

## Short Communication

# Unexploited Value of PCR-Stop Assay for Anticancer Drug Efficacy

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Submitted: 11 January 2017

Accepted: 10 March 2017

Published: 13 March 2017

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**OPEN ACCESS****Keywords**

• PCR-Stop-RT-PCR; Personalized medicine; Cancer; Anticancer drugs

**Abstract**

The basic principle of the PCR-Stop assay is that a DNA polymerase encountering a break or an adduct will fall off the template or stall. Consequently, the amount of amplified DNA is inversely proportional to the levels of DNA damage. Though promising as a potential tool for predicting and monitoring anticancer drug efficacy, the original procedure never developed into a reliable and well accepted clinical test due to a number of technical difficulties including reproducibility and sensitivity. Here, we optimized the original PCR-Stop parameters to be compatible with the sensitivity of Real Time PCR (RT-PCR) and have developed an effective tool to reliably measure the efficacy of VP16 (etoposide) and Doxorubicin, two Topoisomerase II (Topo II) inhibitors. Validation of the predicted PCR-stop-RT-PCR response was performed by evaluation of the levels of histone H2AX phosphorylation ( $\gamma$ H2AX) and correlation with survival assays in tissue culture cells. Moreover, the PCR-Stop-RT-PCR assay was tested on Peripheral Blood Mononuclear Cells (PBMC) of two leukemia patients treated with the Histone Deacetylase Inhibitor (HDACI) Vorinostat<sup>SAHA</sup>. Our results indicate that Vorinostat<sup>SAHA</sup> increased the sensitivity of both patients PBMC to VP16 by about 40%. This assay could be adapted to a variety of anticancer drugs and use for assessment of drug efficacy, suitability or optimization.

**ABBREVIATIONS**

RT-PCR: Real-Time PCR; Topo: Topoisomerase; HDACI: Histone Deacetylase Inhibitor; PBMC: Peripheral Blood Mononuclear Cells; VP16: Etoposide;  $\gamma$ H2AX: Histone H2AX Phosphorylation; TSA: Trichostatin A; DMSO: Dimethyl Sulfoxide; FBS: Fetal Bovine Serum; MEM: Minimum Essential Media; RPMI: Roswell Park Memorial Institute; RFI: Referenced Fluorescence Intensity

**INTRODUCTION**

Most chemotherapeutic regimens include at least one drug targeting DNA or enzymes acting on DNA. These drugs are thus most effective during the replicative phase of the cell cycle and include cross-linking agents such as cisplatin and its derivatives, as well as small molecule inhibitors of topoisomerases. Prior reports have demonstrated that Topo II inhibitors such as VP16 and Doxorubicin, induce prominent DNA cleavage in the P1/P2 promoter of the *c-myc* gene [1]. The determination of the preferred DNA sequence targeted by these drugs allowed the development of a PCR-stop assay to assess the amount of DNA strand breaks generated by the drugs *in vitro* [2]. However, this procedure never developed into a reliable and well accepted clinical test due to a number of technical difficulties. One of the main difficulties was the reproducibility of the results. This was primarily due to the use of non-optimal set of primers. Another limitation was the sensitivity of the technique which was not high enough to detect DNA breaks at clinically relevant doses of

anticancer drugs. This was subsequently improved with the use of radioactive nucleotides to detect the amount of DNA breaks [3], but this brought another set of challenges related to the use and disposal of radioactive material. By optimizing the original PCR-Stop parameters to be compatible with the sensitivity of RT-PCR we have developed an effective tool to reliably measure the efficacy of a variety of Topo II inhibitors (U.S. Patent No. 8,367,340). Validation of the predicted PCR-stop-RT-PCR response was performed by evaluation of the levels of  $\gamma$ H2AX, a primary marker of DNA strand breaks [4], and correlation with survival assays in tissue culture cells. Most importantly the technique indicates that DNA extracted from the PBMC of two leukemia patients treated with the HDACI Vorinostat<sup>SAHA</sup> are more sensitive to VP16. This assay could be adapted to a variety of anticancer drugs targeting the DNA or enzymes acting on the DNA. In addition, because only small amounts of DNA are required (50-100 ng) several drugs testing could be performed simultaneously and optimal drugs or drug combination could be identified for a particular patient.

