Some Insights into the Cellular Responses Against Doxorubicin and Actinomycin D**

Malek Zihlif, [1] Hamza Beano, [1]Mohammad Barouqa, [1] Sameer Shwaiki, [1] Randa Naffa [1]

Abstract

Cellular responses to anti-cancer agents are an important factor in recognizing mechanisms of resistance and in identifying new treatment biomarkers. In this study, we have compared the effect of actinomycin D and doxorubicin on selected genes in the transcription and ubiquitin pathways. The human promyelocytic leukemia cells (HL60) was used as a model system and the chosen genes were POL2A and ELL2 (from transcription machinery) and UBE2DE and CDC (from ubiquitin pathway). The responses of the four targeted genes suggested a degree of biological resistance amongst just those functions that might be expected to be damaged by the drug action. It is as if the cells are trying to up-regulate these functions to offset drug inhibition. For example, doxorubicin up-regulated ubiquitin-related genes suggesting an attempt to remove the trapped cleavable complex by an ubiquitin-dependent mechanism, while actinomycin up-regulated the transcription machinery genes suggesting an attempt to overcome the longed lived complexes that are formed by the actinomycin D on the DNA.

Introduction

DNA intercalating agents are important clinical and experimental cancer drugs. They can be divided into two broad classes; those that intercalate into the Watson-Crick duplex with fast kinetics and poison the enzymes topoisomerase I and II,[1-3] and those that intercalate having dissociation kinetics slow enough to block the passage of RNA polymerases. In this way, the production of full-length transcripts is either slowed down, or ablated. [3, 4] Topoisomerase poisons are generally believed to exert their cytotoxic effects by trapping topoisomerase cleavable complexes in a manner that leads to the formation of lethal DNA double strand breaks[5, 6], but it is possible that the trapped cleavable complexes, or perturbed topoisomerase function, may directly affect transcription, and these effects might contribute to the mechanism of action.[7] This study aims to compare the biological responses of cells to actinomycin D, a representative for the transcription inhibitor group, with doxorubicin, a representative for the topoisomerase inhibitor. These responses will be measured against the ubiquitin pathway and the transcriptional machines which are known to be involved in the biological responses to these drugs.

1. **Department of Pharmacology, Faculty of Medicine, University of Jordan, Amman, Jordan.**
* Correspondence should be addressed to:
Malek Zihlif
P. O. Box: Amman 11942, Jordan.
E-mail: m.zihlif@ju.edu.jo.

The general cellular response to topoisomerase II poisons is now reasonably well described, [8, 9] and may be summarized in the following. The trapped cleavable complex prevents strand passage and the relaxation of DNA supercoiling, both of which have direct biological consequences in their own right.[10, 11] The trapped cleavable complex is also anticipated to cause transcription arrest directly, by collision with a transcribing polymerase, in analogy with this known activity of trapped topoisomerase I cleavable complexes.[12, 13] Cells attempt to remove the trapped cleavable complex by the ubiquitin-dependent mechanism, in which ubiquitin is linked through its carboxyl terminus to the topoisomerase, marking it as a substrate for proteolysis by the 26S proteasome.[14] This process exposes over-lapping DNA double strand breaks that can be repaired by direct relegation, or by homologous recombination or non-homologous end joining. [14]

Advances into the process of transcript elongation by RNA polymerase II have identified this stage as a dynamic and highly regulated step. Transcription elongation is not a smooth continuous process but is characterized by multiple, and specific, pausing events. The duration of these pauses depends on the specific DNA sequence being transcribed and on the transcriptional elongation factors required to enhance transcription through the pause sites.[15] One important example on these elongation factors is the ELL2 that has indistinguishable elongation activities in vitro. Many studies have illustrated that ELL2 stimulates the overall rate of elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites along the DNA templates, [15, 16] The model of actinomycin D prescribed by Sobell (1985) suggests a possible relationship to the phenomenon of pausing by the RNA polymerase enzyme during transcription. [17-19] The involvement of the elongation transcription factors in overcoming the actinomycin D pausing is similar to the way that is used by the cells to defeat the natural pausing.

