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Molecular modes of action of cantharidin in tumor cells

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Abstract

Cancer chemotherapy is often limited by patient's toxicity and tumor drug resistance indicating that new drug development and modification of existing drugs is critical for improving the therapeutic response. Traditional Chinese medicine is a rich source of potential anticancer agents. In particular, cantharidin (CAN), the active principle ingredient from the blister beetle, *Mylabris*, has anti-tumor activity, but the cytotoxic mechanism is unknown. In leukemia cells, cantharidin induces apoptosis by a p53-dependent mechanism. Cantharidin causes both DNA single- and double-strand breaks. Colony-forming assays with knockout and transfectant cells lines showed that DNA polymerase β, but not ERCC1, conferred increased cell survival after cantharidin treatment, indicating that base excision repair (BER), rather than nucleotide excision repair (NER), is important for CAN-induced DNA lesions. Oxidative stress-resistant thymic lymphoma-derived WEHI7.2 variants are also more resistant to cantharidin. These data suggest that cantharidin treatment causes oxidative stress that provokes DNA damage and p53-dependent apoptosis.

Keywords: Apoptosis; Cancer chemotherapy; DNA repair; Natural compounds; Oxidative stress

1. Introduction

Chemotherapy in leukemia is limited by the development of drug resistance in tumor cells and adverse side effects in patients and myelosuppression. The search for novel anti-tumor agents that circumvent these limitations has turned to natural sources, in particular, to compounds used in traditional folk medicines. This approach has already proven successful for drug discovery in the past; camptothecin from *Camptotheca acuminata* and paclitaxel from *Taxus brevifolia* are outstanding examples of compounds derived from traditional Chinese medicine that are currently used as chemotherapeutic agents [1].

We screened medicinal plants and animals used in traditional Chinese and Vietnamese medicine in search of agents with potential anti-tumor activity in a human CCRF-CEM leukemia cell culture model (unpublished data). One of the most cytotoxic compounds in this model system was the extract of the blister beetle, *Mylabris*. The principle active ingredient of *Mylabris* is cantharidin (CAN), a compound that has been used in China as a medicinal agent for 2000 years and for the treatment of cancer, particularly hepatoma [2]. CAN is potentially attractive for the treatment of leukemia because it does not cause myelosuppression [2,3] and is effective against cells exerting the multidrug resistance phenotype [4,5].

CAN and norcantharidin (NCTD), the demethylated cantharidin derivative that also has clinical potential, are protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) inhibitors [6]. This activity appears necessary for the growth inhibition activity of these compounds [6]. Protein phosphatases are involved, among others, in the regulation of multiple cellular processes including signal

Abbreviations: BER, base excision repair; CAN, cantharidin; NCTD, norcantharidin; NER, nucleotide excision repair; OTM, Olive tail moment; PP1/PP2A, protein phosphatase 1/2A

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transduction pathways, cell cycle progression, glucose metabolism, and calcium transport [7]. Thus, although the biochemical target of CAN and NTCD is known, the critical molecular pathways by which CAN and NCTD cause growth inhibition and cell death are unclear.

In this study, we have shown that CAN induces apoptosis in leukemia cells by a p53-dependent mechanism. Treatment with CAN causes increased DNA strand breakage, and increase in DNA repair was related to decreased cellular sensitivity to CAN. Appropriate lines deficient in *POLB* or *ERCC1* genes were used to answer the question, whether base excision repair (BER) or nucleotide excision repair (NER) are important for the removal of CAN-induced DNA lesions. Finally, we show that resistance to oxidative stress causes cross-resistance to CAN.

2. Materials and methods

2.1. Drugs

Cantharidin and methyl methanesulfonate (MMS) were purchased from Sigma–Aldrich.