**MATERIALS AND METHODS****Cell culture and drug treatments**

The human glioblastoma U118 cells and the untransformed human small intestinal epithelial FHs Int 74 cells were purchased from the American Type Culture Collection (Manassas, VA). The human colon carcinoma RKO cells were obtained from Dr. Michael Kastan (Duke University, Durham, NC). The U118 cells

were grown in Minimum Essential Media (MEM) containing 10% Fetal Bovine Serum (FBS, GIBCO-BRL, Gaithersburg, MD), The FHs 74 Int cells were grown in Hybri-Care media (ATCC) containing 10% FBS and the RKO cells were grown in Roswell Park Memorial Institute (RPMI 1640) medium containing 10% FBS in the absence of antibiotics. The cells were grown and treated as described before [5]. Briefly, the cells were either left untreated, treated with HDACIs, either 10 ng/ml Trichostatin A (TSA, Sigma-Aldrich, St-Louis, MO) or 1.25  $\mu$ M Vorinostat<sup>SAHA</sup> (Biomol, Plymouth Meeting, PA) for 4hrs followed by either VP16 or doxorubicin (Sigma-Aldrich) for 1h. For clonogenic survival assays the cells were plated at 2–3  $10^2$  cells/60-mm-diameter Petri dish and exposed to HDACIs before exposure to increasing amounts of either VP16 (0–100 $\mu$ M), or doxorubicin (0–10 $\mu$ M) for 1 h.

PBMC: Blood samples were obtained from patients enrolled in a Phase 1 clinical trial (GCC0447) for relapsed and/or refractory acute leukemias and myelodysplastic syndromes at the University of Maryland Greenebaum Cancer center, Baltimore, MD. All patients signed a University of Maryland (Baltimore, MD) Institutional Review Board (IRB)–approved informed consent form [6]. Peripheral blood (5 mL/time point) was collected before Vorinostat<sup>SAHA</sup> treatment and four hours after the last dose of Vorinostat<sup>SAHA</sup> on day three of 400 mg p.o. Mononuclear cells were purified by gradient sedimentation using ACCUSPIN System-Histopaque-1077 (Sigma-Aldrich). The cells were then cryo preserved in 7.5% dimethyl sulfoxide (DMSO)/ 92.5% FBS and stored in liquid nitrogen vapor until use. The day prior the assay, the cells were thawed and slowly diluted with cold media (RPMI, GIBCO-BRL) and spun at 800 rpm for 10 min. The cells were then re-suspended in fresh media (RPMI-10% FBS) and allowed to stabilize for 4h at 37°C. The samples obtained before Vorinostat<sup>SAHA</sup> treatment ( $10 \times 10^6$  cells) were then treated *in vitro* with either Vorinostat<sup>SAHA</sup> (2.5 $\mu$ g/ml, 4h), VP16 (Sigma-Aldrich, 25  $\mu$ M, 2h) or Vorinostat<sup>SAHA</sup> first followed by VP16. The samples obtained from patients receiving Vorinostat<sup>SAHA</sup> were either used as is (Vorinostat<sup>SAHA</sup> *in vivo*) or treated with VP16 for 2h. The cells were replenished with fresh media at the end of the treatments and genomic DNA was extracted (genomic Prep Mini Spin kit, Pittsburgh, PA) 16h later and assayed by PCR-Stop-RT-PCR as described below.

### PCR-Stop-RT-PCR

Reactions for RT-PCR were set up with Quanti Tect SYBR Green Master Mix (Qiagen, USA) in iCycleriQ 96-well PCR-plates (BIO-RAD Laboratories, CA, USA). Primer sequences used are: Target Forward primer; 5' - CCCTTTATAATGCGAGGGTCT - 3', Target Reverse Primer; 5' - GGCTCTTCCACCCTAGCC - 3', Reference Forward Primer: 5' - TACTTTAGTTAACCAGTGCCAGTCC - 3', Reverse: 5' - ATCTGCGTGGCTACAGATAAGTTAC - 3'. To test the PCR-efficiency of both primer pairs, dilution series of gDNA from untreated cells were amplified with RT-PCR and then the PCR efficiency was calculated from the slope of the standard curve. Each reaction contained 12  $\mu$ L of 2 $\times$  Mastermix, 0.25  $\mu$ M of each primer, 50–100 ng gDNA and nuclease-free water to yield 25  $\mu$ L reaction volume. Primer ratio was 250 nM of primers for a template concentration between 10 and 100 ng. Quantitative RT-PCR assays were carried out in triplicates on a myiQi Cycler (BIO-