In this work, we have compared the effect of actinomycin D and doxorubicin on selected genes in the transcription machinery and in the ubiquitin pathway. The model system was human promyelocytic leukemia cells (HL60) and the chosen genes are POL2A and ELL2 (from the transcription machinery) and UBE2DE and CDC (from the ubiquitin pathway). The result of this work may shed light on the extent of similarity in biological response to topoisomerase poisons and transcription inhibitors. Such studies also help in understanding tahet possible modes of cellular resistance, which, of course, will give researchers a good opportunity to identify new targets for complimentary drugs.

Materials and Methods

Cell Line: HL60, a human promyelocytic leukemia cell line, was obtained kindly from Dr. Mona Hassuneh, Faculty of Science, University of Jordan. HL60 cells were maintained in a complete medium consisting of RPMI medium 1624 (Gibco, USA) supplemented with 2mM L-glutamine, 100units/ml of penicillin/streptomycin and 10% foetal bovine serum, all which were purchased from Gibco (USA) and cultured at 37 °C in the presence of 5% CO_2. Cultures were passaged twice weekly at 70% confluency.

Cell Treatment: Doxorubicin and Actinomycin D were purchased from Sigma (USA) and solubilized in phosphate buffered saline (PBS). The IC_{50} used for doxorubicin treatment is 18 nM according to Kostrzewa-Nowak et al., [20] and the IC_{50} of actinomycine is 1 nM according to Takusagaw et al., [21] The HL60 cells were seeded at a density of 1.5 x 10^5 cells/ml in a T-25 tissue culture flask and incubated for 24 hours before drug treatment with 5 x IC_{50} concentrations for both 8 and 24 hours. The amount of drug and viable number of HL60 cells after treatments are listed in Table (1). The treatments were done in duplicate for each drug. Control cells were treated in the same way, except they did not receive the drug treatments. Following the treatment period, the cells were harvested by centrifugation at 1000 rpm for 10 minutes at 4 °C. The culture medium was removed by aspiration, the cells were washed with ice-cold PBS and the pellets recovered again for RNA extraction.

Table (1): Viability of cells after 8 and 24 hours drug treatments.

Drug	Treatment 5 X IC50 (nM)	No. of cells	% Viable cf control 8 hrs	% Growth cf control 24 hrs
Doxorubicin	90 nM	1.5×10^6	92	80
Actinomycin D	5 nM	1.5×10^6	92	81

RNA Extraction and cDNA Synthesis

Total RNA was extracted using Trizol, LS (Invitrogen, USA). The RNA quality was assessed by the spectrophotometric method (A_{260}/A_{280}). RNA samples were stored at -80°C until used. Complementary DNA (cDNA) was synthesized from 1.0 μg total RNA using a RT-PCR Kit (Promega, USA) in a final volume of 20 μl with random hexamer primers according to the manufacturer's instructions.

Quantitative Real-time PCR (Q-PCR)

Q-PCR was carried out in IQ4 real time PCR (BioRad, USA). The reaction mixture consisted of 1X QuantiTect SYBR Green PCR Master Mix (12.5 μl) (Qiagen, Germany), 1.0 μl of cDNA and primers as indicated in Table (2) in a total volume of 25 μl QPCR mix. The PCR condition for GAPDH (internal reference control), UBE2DE and CDC34 comprised of first incubation at 95°C for 10 minutes, 40 cycles of

denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds. While the PCR condition for GAPDH (internal reference control), POL and ELL comprised of first incubation at 95°C for 15 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 30 seconds. Fluorescence was recorded at the end of extension. A negative control without cDNA template was run simultaneously with every assay. To generate a standard curve, template cDNA from untreated-control HL60 cells was used. Quantification of gene expression was calculated by the standard curve and cycle threshold of each sample. The results of gene expressions were normalized to reference gene expression and the fold exchange was determined in comparing with untreated cell control. Two replicates of this experiment were carried out in which every gene had a duplicated reading thereby generating 4 expression values for every gene. A melt curve analysis was done after QPCR to ensure the specificity of the PCR product.