2.2. Cell lines

Human CCRF-CEM leukemia cells, lymphoblastoid TK6 cells with wild-type p53 and lymphoblastic WTK1 cells with a p53Ile273 mutation [8] were maintained in RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (Gibco BRL) in a 7% CO_2 atmosphere at 37 °C. Chinese hamster ovary (CHO-9) cells with a wild-type *ERCC1* gene, CHO 43-3B cells with a mutated *ERCC1* gene (defect in the 5′ endonuclease function), and CHO 43-3B/ERCC1 cells with mutated *ERCC1* complemented by transfection of a cloned wild-type gene (kindly provided by Dr. R. Wood, Pittsburgh, USA) were cultured in Dulbecco's MEM:F12 medium supplemented with 10% fetal calf serum (Gibco BRL) as described [9]. Cells were passaged twice weekly. All experiments were performed with cells in the logarithmic growth phase.

The *β-pol* null (−/−; Mb19tsA, clone 2B2) and the corresponding wild-type (+/+; Mb16tsA, clone 1B5) cell lines were described previously [10]. They were derived from embryonic tissue of either *β-pol* knockout or wild-type mice. The cells were maintained in DMEM medium as described [10].

The mouse thymic lymphoma-derived WEHI7.2 parental cell line was obtained from Dr. Roger Miesfeld (University of Arizona, Tucson, AZ). Cells were maintained in Dulbecco's Modified Eagle Medium–low glucose (Invitrogen) supplemented with 10% calf serum (Hyclone Laboratories, Logan, UT) at 37 °C in a 5% CO_2 humidified environment. Stock cultures were maintained in exponential growth at a density between 0.02 and 2×10^6 cells/ml. WEHI7.2 cells stably transfected with and overexpressing human Bcl-2 (Hb12), constructed and maintained as described in Lam et al. [11], were also obtained from Dr. Miesfeld. Thioredoxin (THX) overexpressing cells were constructed by stably transfecting human thioredoxin into WEHI7.2 cells, then selecting and maintaining clones as described [12]. THX cells express 1.8-fold more thioredoxin than the parental cells [11]. Catalase overexpressing cells were constructed by stably transfecting WEHI7.2 cells with a vector containing rat catalase as described [13]. CAT38 and CAT2 clones, expressing 1.4- and 2.0-fold parental cell catalase activity, respectively, were selected and maintained in 800 μg/ml G418 (GIBCO-BRL). Hydrogen peroxide-resistant cells (200R) were developed by subculturing parental cells in the presence of fresh H_2O_2 every 3 days as described [14]. This procedure resulted in a population of cells that is 2.8-fold more resistant to 200 μM H_2O_2 than the parental cells. 200R cells were maintained in the presence of 200 μM H_2O_2. Any variant normally grown in the presence of drug was cultured in drug-free medium for 1 week prior to each experiment.

2.3. Measurement of cellular drug response

2.3.1. Cell response to CAN

The in vitro response of CCRF-CEM, TK6, and WTK1 cells to CAN was evaluated as described [5]. Briefly, aliquots of 5×10^4 cells/ml were seeded in 24-well plates and extracts (10 μg/ml) or CAN were added immediately. Cells were counted twice 7 days after treatment. The results are expressed as % vehicle-treated (DMSO) control cell number and represent the net outcome of cell proliferation and cell death.

2.3.2. Clonogenic cell survival assay

The CAN response of the CHO parental cells and the CHO variants was determined by colony-forming assays. Briefly, 500 cells were seeded in 60 mm dishes, incubated at 37 °C for 8 h and then treated with CAN. After 1 week, the colonies were fixed in methanol for 5 min, air-dried, stained (1.25% Giemsa/0.125% Crystal violet), rinsed in water, and counted. Survival is expressed as a percentage of the untreated control and represents the mean of three independent measurements.

2.3.3. MTS assay

The CAN response of the WEHI7.2 parental cells and the WEHI7.2 cell variants was measured using the MTS assay (Promega) as described previously [15]. Briefly, cells were plated at 1.5×10^4 cells per well in 100 μl medium in a 96-well plate and incubated in the absence or presence of the indicated concentrations of CAN for 48 h. Relative absorbance was measured by incubating the cells for 3 h at 37 °C with the MTS solution, prepared and used according to the manufacturer's protocol (Promega), and reading at