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Research Article

Cellular Responses to Anthracyclines Identify Ku70, a DNA Repair Factor that Changes Compartment and Remains Stable in Leukemic Cells

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Abstract

Anthracyclines such as doxorubicin and daunorubicin are anticancer drugs that act by damaging the DNA and used for treating a variety of cancers including adult acute myeloid leukemia. To date, nearly 50 % of acute myeloid leukemia patients show resistance to anthracyclines although the cause is not known. We first investigate if there is a relationship between the expression level of 23 DNA repair genes in three leukemic cell lines (KG-1, HL-60 and Mono-Mac1) and cellular responses to anthracyclines. We observed that the DNA repair genes were all downregulated in these cell lines following exposure to doxorubicin. Further analysis revealed that the general downregulation of the genes was linked to a substantial decrease in the recovery of total RNA raising the possibility that assessment of total RNA, and not specific gene or set of genes, can be used as a simple indicator of cellular responses to anthracyclines. Furthermore, examination of total protein extracts derived from these cell lines revealed for the first time that Ku70 is a key protein that remained stable, while the majority of proteins were loss, upon anthracycline treatment. Importantly, Ku70 redistributes from the cytoplasm to the nucleoli in a time-dependent manner in response to anthracycline exposure. We propose that Ku70 redistribution might play a vital role in predicting cellular response to anthracycline and promoting cell death.

Keywords: Ku70, leukemia, anthracyclines, Gene eXpression Profiler, DNA damage, total RNA
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Introduction

The standard treatment for acute myeloid leukemia (AML) patients is a combination chemotherapy consisting of infusional cytarabine and an anthracycline such as daunorubicin [1]. The anthracycline drug family includes also doxorubicin, idarubicin and epirubicin and these chemotherapeutic agents act by intercalating with the DNA, producing a variety of DNA lesions that include DNA-adducts and DNA interstrand crosslinks [2, 3]. Anthracyclines can also generate reactive oxygen species (ROS) during metabolism leading to oxidative damage to the DNA such as modified bases and strand breaks [4-6]. In general, anthracyclines are highly genotoxic agents that potently inhibit DNA transactions such as DNA synthesis. It has been demonstrated that the efficiency in the repair of anthracycline-induced DNA lesions governs cellular responses to the drug. For example, cells deficient in XPA, a protein that is involved in the recognition of DNA damage in the nucleotide excision repair pathway or DNA polymerase eta (XPV) involved in bypassing thymine dimers, are highly sensitive to doxorubicin [7, 8]. In addition, a recent study showed that the expression level of the base-excision repair protein, the DNA glycosylase OGG1 involved in the repair of the oxidized DNA lesion 8-oxoG, is 7-fold reduced in AML patients [9]. These patients exhibit poor prognosis raising the possibility that OGG1 could play a role in enhancing the toxicity of the drug, perhaps by processing 8-oxoG to more toxic lesions [9]. Moreover, it has been shown that anthracyclines can inhibit the expression as well as the activity of poly (ADP-ribose) polymerase-1 (PARP-1) [10]. Both OGG1 and PARP-1 belong to the base-excision repair pathway, which functions to repair oxidized DNA base lesions, apurinic/apyrimidinc (AP) sites, as well as DNA strand breaks with blocked 3'-termini [11]. Thus, it seems possible that the level of DNA repair genes could influence cellular responses to anthracyclines. So far, no study has been undertaken to determine if there is a correlation between the expression levels of genes belonging to various DNA repair pathways and the sensitivity and resistance of AML cell lines to anthracyclines.

In this study, we set out to investigate the expression level of 23 genes that have been selected from the major DNA repair pathways including base-excision, nucleotide excision, mismatch and recombination. We examined the expression level of these genes in three leukemia cells lines using the Gene Expression Profiler. This state-of-art technology employs RT-PCR combined with capillary electrophoresis and can detect the expression level of at least 30 genes from a single sample (https://www.beckmancoulter.com/). In this report, we found no difference in the expression level of the DNA repair genes between three leukemic cell lines: HL60, Mono-Mac-1 and KG-1 under normal growth conditions. In contrast, exposure to the anthracycline doxorubicin caused a drastic reduction in the DNA repair genes in HL60 and Mono-Mac-1 cell lines, while 10-fold higher drug concentration was required to produce a similar effect in the KG-1 cell line, except for two genes, *APE1* and *PCNA*, which remained partially expressed. Interestingly, anthracycline treatment triggered a substantial decrease in the recovery of total RNA from these cell lines, but which was dependent on the drug concentration. This observation raises the possibility that assessment of total RNA can be used as a simple indicator of cellular responses to anthracyclines. Herein, we also show for the first time that Ku70 is a key protein that remained stable following chronic exposure of the cells to anthracycline. Importantly, Ku70 redistributes from the cytoplasm to the nucleoli in a time-dependent manner in response to anthracycline exposure. We discuss the possible roles for the relocalization of Ku70 in leukemic cells following the drug treatment.

Materials and methods

Cell lines and materials

KG1, HL60 and Mono-Mac1 are human leukemia cell lines, obtained from The Quebec Leukemia Cell Bank (QLCB), Montreal, Canada. PARP1 (F-2), Ku70 (A-9), β- actin (AC-15) and mouse IgG-FITC antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The anthracyclines: Doxorubicin, daunorubicin, idarubicin and epirubicin were purchased from Maisonneuve-Rosement Hospital, Montreal, Canada.

Cell culture

Human leukemia cell lines, KG-1, HL60 and Mono-Mac1 were cultured in RPMI 1640 medium

supplemented with 20% of FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were cultured at 37°C in a humidified atmosphere with 5% CO2.