Table (2): Primers sequences, concentrations and PCR conditions.

Gene	Primer Sequence (5→3')	Concentration	Size of PCR product (bp)	References
UBE2DE	F:agagtgaggagccagacgaca R:ctgcagaacattgtgctggag	0.5μM	103	22
CDC34	F:aagatgtggcaccctaacatctac R:agggagatcacactcaggagaat	0.5μM	155	23
POLA2	F:gctgctggacgtgagtatga R:ggtgtcatggcaggagagat	7.5 pmol	204	24
ELL2	F: tacagtgaaatagacagacggtcattgg R: ccatttagtgttggtggtactctgttc	0.5μM	227	25
GAPDH	F:cgaccactttgtcaagctca R:aggggagattcagtgtggtg	3.0 pmol	202	24

Results

As a prelude to the quantitative real time studies, we first assessed the viability of the cells after 8 and 24 hours. The observed cell viability was 90% in the 8h treatments and approximately 75% in the 24h treatments compared to the control cells. The real-time PCR results showed that

mRNA expression levels for the four genes UBE2DE, CDC34, POL2A, and ELL2 vary with the duration of exposure and also with the used agents. Clearly, the observed values indicated that every agent is specific in its effect. For example, treating the cells with doxorubicin for 8 hours up-regulated the UBE2DE and CDC34 approximately 2 fold and produced no significant

changes in POL2A and ELL2 genes (see Figure 1). Similarly, treating the HL60 cells with actinomycin D, for 8 hours up-regulated POL2 and ELL2, 2.33 and 2.27 fold, respectively, whereas the POL2A and CDC34 scored no significant changes (see Figure 1). In the 24 hour exposure time, the up-regulation effects observed in the 8 hour treatment seems to be diluted. The 24 hour detected expression values for the examined genes are close to their conger in the control samples. Taking the 24 hours as a whole will not indicate significant changes in the expression levels for the tested genes.
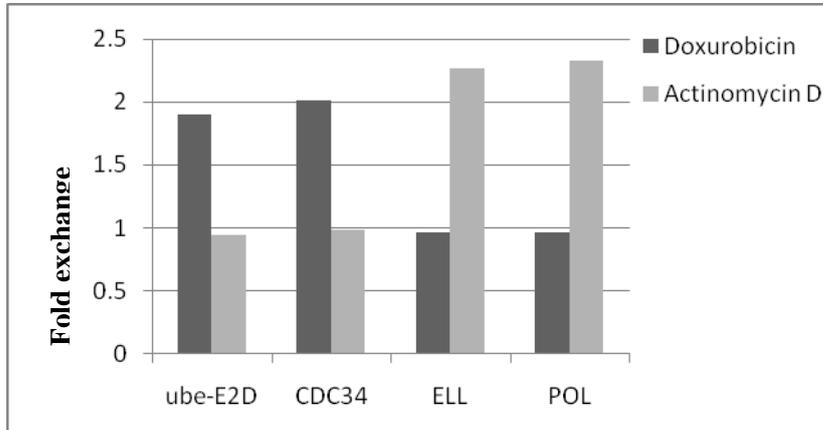

Figure (1): Effect of two drugs on four gene expression (Ube-E2D, CDC34, ELL, POL) after 8 hr treatment.

