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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medicine in search of agents with potential anti-tumor activity in a human CCRF-CEM leukemia cell culture model (unpublished data).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

490 nm using a microplate autoreader (Bio-Tek Instruments). Response was calculated as a percentage of the untreated control absorbance. The EC_{50} represents the mean of three independent determinations.

2.4. Single cell gel electrophoresis (SCGE, comet assay)

The detection of DNA single-strand breaks and alkali-labile DNA sequences by the alkaline comet assay as well as of double-strand breaks by the neutral comet assay was performed according to published protocols [16,17] with slight modifications. Briefly, at various time points after treatment with CAN, subconfluent cells were trypsinized, washed with cold PBS, and kept on ice until assayed. Cells were embedded in 0.1% low melting point-agarose, and microscope slides were immersed in ice-cold lysis solution. For neutral comet assays, slides were incubated in neutral lysis buffer (2.5 M NaCl; 100 mM EDTA; 10 mM Tris; 1% Na-laurylsarcosine, pH 7; 1% Triton X-100; 10% DMSO were added freshly) for 1 h at 4 °C. For alkaline comet assays, the lysis buffer was adjusted to pH 13. After lysis, electrophoresis (25 V) was carried out for 15 min at 4 °C. Electrophoresis buffers for neutral comet assays consisted of 90 mM Tris, 90 mM boric acid, and 2 mM EDTA adjusted to pH 7 and for alkaline comet assays of 300 mM NaOH, 1 mM EDTA adjusted to pH 13. The fixed and ethidium bromide-stained slides were analyzed using fluorescence microscopy. DNA migration (related to the induction of DNA strand breaks) was analyzed using an image analysis system (Kinetic Imaging Ltd., Komet 4.0.2, Optilas) with a defined standard and expressed as an Olive tail moment [18].

2.5. Annexin V apoptosis assay

For quantification of apoptosis induced by CAN, CCRF-CEM cells were stained with annexin V-FITC (Pharmingen) and propidium iodide as described [19]. Double staining of unfixed cells with annexin V-FITC and propidium iodide allows the differentiation between apoptotic and necrotic cells. Cells were harvested after 24–168 h incubation with 2.3 μM CAN (=IC_{50}), washed with cold PBS, stained according to the manufacturer's protocol (Pharmingen), and subjected to flow cytometry on a FACSCalibur (Becton Dickinson). The evaluation of cell populations was performed using a computer-based program (CellQuest, Becton Dickinson).

2.6. Statistical analyses

The mRNA expression values of the genes of interest were selected from the 60 cell line panel from the database of the National Cancer Institute (NCI), Bethesda, MA, USA (http://dtp.nci.nih.gov). The mRNA expression has been determined by microarray analyses as reported [20]. This database was searched for genes involved in human DNA repair [21]. Kendall's τ-test (WinSTAT Program, Kalmia) was used to calculate significance values and rank correlation coefficients as a relative measure for the linear dependency of two variables (mRNA expression for these genes and CAN IC_{50} values). Kendall's τ-test does not depend on a normal distribution of the data. The data were then subjected to a step-up re-sampling multi-comparison procedure to control the false discovery rate (FDR), type I errors (false conclusions of significance), among the significant correlations at a given significance level [22].

3. Results

3.1. Induction of apoptosis by CAN

A decrease in cell number is the net result of growth inhibition and cell death. CAN has been shown to induce apoptosis in adenocarcinoma cells [23]. Therefore, we tested the ability of CAN to induce apoptosis in CCRF-CEM cells. We observed a time-dependent increase in apoptosis reaching 70.3% after 168 h in cells treated with 2.27 μM CAN (Fig. 2). This concentration represents the inhibition concentration 50% (IC_{50}) value measured in growth inhibition assays (data not shown). The apoptotic and necrotic cell fractions of DMSO vehicle-treated cells were similar to untreated controls (<5%) indicating that DMSO was not cytotoxic (Fig. 1). These data demonstrate that CAN induces apoptosis in CCRF-CEM cells.