RAD Laboratories, CA, USA). The cycling conditions were 15-min polymerase activation at 95°C followed by 45 cycles at 95°C for 30 sec, 56°C for 30 sec and 72°C for 20 sec. These parameters avoid the generation of non-specific products. PCR products were measured in real time using SYBR Green Fluorescence. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the fluorescence was detected (Ct). The Ct is proportional to the breaks introduced in the target template; the higher the amount of breakage in the given target template, the higher the Ct measured. PCR product amplified from gDNA with reference primers that target 3' region of c-myc promoter was used as positive (reference) control. The data are expressed as a percentage of the amplification of the targeted site (Topo II) over non-target site (3' sequence downstream of Topo II site).

### Protein Chip assay

$\gamma$ H2AX levels were evaluated by Protein array performed by Zeptosens (Switzerland) on four serial dilutions of the samples. Rabbit IgG and mouse IgG were spotted on each array as controls for the fluorescence detection step of the assay. Signals were detected by an ultrasensitive fluorescence reader based on planer waveguide technology confining illumination to the surface of the microchip. Readout was obtained through a 12-bit CCD camera with a fluorescence based microarray system (532 and 635 nm lasers), which utilizes ZeptoCHIPS™ optimized for protein arrays. Microarray images were analyzed using the analysis software Zep to VIEW Pro 2.0 (Releasej). Background signal intensities was determined for each spot individually and subtracted from the sample spot signal leading to a net signal excitation light intensity used for normalization of the signal intensity expressed as “Referenced Fluorescence Intensity” (RFI). The Mean RFI for each sample was calculated based on an error-weighted linear fit through the RFI values of the 8 sample spots (duplicate spots of the 4 sample dilutions). Error bars of the Mean RFI represent the statistical error of the error-weighted least squares fit. The mouse monoclonal antibody against  $\gamma$ H2AX was from Zeptosens and used at 1/250 dilution.

## RESULTS AND DISCUSSION

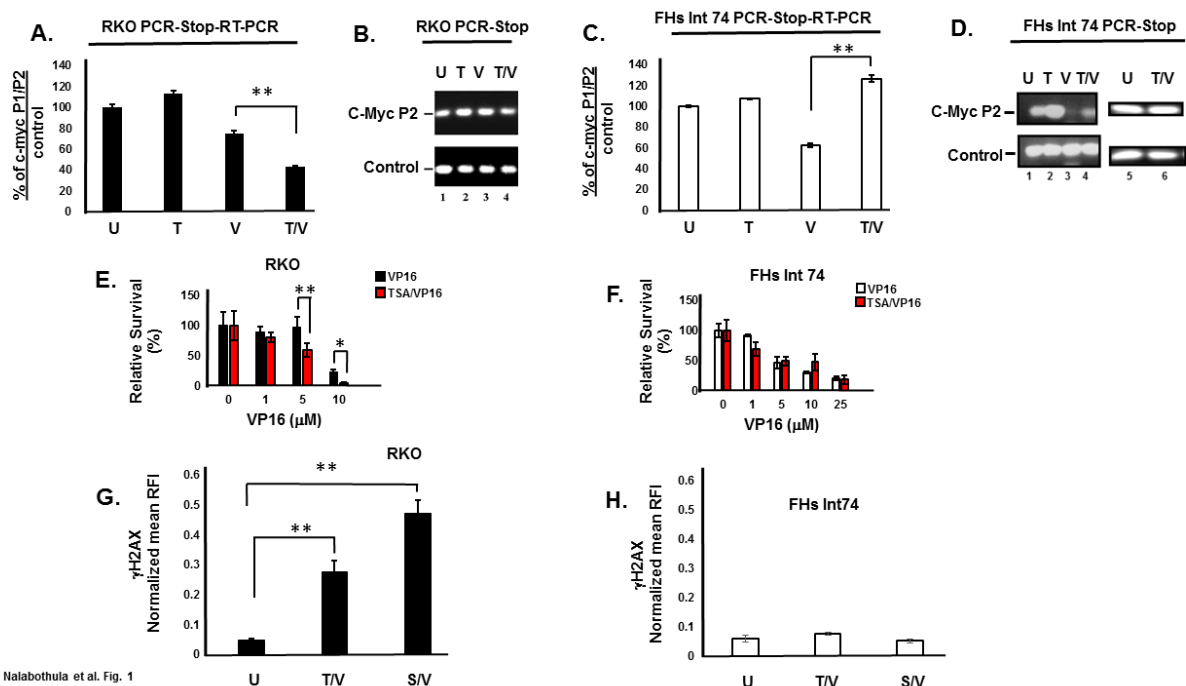
High specificity of RT-PCR relies on the primers capacity to yield a single amplification product. We thus first aimed at evaluating the potential use of the primer sets originally identified to measure the efficacy of Topo II inhibitors by PCR-Stop(2). These primers targeted the region from nucleotide 2392 to 2674 of the human c-myc gene and produce a 283 bp fragment corresponding to the P2 promoter. A 3' end region of the same gene, not targeted by Topo II, was also amplified as negative control. However, once submitted to the sensitivity of RT-PCR these primers performed poorly, producing multiple products with great levels of variability (data not shown). Therefore, we scanned upstream and downstream this region and found a new set of primers targeting the region from nucleotide 2295 to 2556 (U.S. Patent No. 8,367,340) which retain Topo II targeted sites [7] while reproducibly yielding single RT-PCR product. For the same reason we also optimized primers not targeting Topo II sites to be used as internal control and found that the region