Discussion

Cellular responses to anti-cancer agents are an important factor in recognizing mechanisms of resistance and in identifying new biomarkers for cancer treatment. In this study, we hypothesized that the cell responses against anti-cancer agents vary according to their molecular target and reflect a degree of biological resistance amongst those functions that might be expected to be most damaged by drug action. The response variations might lead to a greater understanding of the mechanism of action for anti-cancer drugs and may open a new land for a new therapy that aims to reduce these agents' resistance and toxicity. To prove our hypothesis we investigated the effects of two important intercalating agents, doxorubicin and actinomycin D, on two fundamental cellular machineries, the ubiquitin pathway and the transcriptional machinery. We used a relatively toxic concentration (5 x IC$_{50}$) at two different time points, 8 and 24 hours, aiming to examine the relationship between drug-induced genomic injury and altered targeted genes expression. The choice of 8 and 24 hours is significant as it reflects the early and late cellular response times to the drugs.

The responses of our targeted genes, POL2A, ELL2, UBE2DE, and CDC34, suggest an attempt to up-regulate those functions that may offset drug inhibition, a kind of biological Le Chatelier's Principal. For example, the earlier responses against doxorubicin treatment showed an up-regulation in the ubiquitin pathway genes but not in those related to transcription. On the other hand, treatment with actinomycin D up-regulated its primary target, the transcription machinery, not in those related to the ubiquitin pathway. However, this specific up-regulation was diluted at the later time, in which the cells might give up the resistance options and chose to start the preparation to undergo programmed cell death.

The up-regulation effects of doxorubicin on ubiquitin-related genes suggest an attempt to remove the trapped cleavable complex by an ubiquitin-dependent mechanism, marking it as a

substrate for proteolysis by the 26S proteasome.[13, 14] Figure (3) summarizes the ubiquitin-mediated proteolysis pathway where the proteasome selects only ubiquitin-tagged proteins for degradation, and how it has been up-regulated by doxorubicin. Doxorubicin activates two important genes in these pathways, which code for members of the E2 family. The two E2 enzymes are essential in three of the five tagging cascades. Thus, if the target in Figure (3) is topoisomerase II, we can conclude that three of the five possible cascades for tagging the poisoned enzyme have been up-regulated by doxorubicin as a response to topoisomerase-trapping. These findings concur with those of Kudoh and colleagues that a cluster of genes involved in the ubiquitin-proteasome pathway are prominently up-regulated by doxorubicin treatment. [26] In case of actinomycin D, no significant changes were observed and the genes mostly chose to stay unchanged, the finding that may drive an exclusion of topoisomerase poisoning from the mechanism of action from actinomycin D.

The opposite findings on the transcription related genes reassured our findings and agreed again with our hypothesis. It implies again that the cell up-regulated those functions that might be expected to be most damaged by the drug action. Clearly, the ELL2 and POL2A genes are only up-regulated in the actinomycin D case and not in the doxorubicin case. Here the cells are trying to overcome the longed lived complexes that were formed by the actinomycin D on the DNA by two mechanisms. The first is by up-regulating the gene expression level of POL2A, the main transcription driver that is known to have an important role in controlling the rate of transcription. [15, 16] The second response is through

up-regulating the ELL2 gene that is known to stimulate the overall rate of elongation by RNA polymerase II by suppressing transient pausing by polymerase at many sites along the DNA templates. [15,16] Surely, the major part of this up-regulation will happen in order to overcome the pausing time that results from the longed lived, 300s or more, occupation of actinomycin D into DNA.