MTT assay

The leukemic cell lines were seeded in 96 well plate as $4x10^4$ cells per 200µl of medium per well with increasing concentration of each drug. After 24, 48 and 72 hours of incubation, 10 µl of 5mg / mL of Methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, Ltd, Ontario, Canada) in PBS was added to each well and incubated for another 3 hours at 37°C. The reaction was stopped by adding DMSO (Sigma lifesciences, Canada) in each well to dissolve the MTT crystals. The absorbance of each sample was then determined at 540 nm following the manufactured instructions using ELx808 Absorbance Microplate Reader (Biotek, Winooski, USA). All experiments were performed in triplicate.

RNA extraction for GeXP analysis

mRNA extraction was performed with the RNeasy Mini kit (Qiagen Sciences, Maryland, USA), following user guide lines. The extraction was done after treatment with or without doxorubicin at different time point. Briefly, cells at $10x10^6$ were treated with Doxorubicin (72 hours at 0.5 and 5 µM) for GeXP analysis. After treatment, the mRNA were extracted in triplicate and quantified for the analysis.

Multiplex analysis by GeXP system

Primer design of the set of 23 genes (see Table I and suppl. Table SI) was done using the eXpress Designer module of the express Profiler software (Beckman Coulter). 25 ng RNA were used in a 20 µL reaction volume for RT. Kanamycin RNA, an internal positive control was included. The RT reaction was performed as described before in [12] using the GenomeLab TM GeXP Start Kit (Beckman Coulter). Expression analysis was carried out with the GenomeLab TM GeXP system (Beckman-Coulter) following manufacturer's instructions using the fragment analysis method. The data was normalized to Kanamycin before being expressed as a Mean Normalized Expression.

Western Blot analysis

Equal amounts of protein (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After immunoblotting with anti-PARP1, anti-XRCC6 antibodies, nitrocellulose membranes (Biorad, Hercules, CA, USA) were reprobed with anti-β-actin, ensuring equal protein loading.

Apoptosis assay

Cells (~ 5 X 10^5) were pelleted (1000 rpm for 1 min) and washed with 1 ml of cold 1X PBS. The cells were then incubated for 20 min in the dark with 5 µl of 7-Amino-actinomycin D (7AAD), 1 µl of Annexin V (PharMingen, US) and 10 µl of binding buffer (100 mM Hepes pH 7.4, 140 mM NaCl, 25 mM $CaCl_2$). Controls were incubated either with the binding buffer or with annexin V. Another 400 µl of binding buffer was added to each of the samples before FACScan analysis (FACSCalibur, BD Dickinson).

Coomassie staining and mass spectrometry

Cells were treated with Daunorubicin (5µM) for 72 hours and then proteins were extracted using RIPA buffer. The proteins were quantified and 50 µg were loaded per well in 10% SDS-PAGE gel. The gel was stained with 0.5% Coomassie Blue G-250 prepared in 50% methanol/10% acetic acid for 10 min and destained with 40% HPLC grade methanol/10% acetic acid. After, the band of interest was cut and sends for Identification by Mass spectrometry to Taplin Biological Mass Spectrometry Facility, Harvard Medical School.

RT-PCR analysis

Cells were seeded at $10x10^6$ over night before treatment with daunorubicin for 3, 6 and 72 hours at 5 µM. 1µg of RNA was used per reaction. cDNA synthesis and PCR on Gene Amp PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) were performed with three independent RNA preparations. The primers for GAPDH were the same used for the GeXP analysis (see supp. Table S1). The *XRCC6* and *OCTN2* primers were designed by Primer 3 software. *OCTN2* primers: left primer:

5'-GTGCTGCCACTGTTTGCTTA-3'and right primer 5'-GGACTGCTGCTTCTTGGAAC-3'. XRCC6 primers: left primer 5'- AAAAG ACTGGGCTCCTTGGT-3' and right primer 5'- AGCAGCTCCTGCTTCTTCAG-3'.

Immunofluorescence

Cells growing on 6 well plates were treated with Daunorubicin (5μM) at different time (1, 6 and 24 hours) then washed with PBS cold twice and fixed with FCM Fixation Buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 30 min. The cells were then permeabilized using FCM Permeabilization Buffer 1x (Santa Cruz, CA, USA) for 5 min. Following two washes with PBS, cells were stained with diluted mouse anti-Ku70 in FCM Wash Buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min on ice. After washes, the cells were then stained with FITC-conjugated goat anti- mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were mounted with UltraCruz™ Mounting Medium (Santa Cruz Biotechnology, Santa Cruz, CA, USA) containing 1.5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI), which is used to counterstained DNA. Images were taken using the Microscope confocal LSM 510 META Zeiss with the objectif 100x / 1.4 Plan-Apochromat 1.4 oil.

Statistical analysis

For survival curves analysis at least three independent experiments were performed and the standard deviations were calculated. For GeXP analysis the percentage CV was calculated for each replicate. Only % CV ≤ 20% was taken for analysis.

Results

Leukemic cell lines display varying sensitivities to anthracyclines

To have a better insight into the mechanism of resistance to anthracyclines, we chose three leukemia cell lines and examined the sensitivity towards this drug family. As shown in Figure 1, all three cell lines displayed sensitivity to the anthracyclines after 72 h of treatment with doxorubicin, daunorubicin, idarubicin or epirubicin using MTT assay. However, the KG-1 cell line showed resistance only at lower concentrations (< 0.1 μM) of doxorubicin, daunorubicin, or epirubicin, suggesting that leukemia cells may habour defects that cause differential responses to the drugs. We next checked whether the responses of the leukemia cells towards the anthracyclines would correlate with the cleavage of PARP, a physiological process indicative of cells undergoing apoptosis [13]. In all three sensitive cell lines, PARP was cleaved from the 113 to the 89 kDa fragment upon treatment with daunorubicin (Figure 2). The 89 kDa fragment corresponds to the C-terminal cleaved product of PARP-1(p89) under genotoxic stress[14]. Amongst these cells, KG-1 showed the earliest cleavage of PARP, which occurred at approx. 3 h and completely cleaved by 48 h (Figure 2B), while for HL-60 and Mono-Mac1 cleavage seemed to occur after 3 h and completed by 6 and 12 h, respectively (Figure 2A and C). These data indicate that indeed KG-1 responds differently to the anthracyclines, and that all three cell lines appear to undergo cell death via apoptosis when exposed to anthracyclines, although this process may be delayed in the KG-1 cells.

