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ALCAPs induce mitochondrial apoptosis and activate DNA damage response by generating ROS and inhibiting topoisomerase I enzyme activity in K562 leukemia cell line

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ABSTRACT

Endemic *Alkanna cappadocica* was used to isolate novel antitumor molecules from Turkish landscapes in our previous studies. In this study, deoxyalkannin (ALCAP1), β,β-dimethylacrylalkannin (ALCAP2), acetylalkannin (ALCAP3), and alkannin (ALCAP4) as well as the novel isolated compounds 5-methoxydeoxyalkannin (ALCAP5), 8-methoxydeoxyalkannin (ALCAP6), 5-methoxyacetylalkannin (ALCAP7), 5-methoxy-β,β-dimethylacrylalkannin (ALCAP8) were characterized. The topoisomerase I (topo I) inhibitory activity of ALCAPs was investigated using *in vitro* plasmid relaxation assay and found that ALCAP2, 3, 4 and 7 were potent inhibitors at 2–6 μM concentrations. Further, DNA damage response to ALCAP treatments was also studied by measuring the H2AX$^{(S139)}$ and ATM$^{(S1981)}$ phosphorylations. ALCAP2, 7 and 8 induced the DNA damage and apoptosis, consistently resulted in PARP cleavage at nanomolar concentrations in K562 leukemia cells. Moreover, when the free radical (ROS) generating capacity of the compounds was studied by 2′,7′-dichlorofluorescein-diacetate assay using flow cytometry, we found that a known antioxidant *N*-acetyl-cysteine almost completely abrogated the H2AX$^{(S139)}$ phosphorylations and the caspase 3 cleavage and activation. Thus, γH2AX$^{(S139)}$ foci formation remained higher than the control, and an increase in CHK2$^{(T68)}$ phosphorylation was observed by ALCAP2 and 7 treatments suggested that, these compounds can be potential therapeutics against tumor cell growth because of their unique DNA damaging abilities additional to enzyme inhibition similar to those of doxorubicin.

1. Introduction

Alkannin was isolated from the roots of *Alkanna tinctoria* Tausch, and shikonin, an *R* enantiomer constituting the major compound in red pigment extract, was obtained from *Lithospermum erythrorhizon* previously [1]. Therapeutic studies using shikonin and its derivatives have reported that these compounds are effective for burns, anal ulcers, hemorrhoids, infected crusts, bedsores and external wounds [1].

Because 1, 4 naphthoquinones constitute a class of compounds containing two ketones in the 1 and 4 positions on a naphtalene structure, 5,8-dihydroxy derivatives of this class of organic compounds are named hydroxynaphthoquinones. The biologic and pharmacological activities of alkannin and shikonin, which are from this group, and the derivatives are attributed to chiral pharmaceutical parts [1,2]. In a recent paper, Hsu et al. [3] reported that shikonin triggered apoptosis by procaspase-9 processing, caspase 3 activation and poly-ADP-ribose (PARP1) degradation, consecutively. Mao et al. also reported [4] that shikonin treatment induced apoptosis by rapid generation of reactive oxygen species (ROS) in K562 cells and resulted in marked mitochondrial cytochrome c release. Furthermore, it was also reported that these alterations induced PARP cleavage, activated c-Jun-N-terminal kinase (JNK) and resulted consequent p38 phosphorylations. Scavenging of ROS using *N*-acetyl-cysteine (L-NAC), a well-known antioxidant, completely abrogated the aforementioned effects of shikonin. Overall, the data suggested that a "redox cycle" was the primary cause of cytotoxicity and that a semiquinone radical played a central role in activation of cellular damage cascades [5].

The bioreductive alkylation mechanism could be another putative explanation for the cytotoxic behavior of these compounds [5]. Therefore, several research groups studied shikonin, and its derivatives, and showed that these compounds resulted in apoptotic

Abbreviations: γH2AX, modified and phosphorylated histone 2A; ATM, ataexia telangiectasia mutated; PARP, poly-ADP ribose polymerase; CHK2, checkpoint kinase 2; CPT-11, irinotecan; Doxo, doxorubicin; L-NAC, L-*N*-acetyl-cysteine.

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DNA fragmentation by strongly interacting with topoisomerase (topo) enzymes. Inhibition of topo I and II results in phosphorylated H2AX$^{(S139)}$ foci formation, a well known hallmark of DNA damage in intra S phase. This activates the repair response kinase, ATM$^{(S1983)}$ phosphorylations consequently [6,7]. Compounds activate the DNA damage response [2,5,8–10] result in apoptosis in tumor cells with a high DNA replication rate that can be potential therapeutics to prevent the tumor cell growth. Therefore, the topoisomerase inhibition and the oxidative stress related DNA damage process is not clearly defined in prostate cancer, H2AX$^{(S139)}$ and ATM$^{(S1981)}$ phosphorylations in K562 and PC-3 prostate tumor cells via ALCAP compounds were studied, yet the apoptosis was not induced.

In our study, previously identified and purified derivatives of alkannin [11–13] were investigated for their biologic activities and found that ALCAP2 and 7 are potent activators of ROS generation, result in cytochrome c release from the mitochondria and activate the DNA damage response in K562 cells similar to known topoisomerase II inhibitor doxorubicin but not topoisomerase I inhibiting compound CPT-11. It was also demonstrated that ALCAPs activated H2AX$^{(S139)}$ and ATM$^{(S1981)}$ phosphorylations in comparison to the control, even at nanomolar concentrations, and the ROS mediated DNA damage was abrogated by antioxidant L-NAC. Therefore, we suggest that ALCAPs exert their DNA damaging abilities via the generation of ROS abundantly beside the inhibition of topo I during replication/transcription in K562 leukemia cells.