3.2. DNA damage and repair

CAN and NCTD inhibit growth and induce apoptosis in several cell types; however, the mechanism by which these compounds induce apoptosis is not understood [23–25]. Many of the chemotherapeutic agents currently in use are genotoxic. Treatment of oral cancer KB cells with NCTD caused DNA strand breaks [26] suggesting the possibility that CAN may also work by this mechanism. We tested the

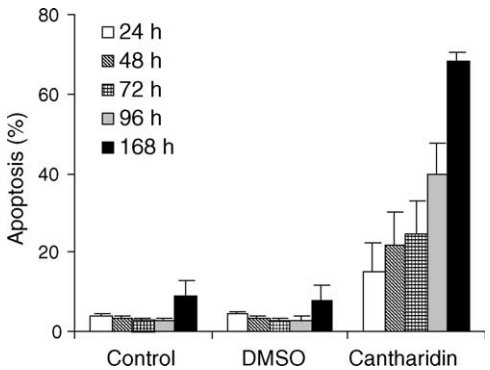

Fig. 1. Induction of apoptosis in CCRF-CEM cells after treatment with. Quantification of apoptotic cells after incubation with 2.27 μM CAN (=IC_{50} concentration) for 24–168 h.

ability of CAN to induce DNA strand breaks in CCRF-CEM cells and found that it increased both DNA single-strand breaks (alkaline comet assay) and DNA double-strand breaks (neutral comet assay) in a time-dependent manner (Fig. 2A). About twice as many DNA single-strand breaks were determined as compared to double-strand breaks measured in the comet assay. The DNA lesions were also correlated to the concentration of CAN (Fig. 3B). CCRF-CEM cells were able to repair CAN-induced DNA strand breaks so that at 24 h post-treatment, the comet tails in the treated cells were of similar length as in the untreated controls (Fig. 2B).

The nuclear polymerase β (PolB) is a key enzyme in the base excision repair [27,28]. To test, whether BER plays a role for survival of CAN-treated cells, we performed colony-forming assays with either *β-pol* knockout (−/−) or wild-type (+/+) embryonic mouse fibroblasts. The dose–response curves in Fig. 3A show that *β-pol* (−/−) cells are hypersensitive to CAN compared to *β-pol* (+/+) cells. The IC_{50} values were 2.9 μM and 3.7 μM, respectively. As control experiment, we treated both cell lines with MMS, which is known to be hypersensitive in *β-pol* (−/−) cells [27,28]. As expected, we found that *β-pol* (−/−) cells were more sensitive to MMS than *β-pol* (+/+) cells. Upon treatment with 0.1 mM MMS, the survival rates for *β-pol* (+/+) and *β-pol* (−/−) were 47.3% and 7.3%, respectively.

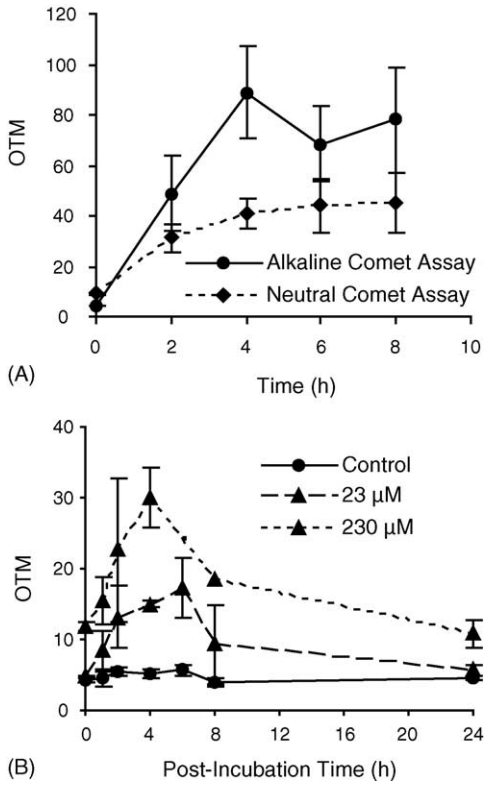

Fig. 2. Induction and repair of DNA stands breaks in CCRF-CEM cells after treatment with 227 μM CAN (=100-fold IC_{50} concentration). (A) Single-strand breaks (alkaline comet assay) and double-strand breaks (neutral comet assay) in CCRF-CEM cells after continuous treatment with CAN. OTM indicates Olive tail moments (see Section 2). (B) Single-strand breaks in CCRF-CEM cells treated for 2 h with different concentrations of CAN then incubated in drug-free medium. CAN was added at time = 0. Values represent the mean ± S.E.M. of three independent experiments.