between nucleotide 8033 and 8273 was more suitable for RT-PCR amplification. A number of other parameters such as the ratio of primers *versus* template, the annealing temperature and extension were also optimized as described in Material and Methods. We used our newly identified primers to assess the efficiency of the Topo II inhibitor VP16 alone and in combination with the HDACi TSA in human colon carcinoma RKO cells. The data shown in (Figure 1A) indicate that treating the cells with VP16 alone decreased the levels of DNA amplification at the c-myc P1/P2 promoter by about 25%. Pre-treating the cells with TSA before exposure to VP16 produced even more DNA breaks (57% breaks; 43% amplification). Similar data were obtained by conventional PCR-Stop assay where amplification products are measured at the end of the PCR cycles on a gel (Figure 1B). However, measuring the efficiency of amplification by conventional PCR is not quantitative, has low sensitivity, low resolution, in addition to a short dynamic range.

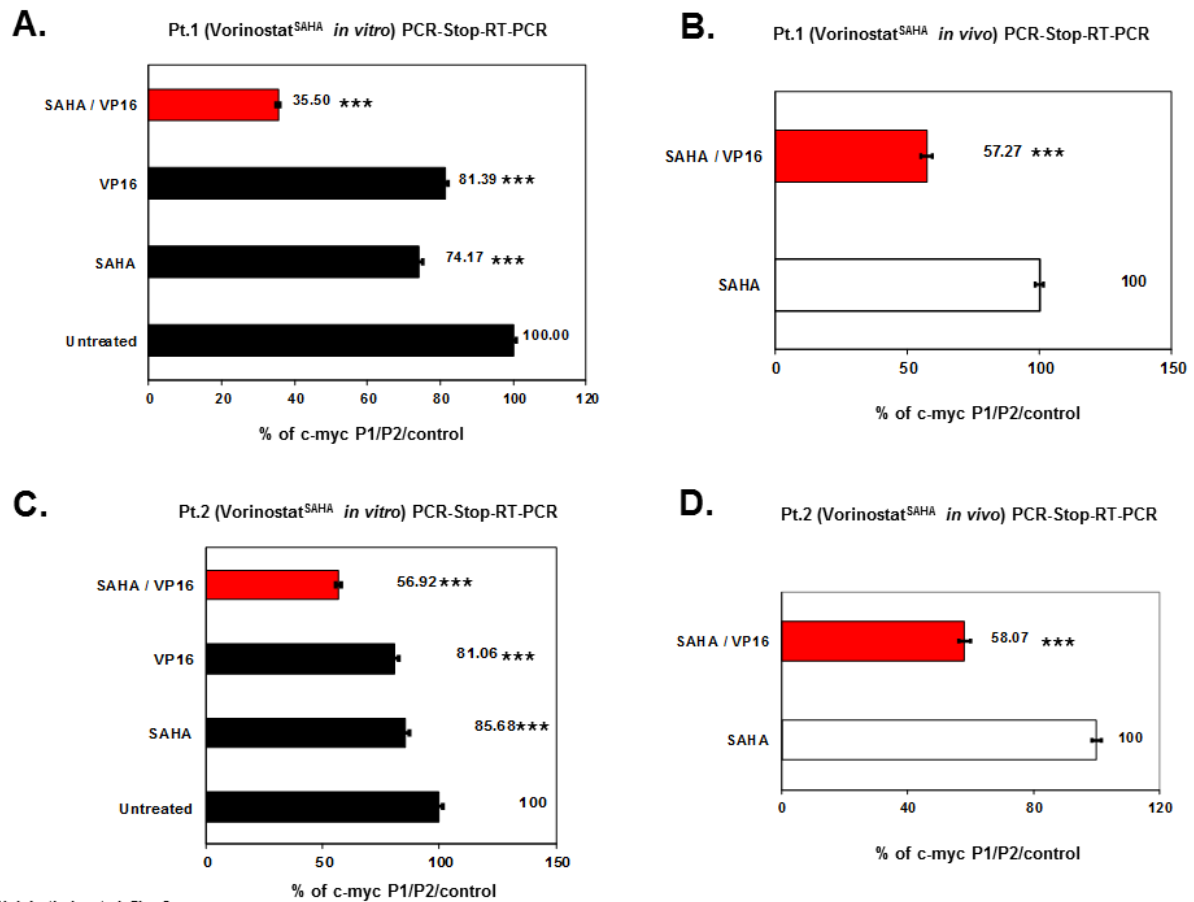
In order to determine whether the PCR-Stop-RT-PCR assay could also measure the well documented HDACi preferential selectivity for cancer cells [5,8] we also used non-transformed cells following treatment with HDACi and VP-16. Our PCR-Stop-RT-PCR data indicate that TSA does not increase the amount of DNA strand break induced by VP16 in non transformed cells but rather protect the cells from VP16 toxicity (Figure 1C). This observation can also be made by conventional PCR-Stop (Figure 1D) but the variability of the data (lanes 4 and 6) and the inability to accurately measure the amplified products emphasize the limitations of the conventional approach.