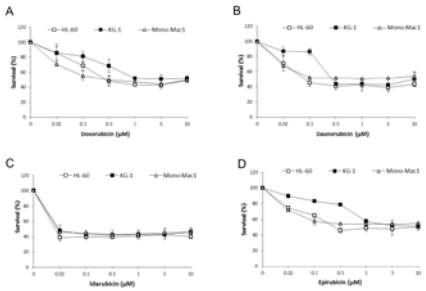

Figure 1 Sensitivities of the leukemia cell lines towards anthracyclines. Cell survival of the cell lines (KG-1, Mono-Mac1 and HL-60) was monitored using the MTT assay and the indicated concentrations of the drugs. Each plot line shows an average of the results of three experiments and the error bars indicate the standard deviation.

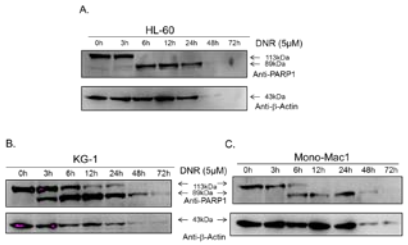

Figure 2 Kinetics of anthracycline-mediated PARP-1 cleavage in the leukemia cell lines. The leukemia cell lines were treated with daunorubicin (DNR) for the indicated time and total protein extracts (50 μg per lane) were resolved by 10% SDS-PAGE followed by Western blot analysis to monitor for PARP-1 cleavage using anti-PARP-1 monoclonal antibody and for normalization with antibodies against β-actin. The results are representative of two independent experiments.

The leukemia cell lines display similar expression pattern of DNA repair genes under normal conditions

Because anthracyclines act by damaging the DNA, we checked for the expression level of genes involved in DNA repair in these leukemia cell lines. We selected 23 genes to be analyzed in the three leukemic cell lines (see Table S1), and these genes belonged to the base-excision, nucleotide-excision, mismatch and recombination DNA repair pathways. To our knowledge, no studies investigated the expression level, in particular, of the base-excision repair (BER) genes in these cell lines. The expression pattern of the 23 genes in the three leukemia cell lines was assessed using a Gene eXpression Profiler (GeXP) where the externally added kanamycin gene, Kan(r), was used for normalization. The base line for normal expression was set at 1.0, above 2.5 for genes that were overexpressed and below 0.5 for genes that were significantly downregulated. In the case of the BER pathway, *APE1* (encoding an AP endonuclease) and *PCNA* (encoding a factor involved in DNA synthesis) were the only genes upregulated in all three leukemia cell lines (Figure 3A). Two other genes, *PARP-1* (Poly ADP ribose polymerase) and *UNG1* (uracil DNA glycosylase) were significantly upregulated in HL-60 and KG-1 (Figure 3A). In contrast, the AML prognostic marker *OGG1* (encoding 8-oxoG DNA glycosylase) was downregulated in two of the cell lines (KG-1 and Mono-Mac1) (Figure 3A). *NEIL2*, another DNA glycoslyase, was downregulated only in Mono-Mac1 cell line (Figure 3A). Thus, under normal growth conditions there appears to be no unique pattern of expression of the BER genes that would distinguish one cell line from the next, for example, both HL60 and Mono-Mac1 exhibited nearly identical sensitivities to the anthracyclines, yet the expression of many BER genes (e.g., *APE1*, *NEIL2*, *PARP1*, *TDG* and *UNG*) were significantly lower in the Mono-Mac1 cells (Figure 3A). When similar analysis was performed for genes belonging to the mismatch, nucleotide excision, and recombinational DNA repair pathways (Figure 3B), again we found no striking differences amount these cell lines (Figure 3B), raising the possibility that the expression levels of DNA repair genes under normal growth conditions cannot be used as an approach to account for subtle differences in cellular responses to anthracyclines.