Taking the finding from a drug resistance point of view implies that the ubiquitin pathway and the transcription machinery can be a valuable target for a new therapy which will hopefully limit the toxicity and improve the efficacy of the tested anticancer agents. For example, developing a drug that can prevent the up-regulation of ubiquitin E2 genes seen in our experiment may help in increasing the efficacy of doxorubicin and so reduce the dose that is given to the patients. This reduction is important because it has been shown that the long term cardiotoxicity is related to a patient's cumulative lifetime dose. [28] A patient's lifetime dose is calculated during treatment, and doxorubicin treatment is usually stopped upon reaching the maximum cumulative dose. Moreover, it has been shown that the effect of cardiotoxicity increases in long term survivors, from 2% after 2 years to 5% after 15 years. Increasing the efficacy will delay reaching this threshold. [28] On the other hand, the up-regulation of POLA2 and ELL2 genes as a response to actinomycin D treatment can also be attractive and can yield an important target for a drug development. Presumably, the new agent will inhibit the transcriptional machinery and can be combined with actinomycin D. This combination can help in decreasing the bone marrow toxicity effect of actinomycin D and enhance the actinomycin anti-cancer spectrum and activity. [29]
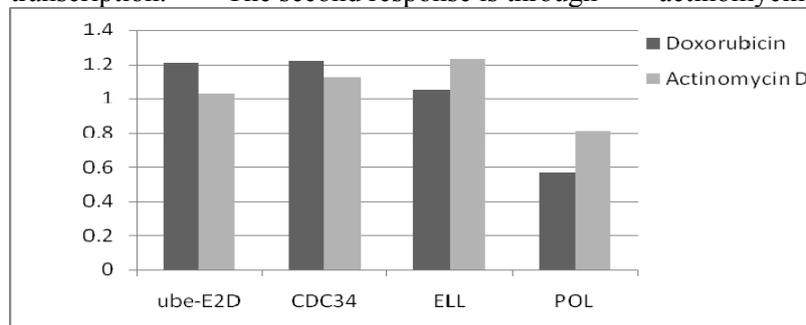

Figure (2): Effect of two drugs on four gene expression (Ube-E2D, CDC34, ELL, POL) after 24 hr treatment.
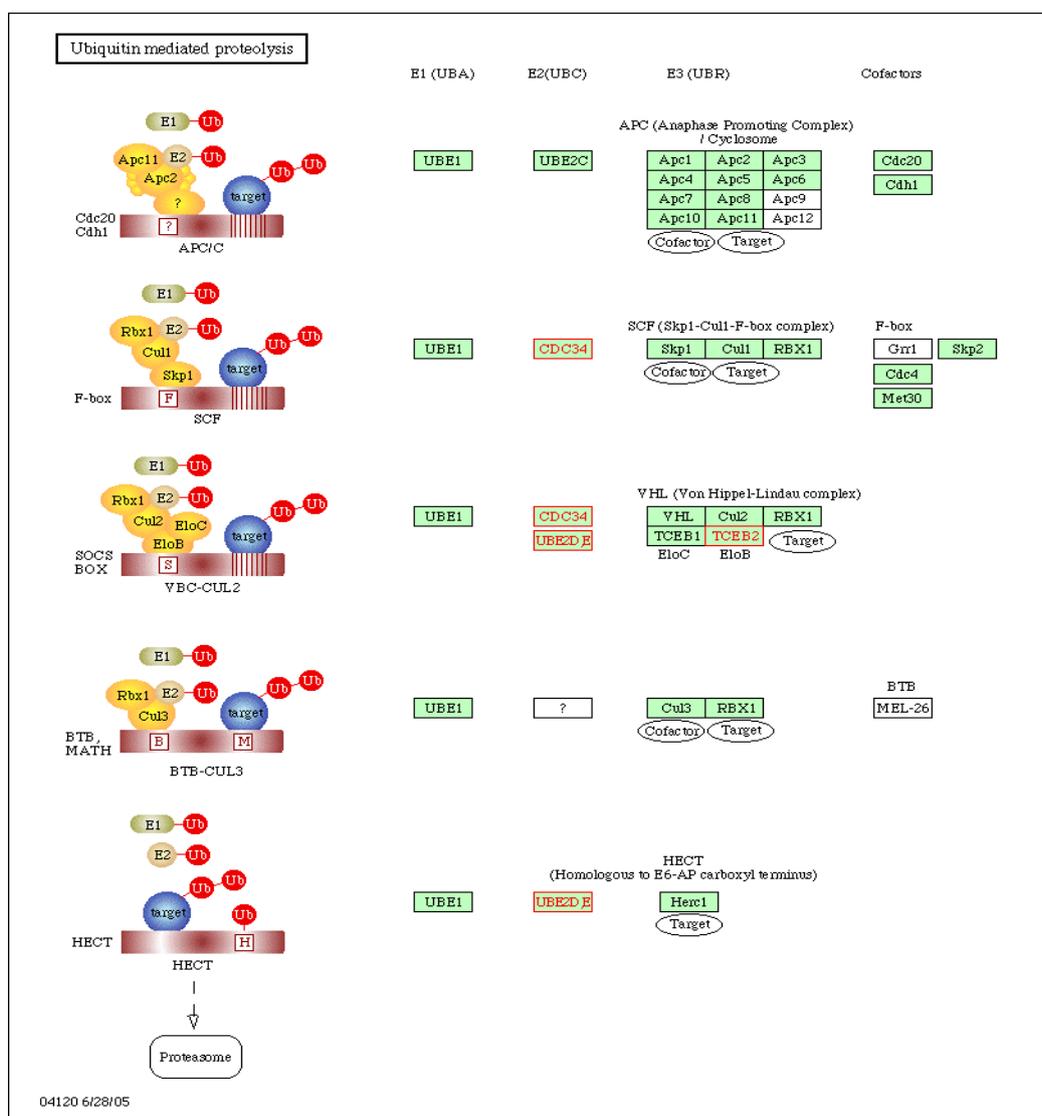
Figure (3): The ubiquitin-mediated proteolysis pathway (DAVID Bioinformatics Resources 6.7).