2. Materials and methods

2.1. Compounds

Eight compounds were isolated from *A. cappadocica*, as described [11–13]. They were dissolved in DMSO and the concentration did not exceed 0.1% in treatments and controls. MTT reagent [3′-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] was purchased from Sigma (St Louis, MO). FITC conjugated anti-annexin V Ab was purchased from BD Pharmingen (San Diego, CA). L-NAC was obtained from Sigma and was freshly prepared prior to each experiment. CPT-11 (10 μM), doxorubicin (1 μM) and H_2O_2 (5 mM) were used as positive controls at different time points.

2.2. Antibodies

ATM, pATM$^{(S1981)}$, PARP1, and pCHK2$^{(T68)}$ antibodies were purchased from Santa Cruz Biotechnology Inc. (Bergheimer, Germany). Beta-actin antibody was purchased from Sigma (Europe), γH2AX$^{(S139)}$ mouse monoclonal antibody from Millipore (USA) and active Caspase 3 antibody from BDBiosciences (San Diego, CA).

2.3. Cell Culture

AU565, DU145, Hela, HT29, MDA-MB231, MCF7, PC-3, and K562 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were propagated in RPMI1640 or DMEM w/o HEPES for L-NAC experiments, supplemented with 5–10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C with 5% CO_2 in a humidified atmosphere.

2.4. Cell Viability

Cytotoxicity of the pure compounds was measured using MTT assay. Briefly, cells were splitted into 96-well plates (4000 cells/well), compounds were added in different concentrations ranging from 0.1 to 10 μg/ml and incubated for 4, 8, 16, 32, 60 and 96 h. Medium was removed 4 h later than adding MTT reagent (0.5 mg/ml) and absorbance of DMSO dissolved blue formazan crystals were read for quantitation. The data obtained was from three independent experiments with duplicates of wells.

2.5. Plasmid supercoil relaxation assays

Reactions were performed in a mixture containing (35 mM Tris–HCl (pH: 8.0), 72 mM KCl, 5 mM MgCl2, 5 mM DTT, 5 mM spermidine and 0.1% BSA) one unit of calf thymus DNA topo I (TAKARA, Otsu-Shiga, JA), 0.25 μg of plasmid DNA (pBR322), and/or the compounds. Plant extract (50 μg/ml) or campthotecin (1 μg/ml) was used as the positive control. The relaxation products were run on 1% agarose gels in TBE buffer (45 mM Tris borate and 1 mM EDTA pH: 8.0) using horizontal gel electrophoresis (5 V/cm). Gels were photographed using a Kodak image station under UV light immediately after being stained with EtBr (0.5 μg/ml). One unit of activity was defined as the activity of removing supercoils from 250 ng plasmid DNA substrate in 30 min at 37 °C. All reactions were performed in DNase-free conditions.

2.6. Immunoblotting

Cells were lysed with ice-cold modified RIPA buffer (1% Nonidet P40, 50 mM Tris–HCl, pH 7.4, 0.25% Na-deoxycholate, 150 mM NaCl), including 1 mM of each NaF, EDTA and Na_3VO_4, complete protease and phosphatase inhibitors and cocktails (Roche, Germany). 6–15% SDS-polyacrylamide gels were used for protein separation and proteins were immobilized onto PVDF membranes (Amersham, UK) using a wet transfer blotter. Membranes were blocked using TBS-T (Tris–Base–Saline containing 0.1% Tween 20) containing either 5% skim milk (w/v) or 1% BSA for phospho-antibodies. Incubations were performed using TBS-T containing 0.5% dry milk at RT for 1 h or at 4 °C overnight. Membranes were developed using ECL plus reagent (Amersham, UK) for 5 min and were photographed using Kodak X-ray films.

2.7. Immunofluorescence (IF) labeling and microscopy

For the detection of γH2AX$^{(S139)}$ foci and expression [14], cells were treated (4–24 h), collected into eppendorf tubes, rinsed in PBS and fixed with 1 ml 4% paraformaldehyde for 1 h at RT. After rinsing, cells were permeabilized with 0.2% triton X-100-containing PBS and blocked for 5 min using 1% BSA in PBS buffer before incubation with primary antibodies. Then the appropriate antibodies were added for 1 h and samples were washed twice with PBS. Secondary antibody incubations were performed at RT for 20 min using Alexafluor594 (anti-mouse) antibody (Invitrogen, Carlsbad, CA). Finally, cells were washed twice and mounted onto coverslips in 30% glycerol containing 0.5 μg/ml DAPI, and analyzed immediately using a Leica DMIL fluorescent microscope (Leica, Germany). Images were captured using Leica software (LAS).

2.8. Flow cytometry and analysis

Cell cycle distribution of γH2AX$^{(S139)}$ was studied using FACSCanto (BD Biosciences, USA), and analysis software FacsDiva 5.0.3. [14]. Briefly, cells in PBS were fixed by adding 100% ethanol (cold) directly after treatment and subsequently labeled with specific (anti-γH2AX$^{(S139)}$) and secondary antibody conjugated with Alexaflour488 (anti-mouse, 1:1000) as described above. Bi-variate analysis was performed using PI staining and γH2AX$^{(S139)}$ expression.