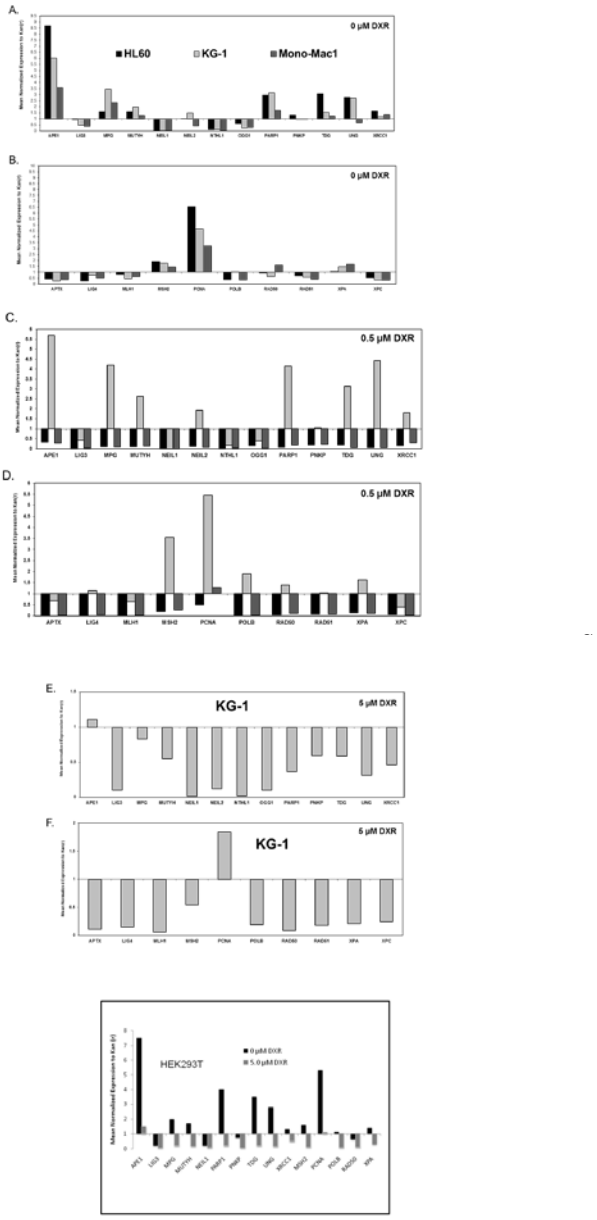

Figure 3 Expression pattern of DNA repair genes in the leukemia cell lines untreated and treated with doxorubicin. Total RNA was extracted from the cells (10x106) treated without (A and B) and with the indicated concentrations (C to F) of doxorubicin for 72 hours. Total RNA (25 ng) from each sample was analyzed by GeXP. A, C and E, genes selected from the BER pathway. B, D and F genes selected from other DNA repair pathways. G, Total RNA was extracted from the control cell line HEK293T treated without and with doxorubicin (5 μM for 72 hours) and the expression levels of the indicated gene and analyzed by GeXP. The data is the average of three independent experiments and each sample was analyzed in triplicate.

Doxorubicin treatment downregulates the expression of the DNA repair genes

We next examined if treatment with anthracyclines could alter the expression of the DNA repair genes thereby influencing the responses of leukemic cells to the drug. Upon treatment with doxorubicin (0.5 µM for 72 h), surprisingly all the BER genes as well as genes belonging to the other DNA repair pathways were completely downregulated in the HL-60 and Mono-Mac1 (Figure 3C and D) as compared to the untreated cells (Figure 3A and B, respectively). In contrast, most of the genes examined were not altered in the KG-1 cell line upon treatment with 0.5 µM of the drug (Figure 3C and D vs. Figure 3A and B, respectively). These data are consistent with the KG-1 cell line showing more resistance to the drug at 0.5 µM (Figure 1A). However, at 10-fold higher concentration of doxorubicin, all the genes were now completely downregulated in the KG-1 cells, except for *APE1* and *PCNA* (Figure 3E and F vs. A and B, respectively). Since nearly all of the DNA repair genes tested were completely downregulated in the cell lines in response to doxorubicin, we suggest that the drug may have a general effect in downregulating gene expression. In fact, this phenomenon was also observed in the kidney carcinoma cell line HEK293T (Figure 3G), suggesting that the downregulation of the DNA repair genes was not specific to the leukemic cells.

Anthracyclines diminish the level of total RNA in the sensitive leukemic cell lines

We assume that if doxorubicin globally blocked gene expression, then the total RNA would be reduced in the cell lines upon drug treatment. As shown in Figure 4, when the same amount of cells were analyzed, the recovery of total RNA from HL60 and Mono-Mac1 was very low upon treatment with 0.5 µM of the drug for 72 h, as compared to the untreated control (Figure 4, lane 1 vs 2 and 4 vs 5, respectively). In fact, the Mono-Mac1 cell line was exquisitely sensitive to the loss of total RNA as compared to HL-60 (Figure 4). In contrast, the KG-1 cell line showed no loss of total RNA following treatment with 0.5 µM of the drug, unless these cells were treated with 10-fold higher drug concentrations (5.0 µM) as compared to the untreated conditions (Figure 4, lanes 8 and 9 vs. 7). Similar results were obtained if the cell lines were treated with another anthracycline such as daunorubicin (data not shown). The observation that the recovery of total RNA correlates with the sensitivities of the leukemia cell lines towards anthracyclines raises the possibility that rapid analysis of total RNA could be an indicator of cellular responses to the drugs.

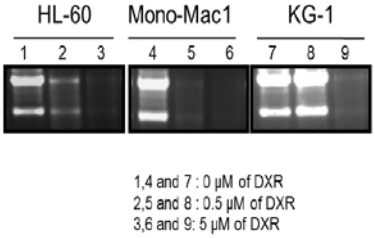

Figure 4 Recovery levels of total RNA from the untreated and doxorubicin-treated leukemia cell lines. Total RNA was extracted from 10x106 cells that were either untreated or treated with the indicated concentrations of doxorubicin (DXR) and equal volume containing the recovered total RNA was loaded per well onto a 1% agarose gel. Lanes 1, 4, 7 and 10 no treatment; lanes 2, 5, 8 and 11 cells were treated with 0.5 µM of DXR and lanes 3, 6, 9 and 12 cells were treated with 5 µM of DXR.

The recovery of total proteins from leukemic cells correlates with RNA level following exposure to daunorubicin

Since the recovery of total RNA was diminished in a dose-dependent manner following exposure to doxorubicin, we checked if this would cause a corresponding decrease in total protein. Briefly, the leukemic cell lines were treated with or without the anthracycline for various times up to 72 h and total proteins were analyzed by standard SDS PAGE stained with Coomassie (Figure 5A). When total soluble extracts were analyzed, there was a general and considerable loss of total proteins following treatment of the three cell lines with any of the anthracyclines (Figure 5A, lanes 2, 4 and 6 vs, 1, 3 and 5, respectively, shown only for daunorubicin). Thus, it would appear that the loss of total protein in these cells correlates with the observed decrease recovery of total RNA in response to anthracyline exposure.