The data obtained by the PCR-Stop-RT-PCR technique are in good agreement with conventional techniques used to measure cell sensitivity to anticancer drugs. For example, the data obtained by clonogenic cell survival indicate a significant increase in cancer (Figure 1E) but not non-transformed cells (Figure 1F) sensitivity to VP-16 in combination with TSA. This increased sensitivity is reflected in the number of DNA strand breaks as measured by the levels of  $\gamma$ H2AX [4] in the treated cancer (Figure 1G) but not non-transformed cells (Figure 1H). Both techniques thus support the PCR-Stop-RT-PCR data and indicate that this new approach can be used to evaluate cells sensitivity to anticancer drugs.

We next used a more clinically relevant HDACi, Vorinostat<sup>SAHA</sup> to determine whether similar data could be obtained. We first measured the effect on  $\gamma$ H2AX and found data similar to TSA, where Vorinostat<sup>SAHA</sup> increased the number of DNA strand breaks when combined with VP-16 in cancer but not non-transformed cells (Figure 1 G-H). The potential clinical application of the PCR-Stop-RT-PCR assay was then tested on genomic DNA extracted from PBMC of two patients enrolled in a Phase 1 clinical trial for Relapsed and/or refractory acute Leukemias and Myelodysplastic Syndromes conducted at the Marlene and Stewart Greenebaum Cancer Center at the University of Maryland, Baltimore [6]. PBMC were obtained either before therapy and treated *in vitro* with Vorinostat<sup>SAHA</sup> and VP16 (Fig. 2 A, C) or four hours after the last dose of Vorinostat<sup>SAHA</sup> on day three of 400 mg p.o. and treated *in vitro* with VP16 (Figure 2 B,D). The PCR-Stop-RT-PCR data indicate that for both patients, cells pre-treated



