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The phytochemical 3,3'-Diindolylmethane decreases expression of AR-controlled DNA damage repair genes through repressive chromatin modifications and is associated with DNA damage in prostate cancer cells

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Abstract

Androgen receptor (AR) is a nuclear receptor transcription factor that plays a central role in normal prostate physiology as well as in prostate cancer biology (PCa). Phytochemicals like 3,3'-Diindolylmethane (DIM) have emerged as promising therapeutic agents against PCa. DIM has been shown to influence both AR activity and other epigenetic regulators in PCa cells. However, it is not known if DIM contributes to PCa suppression via epigenetic regulation of AR target genes. Here we assessed epigenetic regulation of important AR-target genes in LNCaP PCa cells treated with DIM. DIM led to epigenetic suppression of AR-target genes involved in DNA repair (*PARP1*, *MRE11* and *DNA-PK*) that coincided with an increase in DNA damage. Decreased AR-target genes expression was accompanied by an increase in repressive chromatin marks and loss of AR occupancy and recruitment of EZH2 to their regulatory regions. In addition, decreased expression of DNA repair genes was associated with an increase in DNA damage (γ H2Ax) and up-regulation

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Conflict of Interest

The authors declare no conflict of interest.

of genomic repeat elements *LINE1* and *α -satellite*. Together our results suggest that the dietary phytochemical DIM suppresses AR-dependent gene transcription through epigenetic modulation, leading to DNA damage and genome instability in PCa cells.

Keywords

DIM; prostate cancer; AR; epigenetic repression; DNA damage

1. Introduction

The androgen receptor (AR) plays a critical role in prostate cancer (PCa) biology and is an important therapeutic target for cancer treatment [1,2]. In advanced PCa, AR signaling can become androgen-independent and drive aggressive cell proliferation essential for progression of castration-resistant prostatic adenocarcinoma [3]. Advancement to androgen-independent growth removes androgen deprivation therapy as a treatment option and is associated with poor outcome. Alternative strategies for antagonizing AR signaling could be useful for treatment of advanced disease.

AR regulates the expression of many genes, including a subset of those involved in responding to DNA damage when the genome is disrupted [4,5]. AR binds to specific DNA regulatory regions termed androgen response elements (AREs) and recruits chromatin modifying enzymes and transcriptional regulators to influence AR-target gene expression [6]. Recent work has shown that the histone-methyltransferase enhancer of zeste homolog 2 (EZH2) and associated proteins are recruited to ARE sites and repress AR-target gene expression in PCa [7]. This suggests that targeting the outcome of AR activity in PCa cells (i.e. AR-dependent gene regulation) by influencing chromatin-modifiers and ARE occupancy as opposed to the AR itself could be an effective strategy to slow PCa progression. Indeed, chromatin-modifiers are an attractive target for therapeutic development and several compounds have been approved for use in the clinic [8].

Epidemiological studies have shown the consumption of high levels of cruciferous vegetables, such as broccoli and Brussels sprouts, to be inversely associated with PCa risk, particularly during early stages [9,10]. 3,3'-Diindolylmethane (DIM) is a phytochemical derived from cruciferous vegetables that has been shown to induce cell-cycle arrest and apoptosis in PCa cells while sparing normal cells [11,12]. Although DIM has been shown to lead to alterations in the AR signaling pathway in PCa cells, no studies have explored a potential chromatin-dependent mechanism of action of DIM on AR-target genes.

We have previously shown that DIM treatment leads to a global decrease in histone deacetylase (HDAC) activity in PCa cells [13]. Recent work has also shown that DIM influences DNA methylation through its effect on DNA-methyltransferase (DNMT) expression, reversing some cancer-associated DNA methylation alterations involved in cancer progression [14]. Modification of local chromatin structure and promoter occupancy at ARE sites in AR-responsive genes may therefore be an as yet unappreciated contributor to PCa suppression in response to DIM treatment.