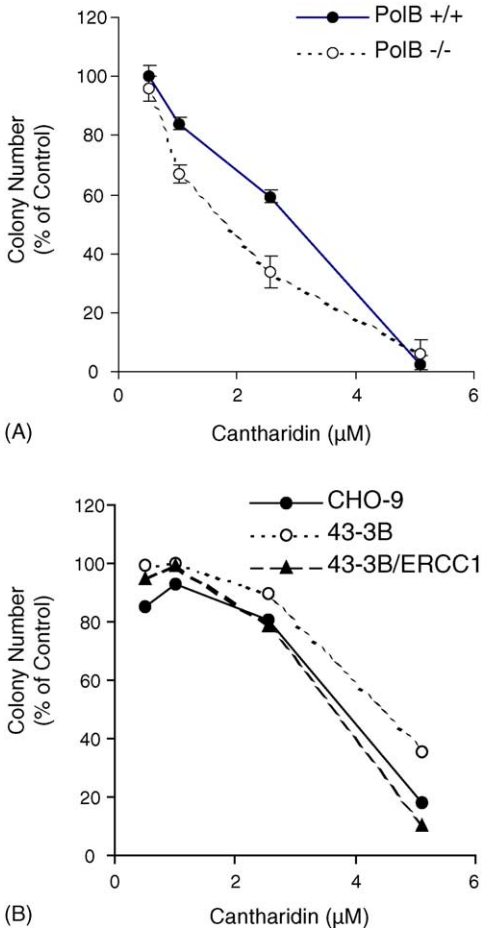

Fig. 3. Effect of *PolB* and *ERCC1* on survival after continuous treatment with CAN. (A) Survival curves for wild-type *β-pol* (+/+) and knockout *β-pol* (−/−) cells. (B) Survival curves for CHO-9 (wild-type ERCC1), 43-3B (ERCC1-deficient CHO mutant), and 43-3B/ERCC1 (43-3B cells transfected with wild-type ERCC1) cells. Values represent the mean ± S.E.M. of three independent experiments.

ERCC1 is a key protein in NER [29,30]. To determine whether NER was critical for survival after CAN treatment, we compared the survival curves of CHO-9 cells (expressing wild-type *ERCC1*), 43-3B (*ERCC1*-deficient CHO mutant), and 43-3B/ERCC1 (43-3B transfected with wild-type *ERCC1*) cells after drug treatment. The dose–response curves were similar for all three cell lines (Fig. 3B) suggesting that NER does not play a major role in repair of CAN-induced DNA damage. Because UV-C-induced DNA lesions are known to be repaired by NER [9], clonogenic survival in these cell lines after UV-C light irradiation was compared in order to control the function of *ERCC1*. Survival was 29% for CHO-9, 0.5% for 32-3B, and 14.8% for 43-3B/ERCC1 cells after UV-C treatment (10 J/m^2) as compared to untreated controls, indicating that *ERCC1* is functioning in the complemented cell line.

Table 1
Relationship of the cellular CAN response to the genes involved in the major human DNA repair pathways

RR	BER		NER		MMR	NHEJ	HR
ALKBH	ADPRT	POLD2	CCNH	POLD1	MLH1	DNAPK	MRE11A
CRY1	APEX2	POLD3	CDK7	POLD2	MLH3	FEN1	NBS1
CRY2	CAMK1	POLE	CSA, CKN1	POLD3	MSH2	G22P1	PIR51
MGMT	FEN1	RFC1	DDB1	POLE	MSH5	KIP2	RAD50
	LIG1	RFC2	DDB2	POLR2A	MSH6	LIG4	RAD51
	LIG3	RFC3	DOK4	POLR2B	MUTYH	MRE11A	RAD51C
	MBD4	RFC4	ERCC1/XPD	POLR2C	PMS2	NBS1	RAD52
	MPG	RFC5	ERCC2/XPD	POLR2D	POLD1	RAD50	RPA1
	NEIL1	TDG	ERCC3/XPB	POLR2E	POLD2	XRCC4	RPA2
	NEIL2	UDG	ERCC5	POLR2F	POLD3	XRCC5	RPA3
	OGG1	UNG	GTF2H1	POLR2G	TREX1		XRCC2
	PCNA	UNG2	GTF2H2	POLR2H			XRCC3
	POLB	USP14	GTF2H3	POLR2I			
	POLD1	XRCC1	GTF2H4	POLR2J			
			IGFBP7	POLR2K			
			LIG1	POLR2L			
			MNAT1	XPA			
			PCNA	XPC			

The gray fields show significant positive correlations ($P < 0.01$; Kendall's τ-test) between the mRNA expression of the indicated gene of 60 NCI cell lines and the IC_{50} values for CAN. The expression values obtained by microarray analyses have been normalized and deposited in the NCI's database (http://dtp.nci.nih.gov). For normalization, reference probes were made by pooling equal amounts of mRNA from logarithmically growing HL-60, K562, NCI-H226, COLO205, SNB-19, LOX-IMVI, OVCAR-3, OVCAR-4, CAKI-1, PC-3, MCF7, and Hs578T cell lines. Test and reference probes were combined, denatured, and hybridized overnight to Synteni microarrays. Arrays were scanned using a laser-scanning microscope. The ScanAlyze program was used to analyze the microarray images. RR, reversion repair; BER, base excision repair; NER, nucleotide excision repair; MMR, mismatch repair; NHEJ, non-homologous end joining; HR, homologous recombination.

