellular PARP and β-Actin levels were determined by immunoblotting.

cers. However, none of them have gone beyond phase II trials yet due to minimal therapeutic effects at the tolerable doses [32]. It is evident that DNA intercalators may kill cells through a number of different mechanisms. At lower dose, they may activate DNA repair machinery, and tumor cells with defects in their DNA repair system may be more sensitive to these DNA intercalators. Alternatively, compounds such as korkormicin A may function as potent activators of p53 system at relative low doses. Tumors that retain the wild type p53 and p53-responsive genes are likely to be more susceptible to korkormicin A-induced apoptosis, whereas normal cells mainly undergo reversible growth arrest. It is worth noting that one of the compounds that block the interaction of p53 with Hdm2 has gone through preclinical studies and entered clinical trials [18,33]. Given the tremendous progress in analyzing tumor genome, gene expression, and proteomics, it is likely that future preclinical or clinical studies of korkormicin A and related compounds will be based on the critical molecular defect of individual tumor or tumor cell lines. It is also conceivable that combining with PARP inhibitor or agents that inhibit p53 ubiquitination would significantly enhance their therapeutic effects.

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