**Figure 1** PCR-Stop-RT-PCR to measure anticancer drug efficacy. A) PCR-Stop-RT-PCR amplification of c-myc P1/P2 promoter expressed as a % of amplification of the targeted site over the untargeted site of the untreated samples in RKO cells. U; untreated, T; TSA, V; VP16, T/V: TSA follow by VP16. B) Conventional PCR-Stop run on a polyacrylamide gel and stained with ethidium bromide. C) Same as A) but performed on untransformed human small intestinal epithelial FHs Int 74 cells. D) Same as B) performed on FHs Int 74 cells. E-F) Colony survival assays of E) RKO and F) FHs Int-74 cells exposed to the indicated drug treatment. G-H) Protein chip array of H2AX levels in G) RKO and H) FHs Int-74 cells treated with the indicated drug combination. \*\* p<0.05.



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**Figure 2** PCR-Stop-RT-PCR performed on PBMC of two leukemia patients enrolled in a Phase 1 clinical trial for Relapsed and/or refractory acute Leukemias and Myelodysplastic Syndromes conducted at the Marlene and Stewart Greenebaum Cancer Center at the University of Maryland, Baltimore. The samples were obtained before (A, C, black bars) and after Vorinostat<sup>SAHA</sup> treatment *in vivo* (B, D, white bars). The A and C samples were treated *in vitro* with either Vorinostat<sup>SAHA</sup> (2.5  $\mu$ M, 4h), VP16 (25  $\mu$ M, 2h) or Vorinostat<sup>SAHA</sup> followed by VP16 (Vorinostat<sup>SAHA</sup>/VP16, red bars). The genomic DNA was extracted and 100 ng was assayed by PCR-Stop-RT-PCR as described in the text. The B and D samples were collected from patients who received Vorinostat<sup>SAHA</sup> orally (400 mg) for three days, PBMC samples were then extracted and frozen. After equilibration the cells were either used as is (Vorinostat<sup>SAHA</sup> *in vivo*, B, D) or treated *in vitro* with VP16 (25  $\mu$ M for 2h). The genomic DNA was extracted and analyzed as described above. Amplification of the c-myc P1/P2 promoter is expressed as a percentage of the untreated sample. \*\*\* $p < 0.005$  as compared to untreated samples.

*in vitro* with Vorinostat<sup>SAHA</sup> are more sensitive to VP16 induced DNA strand breaks than cells treated with VP16 alone (Figure 2 A,C). Patient 1 (Figure 2 A) is the most sensitive with 45% more breaks occurring at the Topo II site when cells are pre-treated *in vitro* with Vorinostat<sup>SAHA</sup>. The data also indicate that *in vivo* administration of Vorinostat<sup>SAHA</sup> sensitizes the PBMC to VP16 in a similar fashion in both patients with more than 40% more DNA strand breaks occurring at the Topo II sites compared to Vorinostat<sup>SAHA</sup> alone.