DNA repair involves a large number of genes [21]. If DNA repair is critical in the mechanism of CAN-induced apoptosis, one prediction is that cells with increased capacity for the repair of critical lesions should be more resistant to CAN. To test this prediction, we correlated the IC_{50} values for CAN with the baseline mRNA expression of various DNA repair genes whose expression was quantified in 60 cell lines of different tumor origins using the database of the National Cancer Institute, USA (http://dtp.nci.nih.gov). Eighty-nine genes in six different DNA repair pathways were analyzed by Kendall's τ-test and FDR algorithm [22] to minimize the number of false positive correlations. mRNA expression of 15 genes correlated significantly with IC_{50} values for CAN (Table 1). These genes are involved in the base excision repair and DNA double-strand break repair (NHEJ, HR), indicating that CAN-induced DNA lesions may be repaired via these pathways. None of the genes of the NER pathway was among the panel of correlating genes supporting the previous finding with *POLB* and *ERCC1*-deficient cells.

One of the major functions of p53 is to cause apoptosis under conditions where DNA damage is severe [31]. To test whether p53 is involved in CAN-induced apoptosis, we compared the CAN response in human TK6 leukemia cells carrying wild-type p53 and WTK1 leukemia cells with mutated p53. Cells with wild-type p53 were more sensitive to CAN than those with a mutated p53 (Fig. 4). The IC_{50} values for TK6 and WTK1 cells were 2.5 μM and 7.4 μM, respectively. These data suggest that CAN induces apoptosis in a p53-dependent way.

3.3. Oxidative stress by CAN

One possible mechanism by which CAN could cause DNA damage is through increase in oxidative stress. A previous study suggests that treatment with several analogs

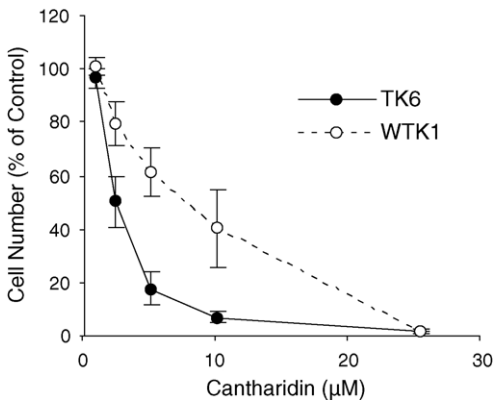

Fig. 4. Effect of the p53 status on the CAN response. Cell number after continuous CAN treatment in TK6 (wild-type p53) and WTK1 (mutated p53) cell cultures compared to untreated control cultures. Values represent the mean ± S.E.M. of three independent experiments.

Table 2
Comparison of the CAN response in WEHI7.2 cells and the oxidative stress-resistant WEHI7.2 variants

	Mean (±S.E.M.)	Degree of resistance
WEHI7.2	42.22 (±7.10)[a]	
THX	112.05 (±20.29)	2.65[b]
CAT2	81.23 (±11.24)	1.92
CAT38	109.50 (±34.99)	2.59
200R	54.41 (±8.21)	1.29
Hb12	120.27 (±8.66)	2.85

[a] EC_{50} values (μM) of parental WEHI7.2 cells and cells stably transfected with expression vectors carrying cDNAs for thioredoxin (THX), catalase (CAT2, CAT38), or Bcl-2 (Hb12) as well as cells selected for H_2O_2 resistance (200R).

[b] The ratio of EC_{50} values of transfectant and H_2O_2-resistant cells to the EC_{50} value in the parental cells.

of CAN increases xanthine oxidase activity which would result in an increased production of reactive oxygen species (ROS) [32]. ROS are well known DNA damaging agents [33]. To test whether oxidative stress contributes to CAN toxicity, we compared the CAN response in several WEHI7.2 thymic lymphoma variants resistant to oxidative stress. The oxidative stress-resistant WEHI7.2 variants, which included cells overexpressing catalase (CAT38 and CAT2), cells selected for resistance to hydrogen peroxide (200R) and cells that overexpress thioredoxin, a small protein that transfers reducing equivalents, were all more resistant to CAN than the parental cells (Table 2). WEHI7.2 cells overexpressing Bcl-2 (Hb12) were also more resistant to CAN (Table 2).