It is noteworthy that the loss of total proteins from the control cell line HEK293T was substantially less when

compared to the leukemia cells following the same treatment conditions (see Figure 5D), suggesting that the leukemia cell lines may exhibit a distinct response from HEK293T.

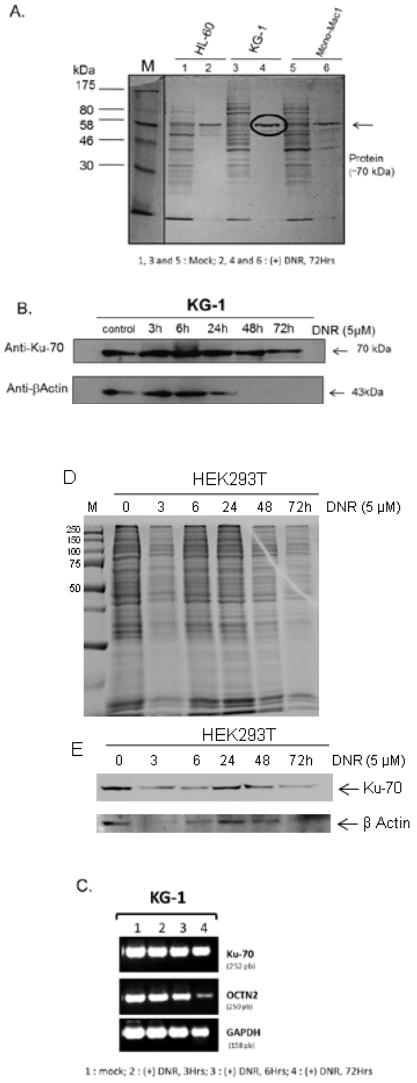

Figure 5 Ku70 is not inducible, but remains the major polypeptide detected in the leukemia cell lines following daunorubicin treatment. A, the leukemia cell lines were untreated and treated for 72 hours with 5 μM of daunorubicin (DNR) and total protein extracts analyzed on SDS-PAGE stained with Coomassie. B, Western blot showing the presence of Ku70 in the KG-1 cell line after 72 hours treatment with DNR. The cell line was treated as above for various times and total cell extract analyzed by Western probed with anti-Ku70 and anti-β-actin, the control protein. C, RT-PCR showing that Ku70 expression is not induced by DNR. C, Cells were treated for various time (0, 3, 6 and 72 hours) with 5 μM of DNR and total RNA isolated for RT-PCR analysis. OCTN2 and GAPDH were used as controls. D and E, The control cell line HEK293T was treated with DNR and total protein analyzed by SDS-PAGE stained with Coomassie and by Western blot as in panels A and B, respectively.

Ku70, a major protein that remains stable in the three cell lines in response to daunorubicin

From the above data, we observed that not all the proteins completely disappeared following drug treatment of the three cell lines. A prominent polypeptide of nearly 70 kDa in size remained stable throughout the treatment and reproducibility seen in HL-60, KG-1 and Mono-Mac1 cells (Figure 5A, lanes 2, 4 and 6). We excised this polypeptide and determined its sequence by mass spectrometry (see Materials and Methods). The major protein identified (15 peptides 30.5 % coverage of the entire protein) corresponded to sequences that matched XRCC6/Ku70 (SWISS-PROT: P12956). Ku70 is a 70 kDa protein that plays multiple roles including the ability to repair DNA double strand breaks and prevent apoptosis [15, 16]. Western blot analysis revealed that indeed Ku70 remained stable following exposure to daunorubicin over 72 h, while the control protein β-actin was completely loss after 48 h (Figure 5B). There was no significant change in Ku70 protein level during the 72 h duration of daunorubicin exposure (Figure 5B). Moreover, we observed no induction of Ku70 gene expression following daunorubicin exposure, while a control gene OCTN2 encoding an organic cation transporter was downregulated (Figure 5C). These data suggest that the stability of Ku70 is not a result of enhanced gene expression. In support of this, Ku70 protein level was also not inducible in the HEK293T following exposure to daunorubicin (Figure 5E).

Ku70 relocalizes from the cytoplasm to the nucleoli upon anthracycline exposure

Besides Ku70 role in non-homologous end joining of DNA double strand breaks, it is also involved in several other biological processes [17, 18]. Ku70 has been reported to form a complex with the pro-apoptotic protein BAX in the cytoplasm and prevent cell death. We

reason that daunorubicin treatment may induce disassociation of the Ku70-BAX complex and promote Ku70 redistribution. Under normal condition, Ku70 was found to be distributed in the cytoplasm and the nucleus in the leukemic cell lines (Figure 6A, showing data for KG-1 cells). The localization of Ku70 in the nucleus was not surprising due to its involvement in DNA repair. Following treatment of the cells with daunorubicin for 1 hr, there was no change in the cellular distribution of Ku70. However, after 6 h treatment there was an increase in Ku70 associated foci in the nucleus, and which appeared to coincide with the kinetics of PARP cleavage (Figure 6). Interestingly, after 24 h treatment most of the Ku70 protein was localized to the nucleus and seemed to concentrate in the nucleoli (Figure 6B). After 24 h of drug treatment, the nuclei of these cells became rounded, a morphological shape that is indicative of cells undergoing death. In fact, nearly 45 and 95 % of these cells were stained positive with annexin V after 24 and 72 h, respectively, following exposure to daunorubicin (Figure 7), consistent with the cells undergoing cell death. Similarly, the control cells HEK293T were subjected to cell death upon treatment with daunorubicin (Figure 7). We interpreted these data to suggest that the movement of Ku70 from the cytoplasm to the nucleus may serve to set BAX free, allowing it to trigger apoptosis.