In the present study we show that DNA repair genes that are transcriptional targets of AR (AR-target DNA repair genes) are epigenetically repressed in LNCaP PCa cells following DIM treatment. Furthermore, we show an increase in chromatin marks characteristic of heterochromatin and gene silencing, which coincide with loss of AR occupancy, and recruitment of EZH2 to their regulatory regions. Decreased expression of DNA repair genes is associated with genome destabilization and an increase in DNA damage in LNCaP PCa cells.

2. Materials and methods

2.1 Cell Culture and Treatment Conditions

Human androgen-dependent prostate cancer cells (LNCaP) were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI1640 media supplemented with 10% fetal bovine serum at 5% CO₂ and 37°C. DIM (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) with a stock concentration of 100µM. Cells were treated with a DIM dose of 15 µM and collected at 48 hours post-treatment for all experiments unless otherwise indicated. These conditions were chosen because they were previously shown to decrease cell growth, decrease HDAC activity, and cause changes in DNA methylation in LNCaP cells [11,13,14]. In addition the DIM concentration was chosen because it was physiologically attainable via supplementation [15,16].

2.2 Western blot analysis

Protein was extracted from cultured cells using RIPA-lysis buffer (50mM Tris-HCl pH8, 150mM NaCl, 1mM EDTA, 0.1% SDS, 0.5% Deoxycholate, 1% NP-40), supplemented with proteinase inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). The protein extracts were quantified using the DC protein assay (BioRad, Hercules, CA, USA). For immunoblot analysis, 70µg of protein was resolved by SDS PAGE 4–12% Bis-Tris gels (Life Technologies) and blotted to PVDF membrane (BioRad) using NuPAGE reagents and equipment (Life Technologies, Carlsbad, CA, USA). Blots were blocked in 5% BSA PBS-Tween buffer for 1h. Membranes were then incubated with primary antibodies recognizing androgen receptor-D6F11 (#5153, Cell Signaling, Danvers, MA, USA), γH2Ax-Ser139 (sc: 101696, Santa Cruz Biotechnology, Dallas, TX, USA), or fibrillarin-H140 (sc:25397, Santa Cruz) overnight at 4°C. Blots were then washed and incubated with goat anti-rabbit IgG-HRP as secondary antibody for detection (Santa Cruz). Protein bands were then visualized using Super Signal WestFemto Reagent Substrate (Thermo Scientific) and developed on the AlphaInnotech FluorChem 8900 system (ProteinSimple, San Jose, CA, USA). Densitometry of each specific protein band was determined using AlphaInnotech FluorChem 8900 software and normalized to its corresponding fibrillarin band.

2.3 mRNA Expression by real-time qPCR

Total RNA from treated cells was isolated using Trizol reagent (Life Technologies). cDNA was prepared with 1µg of total RNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies). The qPCR was performed using the corresponding sets of primers (Sup-Table1). Each set of primers was validated for qPCR and the slope of the

standard curve for each pair of primers was -3.20 on average with a corresponding primers efficiency of 96%. Real time PCRs were done using Fast SYBR Green Mastermix (Life Technologies) on a 7900 HT Fast Real-Time PCR System (Life Technologies). Gene expression was calculated using comparative C_T method [17] in which the target gene expression of each gene was analyzed respect to the expression to the housekeeping gene *GAPDH*.

2.4 Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were done as previously described with slight modifications [14]. Briefly, treated cells were cross-linked with 1% formaldehyde for 10 min, washed three times with ice-cold PBS, harvested in SDS-lysis buffer and then the chromatin was sonicated. Protein-DNA complexes were immunoprecipitated using antibodies specific against H3K27me3 (EMD Millipore, Darmstadt, Germany), H3K4me3 (Abcam, Cambridge, MA, USA), H3K9me3 (Abcam), H3K9Ac (Abcam). Normal-IgG (Santa Cruz) was used as negative control. The complexes were recovered with protein A/G agarose beads (Life Technologies) and washed sequentially in the following buffers: RIPA, High-salt, LiCl and TE. To obtain the DNA, the washed complexes were incubated with RNAase, ProteinaseK, and then the crosslinking was reversed by incubating at 65°C over night. Finally, phenol-chloroform extraction was performed. DNA was amplified using real time PCR with the corresponding sets of primers (Sup-Table1). Each set of primers was validated for qPCR and the slope of the standard curve for each primer pair was -3.27 on average with a corresponding primers efficiency of 98%. ChIP-qPCR reactions were performed in triplicate using Fast SYBR Green Mastermix (Life Technologies). A working input DNA dilution was selected from standard curves and the C_T values from the immunoprecipitated DNA was measured relative to the C_T from input DNA dilution in order to get the %input [14]. ChIP assays were done in triplicate for the data analysis.