4. Discussion

CAN is an attractive drug candidate for the treatment of leukaemia, because it does not cause myelosuppression in patients [2] and is effective against cells with a multidrug resistance phenotype [4,5]. A drawback of CAN is its acute toxicity due to its effect on mucus membranes and the urinary tract [2,26]. Understanding the mechanism of CAN action will help in the derivation of related compounds that have reduced toxicity while preserving the anti-tumor effect.

The strength of this study is that it allows us to build a testable model for the sequence of events whereby CAN kills lymphoid cells. The data suggest that CAN treatment increases oxidative stress resulting in DNA damage followed by p53-dependent apoptosis. CAN is the principle active ingredient of *Mylabris* and causes apoptosis in CCRF-CEM cells accounting for the toxicity seen in the *Mylabris* extract. CAN is a well-documented cytotoxin in many tumor cell lines [6,34]; however, the mechanism of CAN-induced toxicity is not understood in these cell lines. The data in the current study suggest a mechanism by which CAN could induce apoptosis.

Determining the critical event(s) responsible for CAN-induced toxicity has been difficult. CAN is a PP1 and PP2A inhibitor [6]. PP1 and PP2A modulate a large number of cellular processes by counteracting the activity of kinases to provide the critical on/off switch for many pathways [7]. Cell cycle progression is one process where the increases and decreases of both kinases and phosphatases are necessary to complete the cycle. Studies from a number of laboratories indicate that CAN and NCTD treatment results in a G_2/M cell cycle block in many cell types [3,24,25,34,35]. However, several of these studies suggest that cell cycle blockade is not the cause of CAN-induced apoptosis [24,25,34].

Here, we show that CAN treatment causes DNA strand breaks in CCRF-CEM cells. DNA strand breaks have also been documented in oral cancer KB cells after NCTD treatment [26]. A correlation between an increase in the mRNA level for several DNA damage repair genes in the 60 cell line panel and resistance to CAN argues for DNA damage as a mechanistic component of CAN-induced apoptosis. This is consistent with a role for p53 in CAN-induced apoptosis seen in this study because one of the major functions of p53 is to induce apoptosis when DNA damage excesses a threshold [31]; p53 also plays a role in NCTD-induced apoptosis in glioblastoma cells [24]. Phosphorylation of p53 stabilizes the protein [31,36], thus inhibition of phosphatases may enhance the ability of p53 to exert its effect. The ability of Bcl-2 to protect against CAN-induced apoptosis seen in this study indicates that the DNA damage-triggered mitochondrial pathway is also involved. Mitochondrial dysfunction and activation of caspases involved in the intrinsic (mitochondrial) pathway of apoptosis have been seen in other cell types after CAN or NCTD treatment as well [25,34].

The cross-resistance of the oxidative stress-resistant WEHI7.2 variants to CAN suggests that CAN treatment causes oxidative stress which plays a role in CAN-induced apoptosis. Analogs of CAN increase xanthine oxidase activity which would increase intracellular ROS [32]. It is, therefore, tempting to speculate that oxidative stress is involved in the induction of DNA damage by CAN. Increase of endogenous ROS level has repeatedly been shown to cause significant DNA breakage [33].

Resistance to oxidative stress, increased of Bcl-2 or the presence of wild-type p53 have a modest affect on CAN-induced toxicity. Mutational inactivation of PolB, but not of ERCC1, key enzymes of BER and NER, respectively, exerted an effect on CAN cytotoxicity. This suggests that CAN induces non-bulky DNA lesions that are repaired by BER but not by NER. Lesions induced by oxidative stress are rather repaired by BER and NHEJ [21]. This suggests the possibility that multiple mechanisms are responsible for CAN-induced toxicity. In this study, for example, p53 status affected the IC_{50} of CAN; however, cells with mutated p53 still died. A p53-independent mechanism of CAN-induced cytotoxicity has been seen in hepatoma cells [25]. Because CAN and NCTD inhibit phosphatases that are critical to many cell processes, it would not be

surprising that alterations in more than one pathway are critical for apoptosis. The microarray data comparing CAN treated and untreated HL-60 cells suggests CAN affects multiple pathways [37]. Given that multiple mechanisms are involved in CAN-induced toxicity, the drug will likely be most effective against cancer cells with a specific phenotype. It is worth analyzing whether this would be a cancer cell that has acquired mutations in DNA repair pathways, but retained wild-type p53 and sensitivity to oxidative stress.

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