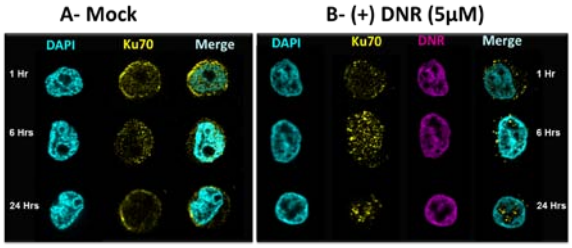

Figure 6 Relocalization of Ku70 in the KG-1 cell line after DNR exposure. KG-1 cells were cultured at 1x106 in RPMI media without (A) and with 5 µM of DNR (B). Samples were taken at different times (1, 6 and 24 hours) and processed for immunofluorescence analysis using confocal microscopy. Ku70 cellular distribution was monitored by the fluorophore fluorescein isothiocyanate (FITC). Excitation lines, DNR (543 nm, HeNe laser); DAPI (405 nm, diode laser); and FITC (488 nm, Argon laser).

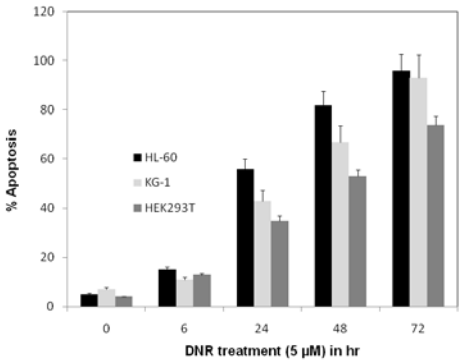

Figure 7 DNR treatment triggers apoptosis in the leukemia and HEK293T cells. Cells were treated with DNR for the indicated time followed by FACS analysis to determine the fraction of cells staining positively with annexin V. The data is an average from three experiments and the error bars indicate the standard deviation.

Discussion

The goal of our study was to investigate whether the expression levels of genes from various DNA repair pathways correlate with the cellular responses of leukemia cells towards anthracyclines. We focused specifically on anthracyclines as these are key anticancer agents used for treating AML, and which act by intercalating with the DNA to block several DNA transactions [2]. The prediction is that upregulation of DNA repair genes could be linked to AML patients who are resistant to induction-therapy with anthracycline. Herein, we found no convincing evidence, under normal growth conditions, that DNA repair genes are differentially expressed in the three cell lines, HL-60, KG-1 and Mono-Mac1. Moreover, we observed that in response to anthracyclines all of the tested DNA repair genes, except for *APE1* and *PCNA*, were sharply downregulated even in the cell line KG-1 that showed resistance at lower drug concentrations, but required a higher dose to observe the phenomenon. The finding that nearly all of the DNA repair genes displayed a pattern of downregulation, and none showing upregulation, in the leukemia cells strongly suggests that resistance towards anthracyclines is unlikely to be associated with the expression levels of these genes. However, we have not investigated further whether either *APE1* or *PCNA* or both of these genes would contribute directly to cause the resistance of KG-1 cells to low doses of anthracyclines. It

is noteworthy that PCNA encodes a clamp protein essential for DNA synthesis and which plays a central role in multiple DNA repair pathways [19, 20]. In fact, PCNA has been shown to interact with a number of DNA repair proteins such as KU70, MSH3, MSH6, MUTHY, FEN1, XRCC1 as well as others [21-26], strongly suggesting that PCNA could be a key factor governing resistance to anthracyclines. Nonetheless, we cannot exclude alternative possibilities that could explain the persistent presence of *APE1* and *PCNA* in the resistant KG-1 cell line. For example, the transcripts of both genes could be more stable or induced in response to anthracyclines in this cell line and may have no direct consequences on drug resistance.

Because anthracyclines are powerful block to many DNA transactions including transcription [27] and that many of the genes examined above were all severely downregulated, we reasoned that the recovery of total RNA would likely be affected. Indeed we observed that the recovery of total RNA was greatly reduced when the more sensitive cell lines such as HL-60 and Mono-Mac1 were treated with low doses of anthracyclines, but not from the resistant cell line KG-1. As such, it seems that the recovery of total RNA might be a better global and facile marker to monitor the responses of leukemic cells towards anthracyclines as opposed to examining the expression pattern of a subset of genes, particularly those involved in DNA repair. We propose that a simple protocol involving treatment of cultured patients' blast cells with anthracyclines followed by analysis of RNA quality by a bioanalyzer would be an ideal approach to rapidly assess the drug responsiveness of the patients' cells. This approach should represent a major advantage in terms of the rapidity in determining in advance whether a given patient would respond to drug treatment as opposed to starting the induction-therapy (lasting 7 days) and waiting another 21 days to monitor the response. Establishing this *in vitro* approach has the benefit of providing the clinician with a direct tool to evaluate the outcome of patients' responses and whether alternative regimen would be required.