2.5 Immunofluorescence

LnCap cells grown on glass coverslips were fixed in methanol at 20°C for 10 minutes, washed three times with PBS and then incubated in 4% paraformaldehyde for 15 minutes. Cells were permeabilized with 0.4% Triton X-100 in PBS for 20 minutes and then washed twice in PBS. Samples were blocked in 5% BSA (Bovine Serum Albumin, Sigma-Aldrich) in PBS supplemented with 0.1% Igepal Ca-630 (Sigma-Aldrich) for 2 hr. Cells were probed with anti- γH2Ax phos-Ser139 antibody at 1:50 (sc-101696, Santa Cruz), then anti-rabbit IgG AlexaFluor 555 secondary antibody at 1:200 (Life Technologies). Nuclei were stained with DAPI. Coverslips were mounted in ProLog Gold (Life Technologies) and observed on a Zeiss LSM 780 NLO Confocal Microscope System. The number of γH2Ax foci per nucleus was determined using ZEN blue software from Zeiss LSM 780. One hundred and fifteen cells were analyzed for each condition in triplicate.

3. Results

3.1 DIM decreases AR-target gene expression by reducing AR-protein levels at promoters and ARE regions

Previous studies have shown that DIM down-regulates AR protein level in androgen-responsive LNCaP PCa cells [11,12,18]. We also found that AR protein levels are reduced in LNCaP cells following 15 μ M DIM treatment for 48h (Fig. 1A). Next, we evaluated whether a decrease in AR-protein level leads to a reduction in the expression of AR-target genes. We began by examining the well-characterized AR-target genes *PSA* and *TMPRSS2* [3]. We also evaluated the AR-target DNA repair genes *PARP1*, *MRE11* and *DNA-PK* identified in a previous AR ChIP-Seq study, in which androgen receptor signaling was shown to regulate DNA repair in PCa [4]. In particular, *DNA-PK* has been shown to be a key target of AR after damage, controlling AR-mediated DNA repair and cell survival after genotoxic insult [4,5]. After 48h treatment, DIM reduced the expression of the AR-target genes *PSA*, *TMPRSS2*, *PARP1*, *MRE11* and *DNA-PK* (Fig. 1B). We observed no change in the expression of *SAFB1*, a DNA-repair gene not controlled by AR (Fig. 1B). We next examined AR protein occupancy at the promoters and AREs of these genes by chromatin immunoprecipitation (ChIP) assays and quantitative PCR (qPCR). AR levels were reduced at the promoter regions of *PSA*, *PARP1*, *MRE11* and *DNA-PK* (Fig. 1C,D). Together these results suggest that DIM down-regulates the expression of AR-target genes, including a subset of DNA-repair genes, by reducing AR protein levels at promoters and ARE regions.