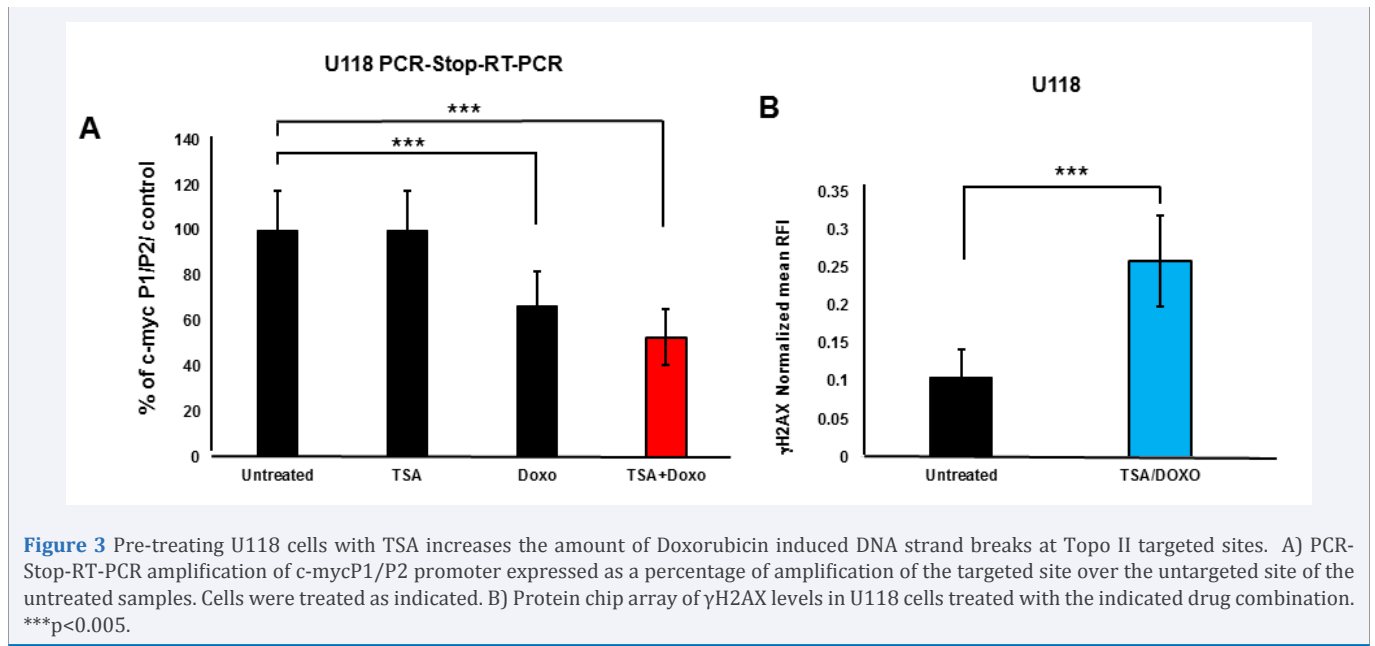
To verify whether the PCR-Stop-RT-PCR assay was cell type specific we treated the human glioblastoma U118 cells with HDACI before exposure to Doxorubicin. This treatment resulted in an increased number of DNA breaks (reduced amplification) at the Topo II targeted sites compared to untreated cells (Figure 3A). The increased cleavage of DNA was also accompanied by increased levels of  $\gamma$ H2AX (Figure 3B). Similar data were obtained with acute promyelocytic leukemia HL-60 cells exposed

to a similar drug combination (data not shown). These data indicate that the assay is not limited to a single cell type and can be applied to more than one drug targeting the Topo II sites.

## CONCLUSION

We have described a simple and reliable technique to measure the potential efficiency of anticancer drugs targeting the DNA or enzymes acting on the DNA such as the Topoisomerases. The availability of a tool that could assess *a priori* the effectiveness of chemotherapy regimen could have a significant impact on overall therapy selection and outcome for cancer patients. While selective for the Topo II targeting drugs the assay described here may have several immediate clinical applications for a number of solid and hematopoietic tumor treatments utilizing these drugs alone or in combination. An advantage of the assay is that in addition to quantifying the amount of damage inflicted to the DNA it also indicates the location of the damage and by consequence





whether the drug is appropriately reaching its target. A more practical advantage is the small amount of DNA (50-100 ng) required to perform the assay. This could allow for testing of multiple drugs combination and doses *in vitro*. The method described here could be used to assess the efficacy of a particular treatment as well as to determine the optimal dose, which could help reduce side effects associated with chemotherapy. This could facilitate selection of patients for a given treatment and help redirect unlikely responders to alternative treatments. Furthermore, the PCR-Stop-RT-PCR assay could be adapted to a variety of anticancer drugs. For example, Topo I cleavage sites have been mapped to the Hsp70 gene *in vivo* [9] and cisplatin-based compounds preferentially form intra-strand cross links between guanine residues [10].

## ACKNOWLEDGEMENTS

This work was supported in part by the National Institutes of Health (NIH/NCI, RO1 1CA116491-01 (FC)) and the Marlene and Stewart Greenebaum Comprehensive Cancer Center.

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Nalabothula N, Ross DD, Carrier F (2017) Unexploited Value of PCR-Stop Assay for Anticancer Drug Efficacy. JSM Clin Oncol Res 5(1): 1050.