A novel observation emanating from our studies is the identification of Ku70 as a predominant protein that remained stable in drug sensitive leukemic cells, while the level of the majority of proteins were vanishingly low following exposure to anthracyclines. Of note, Ku70 cellular distribution gradually shifted from the cytoplasm to the nucleoli and the earliest time of distribution coincides with PARP cleavage. Ku70 is the p70 subunit protein of the Ku70/80 complex which is involved in the non-homologous end joining DNA repair pathway that functions in the repair of DNA double-strand breaks[18]. It has been shown to have multiple cellular location including nuclear [28-33], plasma membrane [34], cytoplasmic [32], and both nuclear and cytoplasmic [35, 36], indicative of roles other than that in DNA repair. In the cytosol, Ku70 has been shown to form a complex with CLU (clusterin) and the proapoptotic Bcl-2-associated X protein (BAX) [37]. This complex of Ku70-CLU-BAX has been shown to have increase levels in the cytosol of highly aggressive human colon cancers that resist cell death [38, 39]. It is believed that Ku70 sequesters BAX preventing it from translocating to the mitochondria where it promotes the release of cytochrome C leading to the activation of caspases [40]. We reason that in response to anthracyclines, Ku70 may be modified and disassociate from BAX setting this latter protein free to induce apoptosis. We further suggest that the displaced Ku70 translocates to the nucleolus and may perform a role in this organelle. The nucleolus contains several key factors that function in sensing and repairing damage DNA and these include ATM, ATR, BLM, MRE11, PARP1, TOPBP1, WRN, XRCC1 and Ku70/80 proteins [41]. Whether Ku70 relocalization is required to participate in the surveillance and repair of rDNA or suppress expression of this region in order to limit ribosomal biogenesis and trigger cell death will require additional experimentation.

In summary, we have provided for the first time compelling evidence that shows a novel localization of Ku70 in the nucleoli of leukemic cells upon anthracycline treatment. We speculate that the relocalization of Ku70 to the nucleoli is unlikely to function in DNA repair, but participate in a process of cell death since these cells are undergoing apoptosis as determined by PARP cleavage. An interesting avenue to pursue is the mechanism that causes Ku70 to redistribute in response to anthracycline exposure in leukemic cells.

Appendix A. Supplementary data

Supplementary data associated with this article_can be

found, in the online version, at 66-139-1-SP.xls

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References

1. Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, Dombret H, Fenaux P, Grimwade D, Larson RA, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppele GJ, Sanz MA, Sierra J, Tallman MS, Lowenberg B, Bloomfield CD. Diagnosis and management of acute myeloid leukemia in adults: Recommendations from an international expert panel, on behalf of the european leukemianet. ***Blood***. 2010, 115:453-474
2. Fornari FA, Randolph JK, Yalowich JC, Ritke MK, Gewirtz DA. Interference by doxorubicin with DNA unwinding in mcf-7 breast tumor cells. ***Mol Pharmacol***. 1994, 45:649-656
3. Momparler RL, Karon M, Siegel SE, Avila F. Effect of adriamycin on DNA, rna, and protein synthesis in cell-free systems and intact cells. ***Cancer Res***. 1976, 36:2891-2895
4. Childs AC, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment in vivo causes cytochrome c release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and bcl-2:Bax ratio. ***Cancer Res***. 2002, 62:4592-4598
5. Forrest RA, Swift LP, Rephaeli A, Nudelman A, Kimura KI, Phillips DR, Cutts SM. Activation of DNA damage response pathways as a consequence of anthracycline-DNA adduct formation. ***Biochem Pharmacol***. 2012,
6. Spencer DM, Bilardi RA, Koch TH, Post GC, Nafie JW, Kimura K, Cutts SM, Phillips DR. DNA repair in response to anthracycline-DNA adducts: A role for both homologous recombination and nucleotide excision repair. ***Mutat Res***. 2008, 638:110-121
7. Moraes MC, de Andrade AQ, Carvalho H, Guecheva T, Agnoletto MH, Henriques JA, Sarasin A, Stary A, Saffi J, Menck CF. Both xpa and DNA polymerase eta are necessary for the repair of doxorubicin-induced DNA lesions. ***Cancer Lett***. 2012, 314:108-118
8. Saffi J, Agnoletto MH, Guecheva TN, Batista LF, Carvalho H, Henriques JA, Stary A, Menck CF, Sarasin A. Effect of the anti-neoplastic drug doxorubicin on xpd-mutated DNA repair-deficient human cells. ***DNA Repair (Amst)***. 2010, 9:40-47
9. Liddiard K, Hills R, Burnett AK, Darley RL, Tonks A. Ogg1 is a novel prognostic indicator in acute myeloid leukaemia. ***Oncogene***. 2010, 29:2005-2012
10. Zaremba T, Thomas H, Cole M, Plummer ER, Curtin NJ. Doxorubicin-induced suppression of poly(adp-ribose) polymerase-1 (parp-1) activity and expression and its implication for parp inhibitors in clinical trials. ***Cancer Chemother Pharmacol***. 2010, 66:807-812
11. Noren Hooten N, Kompaniez K, Barnes J, Lohani A, Evans MK. Poly(adp-ribose) polymerase 1 (parp-1) binds to 8-oxoguanine-DNA glycosylase (ogg1). ***J Biol Chem***. 2011, 286:44679-44690
12. Poschmann J, Drouin S, Jacques PE, El Fadili K, Newmarch M, Robert F, Ramotar D. The peptidyl prolyl isomerase rrd1 regulates the elongation of rna polymerase ii during transcriptional stresses. ***PLoS One***. 2011, 6:e23159
13. Andrabi SA, Kim NS, Yu SW, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD, Poirier GG, Dawson VL, Dawson TM. Poly(adp-ribose) (par) polymer is a death signal. ***Proc Natl Acad Sci U S A***. 2006, 103:18308-18313
14. Woodhouse BC, Dianov GL. Poly adp-ribose polymerase-1: An international molecule of mystery. ***DNA Repair (Amst)***. 2008, 7:1077-1086
15. Roberts SA, Strande N, Burkhalter MD, Strom C, Havener JM, Hasty P, Ramsden DA. Ku is a 5'-drp/ap lyase that excises nucleotide damage