3.2 Repressive chromatin code in PSA promoter and ARE regions after DIM treatment

Chromatin modifications such as histone H3 lysine 27 trimethylation (H3K27me3), H3K9me3 and H3K4me3 play an important role in gene expression and regulation [19]. We tested whether the decrease in PSA gene expression coincides with a change in histone modifications in its proximal and distal regulatory regions. ChIP assays and qPCR were performed to examine H3K27me3, H3K9me3 and H3K4me3 levels at the PSA gene proximal promoter (–120 bp), an ARE site within the promoter (–170 bp) and the ARE-enhancer (–4162 bp) regions of *PSA* after 48h of DIM treatment (Fig. 2A). We found a decrease in the active mark H3K4me3 in the proximal promoter and ARE-promoter regions of the *PSA* gene (Fig. 2B,C). Similar decrease of H3K4me3 was observed at 24h and 72h post-DIM treatment at proximal promoter (Fig. S1b). However, we unexpectedly noted an increase in H3K4me3 at the distal ARE-enhancer region after 48h treatment (Fig. 2D). In contrast we noted an increase in the repressive chromatin marks H3K9me3 and H3K27me3 after DIM treatment in all three regulatory regions of *PSA* (Fig. 2B–D, Fig.S1b). Overall, our data is consistent with chromatin remodeling and gene repression following DIM treatment.

3.3 Increase in the repressive-chromatin mark H3K27me3 at the promoter region of DNA repair AR-target genes following DIM treatment

To test whether the DIM-mediated alterations in chromatin dynamics observed at the *PSA* promoter also occurs at the AR-target DNA repair genes [4,5], we evaluated H3K27me3 and H3K4me3 at the promoter regions of *PARP1*, *MRE11* and *DNA-PK*. We observed a marked increase in H3K27me3 at the promoter region of all three genes after DIM treatment (Fig.

3A–C). Interestingly, we observed no significant change in H3K4me3 after DIM treatment (Fig. 3A–C). To further confirm this repressive switch, we evaluated the repressive mark H3K9me3 at the promoter region of *DNA-PK*. Similar to what we observed in the *PSA* regulatory regions (Fig. 2), H3K9me3 was also increased at the promoter region of *DNA-PK* (data not shown). These results together suggest that the DIM-induced repression of AR-target DNA repair genes may occur principally via bivalent marks H3K27me3 and H3K4me3 as was observed recently with other AR-target genes [20]. In summary these results suggest that the chromatin mark H3K27me3 contributes to gene repression of DNA repair AR-target genes in response to DIM.

3.4 Increase in EZH2 occupancy at DNA repair AR-target genes following DIM treatment

The repressive histone mark H3K27me3 is catalyzed by the histone-methyltransferase EZH2, a constituent of the polycomb repressive complex 2 (PRC2) [21]. Interestingly, recent work has shown that EZH2 and associated proteins are recruited to *PSA*-ARE promoter site and affect its expression in PCa [7]. We next tested whether DIM leads to recruitment of EZH2 to the promoter regions of our AR-target genes of interest. We found a significant increase in the recruitment of EZH2 at the *PSA-ARE* promoter, *PSA-ARE* enhancer, as well as at the promoter regions of *DNA-PK*, *MRE11* and *PARP1* genes (Fig. 4). DIM may therefore suppress DNA repair AR-target genes through recruitment of polycomb repressive chromatin proteins to their gene regulatory regions.

3.5 DIM treatment causes an increase in the DNA damage in LNCaP cells

DIM increases DNA damage by inducing double strand breaks (DSB) in colon cancer cells [22]. To test whether the observed down-regulation of DNA repair genes (*DNA-PK*, *MRE11* and *PARP1*) following DIM treatment leads to an increase in DNA damage in LNCaP PCa cells, we analyzed phosphorylated-H2Ax (γ H2Ax) as a marker of DSB and genome instability [23]. We noted a concentration-dependent increase in γ H2Ax in LNCaP cells treated with DIM for 48h (Fig. 5A). Analysis of γ H2Ax by confocal microscopy also revealed a significant increase in the number of γ H2Ax foci per cell (Fig. 5B). Previous studies have drawn a proportional relation between the number of γ H2Ax foci to the retrotransposition frequency and increased expression of repeat elements such as *LINE1* by oxidative stress and irradiation [24,25]. Since it is known that DIM produced oxidative stress in different cancer cells [22,26], we next tested whether the increase in γ H2Ax foci by DIM leads to a change in *LINE1* and peri-centric heterochromatin repeat sequence (*α -satellite*) expression in LNCaP cells. We found that the expression of both repeat sequences were up-regulated after DIM treatment in a concentration-dependent manner (Fig. 5C). Altogether, these results suggest that the down-regulation of DNA repair AR-target genes after DIM treatment leads to an increase in DSB DNA damage and possible genome instability in LNCaP cells.