Conclusion

The results of this study agree with our hypothesis. Cell responses depend on the type of cellular insults and these responses may reflect a degree of biological resistance amongst the functions that might be expected to be the most damaged by drug action.

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بعض الدلائل لكيفية استجابة الخلايا السرطانية لدوائي دوكسوروبسين وأكتينوماسين د

مالك الزحلف،[1] حمزة بينو، [1] محمد باروقة، [1] سامير شويكي،[1] رندا نفاع[1]

1– قسم علم الأدوية، كلية الطب، الجامعة الأردنية، عمان، الأردن

الملخص

تعد استجابة الخلايا لأدوية السرطان عاملاً مهماً للتعرف على طريقة عمل الأدوية السرطانية وكذلك على كيفية مقاومة تلك الخلايا لعمل هذه الأدوية. في هذه الدراسة قمنا بدراسة تأثير دوائي دوكسوروباسين وأكتينوماسين د على سبيلين حيويين مهمين في الخلية وهما ubiquitin pathway and the transcriptional machinery، وقد عرضنا خلايا اللوكيميا (HL60) لجرعة سامة نسبياً لمدة 8 و24 ساعة. وتم اختيار جينين مهمين لتمثيل كلٍ من السبيلين الحيويين. النتائج اظهرت أن التغير في كمية الـmRNA للجينات تعكس درجة جيدة من لمقاومة البيولوجية في السبل المتوقع انتفاضها لإنقاذ الخلية. على سبيل المثال تعريض الخلايا للدوكسوروبسين د أدى إلى زيادة التمثيل لجيني داخل الخلية فقط لتلك الجينات الممثلة لـ ubiquitin pathway والتي تعمل كما هو مثبت على إزالة تأثير ذلك الدواء من على المادة الوراثيةداخل الخلية. بينما زاد التمثيل الجيني داخل الخلية للجينات الممثلة لtranscriptional machinery بعد تعريض الخلايا للأكتينوماسين د كمحولة لزيادة فاعلية الـtranscription ، علماً بأن لأكتينوماسين د يعمل على إيقاف الـtranscription في الخلية دفعاً اياها إلى اختيار ما يسمى بالموت التلقائي.

الكلمات الدالة: دوكسوروباسين، أكتينوماسين د، Ubiquitin Pathways, Transcription Pathways.