near broken ends. ***Nature***. 2010, 464:1214-1217

16. Sawada M, Sun W, Hayes P, Leskov K, Boothman DA, Matsuyama S. Ku70 suppresses the apoptotic translocation of bax to mitochondria. ***Nat Cell Biol***. 2003, 5:320-329
17. Muller C, Paupert J, Monferran S, Salles B. The double life of the ku protein: Facing the DNA breaks and the extracellular environment. ***Cell Cycle***. 2005, 4:438-441
18. Featherstone C, Jackson SP. Ku, a DNA repair protein with multiple cellular functions? ***Mutat Res***. 1999, 434:3-15
19. Essers J, Theil AF, Baldeyron C, van Cappellen WA, Houtsmuller AB, Kanaar R, Vermeulen W. Nuclear dynamics of pcna in DNA replication and repair. ***Mol Cell Biol***. 2005, 25:9350-9359
20. Shivji KK, Kenny MK, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. ***Cell***. 1992, 69:367-374
21. Clark AB, Valle F, Drotschmann K, Gary RK, Kunkel TA. Functional interaction of proliferating cell nuclear antigen with msh2-msh6 and msh2-msh3 complexes. ***J Biol Chem***. 2000, 275:36498-36501
22. Parker A, Gu Y, Mahoney W, Lee SH, Singh KK, Lu AL. Human homolog of the muty repair protein (hmyh) physically interacts with proteins involved in long patch DNA base excision repair. ***J Biol Chem***. 2001, 276:5547-5555
23. Henneke G, Koundrioukoff S, Hubscher U. Phosphorylation of human fen1 by cyclin-dependent kinase modulates its role in replication fork regulation. ***Oncogene***. 2003, 22:4301-4313
24. Gary R, Ludwig DL, Cornelius HL, MacInnes MA, Park MS. The DNA repair endonuclease xpg binds to proliferating cell nuclear antigen (pcna) and shares sequence elements with the pcna-binding regions of fen-1 and cyclin-dependent kinase inhibitor p21. ***J Biol Chem***. 1997, 272:24522-24529
25. Fan J, Otterlei M, Wong HK, Tomkinson AE, Wilson DM, 3rd. Xrcc1 co-localizes and physically interacts with pcna. ***Nucleic Acids Res***. 2004, 32:2193-2201
26. Matheos D, Ruiz MT, Price GB, Zannis-Hadjopoulos M. Ku antigen, an origin-specific binding protein that associates with replication proteins, is required for mammalian DNA replication. ***Biochim Biophys Acta***. 2002, 1578:59-72
27. Pigram WJ, Fuller W, Hamilton LD. Stereochemistry of intercalation: Interaction of daunomycin with DNA. ***Nat New Biol***. 1972, 235:17-19
28. Koike M, Miyasaka T, Mimori T, Shiomi T. Subcellular localization and protein-protein interaction regions of ku proteins. ***Biochem Biophys Res Commun***. 1998, 252:679-685
29. Koike M, Shiomi T, Koike A. Ku70 can translocate to the nucleus independent of ku80 translocation and DNA-pk autophosphorylation. ***Biochem Biophys Res Commun***. 2000, 276:1105-1111
30. Koike M, Awaji T, Kataoka M, Tsujimoto G, Kartasova T, Koike A, Shiomi T. Differential subcellular localization of DNA-dependent protein kinase components ku and DNA-pkcs during mitosis. ***J Cell Sci***. 1999, 112 (Pt 22):4031-4039
31. Mimori T, Hardin JA, Steitz JA. Characterization of the DNA-binding protein antigen ku recognized by autoantibodies from patients with rheumatic disorders. ***J Biol Chem***. 1986, 261:2274-2278
32. Bakalkin G, Yakovleva T, Hurd YL, Nussenzweig A, Li GC, Terenius L. Autoantigen ku in the brain. Developmentally regulated expression and subcellular localization. ***Neuroreport***. 1998, 9:2147-2151
33. Yaneva M, Jhiang S. Expression of the ku protein during cell proliferation. ***Biochim Biophys Acta***. 1991, 1090:181-187
34. Prabhakar BS, Allaway GP, Srinivasappa J, Notkins AL. Cell surface expression of the 70-kd component of ku, a DNA-binding nuclear autoantigen. ***J Clin Invest***. 1990, 86:1301-1305
35. Fewell JW, Kuff EL. Intracellular redistribution of ku immunoreactivity in response to cell-cell

contact and growth modulating components in the medium. ***J Cell Sci***. 1996, 109 (Pt 7):1937-1946

36. Grawunder U, Finnie N, Jackson SP, Riwar B, Jessberger R. Expression of DNA-dependent protein kinase holoenzyme upon induction of lymphocyte differentiation and v(d)j recombination. ***Eur J Biochem***. 1996, 241:931-940
37. Pucci S, Mazzarelli P, Sesti F, Boothman DA, Spagnoli LG. Interleukin-6 affects cell death escaping mechanisms acting on bax-ku70-clusterin interactions in human colon cancer progression. ***Cell Cycle***. 2009, 8:473-481
38. Pucci S, Bonanno E, Pichiorri F, Angeloni C, Spagnoli LG. Modulation of different clusterin isoforms in human colon tumorigenesis. ***Oncogene***. 2004, 23:2298-2304
39. Pucci S, Mazzarelli P, Nucci C, Ricci F, Spagnoli LG. Clu "in and out": Looking for a link. ***Adv Cancer Res***. 2009, 105:93-113
40. Mazzarelli P, Pucci S, Spagnoli LG. Clu and colon cancer. The dual face of clu: From normal to malignant phenotype. ***Adv Cancer Res***. 2009, 105:45-61
41. Moore HM, Bai B, Boisvert FM, Latonen L, Rantanen V, Simpson JC, Pepperkok R, Lamond AI, Laiho M. Quantitative proteomics and dynamic imaging of the nucleolus reveal distinct responses to uv and ionizing radiation. ***Mol Cell Proteomics***. 2011, 10:M111 009241