4. Discussion

Epigenetic plasticity has been intensely studied in cancer biology and has led to the identification of new molecular targets against cancer [8]. Indeed, dietary phytochemicals such as DIM impact important epigenetic processes in cancer cells, making them attractive

compounds for therapeutic development. Previous work in our lab showed that DIM decreases global HDAC activity in LNCaP cells [13]. Furthermore, we recently showed that DIM can influence DNA methylation in prostate cancer cells [14]. These studies suggest that altered chromatin dynamics may contribute to cell-cycle arrest and induction of apoptosis in response to DIM in PCa cells [11,12,18].

In the present work we observed a decrease of AR protein level after DIM treatment (Fig.1), which has been previously reported [11,12,18]. Interestingly, despite a decrease in protein level, *AR* expression was up-regulated with no significant change in chromatin modifications at its promoter region (Fig. S2). This suggests AR-protein instability following treatment. Further work will be needed to characterize how DIM treatment results in AR turnover.

A decrease in *PSA* expression in response to DIM has been characterized and is reflective of a decrease in AR protein [12,18]. Our results similarly observed a decrease in *PSA* gene expression after DIM treatment (Fig. 1, Fig.S1a). Importantly, we also observed decreased expression of a subset of AR-target DNA repair genes *PARP1*, *MRE11* and *DNA-PK* following DIM treatment and are the first to show depletion of AR from regulatory *cis* elements at their promoters (Fig. 1C–D). Many studies have characterized the connection between chromatin modifications such as histone methylation (H3K9me3, H3K27me3, H3K4me3) and transcriptional regulation [19]. More specifically, previous studies have reported that H3K27me3 and H3K4me3 play a key role in transcriptional regulation of AR-target genes [7,20]. We found that down-regulation of *PSA* expression after DIM treatment correlated with an increase in repressive-chromatin marks H3K9me3 and H3K27me3 and a decrease in the open mark H3K4me3 at its regulatory regions (proximal-promoter and ARE-promoter) (Fig. 2). Our results also suggest that the down-regulated expression of the DNA repair AR-target genes *PARP1*, *MRE11* and *DNA-PK* after DIM treatment is associated principally with the gene repression mark H3K27me3 at their promoter regions (Fig.3).

Another important discovery of this present study is that DIM leads to an increase in the binding of EZH2 to promoter regions of these DNA repair AR-target genes that correlates with an increase in H3K27me3 and a decrease in their gene expression (Fig. 4). This is consistent with previous findings that show that EZH2 can act as a transcriptional repressor of AR-target genes [7,20]. Further work will be needed to identify the full complement of proteins that participate in AR-target gene repression in response to DIM and could identify new protein targets for therapeutic development.

DIM-induced recruitment of EZH2 to the regulatory regions of the DNA repair genes was associated with their decreased expression and an increase in global and local DNA damage as indicated by increased γ H2Ax levels (Fig. 5A–B). In addition, we also noted an increase in the expression of the *LINE1* and *α -satellite* repeat elements that are indicators of genome instability in response to stress. Together these data suggest a novel mechanism of action by which DIM interferes with AR signaling via DNA repair in LNCaP PCa cells: epigenetic repression of AR-target DNA repair genes that leads to DNA damage and genome instability. DIM has been shown by our group and others to affect multiple epigenetic processes. Further work characterizing how DIM influences the timing and coordination of

different epigenetic responses will provide a fuller understanding of why this dietary phytochemical is an effective agent in suppressing PCa growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AR	androgen receptor
DIM	3,3'-Diindolylmethane
ARE	androgen response element
PSA	prostate specific antigen

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Figure 1.

Decreased AR-target gene expression and AR occupancy in promoters and ARE elements following DIM treatment. A) AR protein levels were analyzed by western blot in LNCaP cells treated with DIM or DMSO. Fibrillar protein was probed as a loading control. B) Relative *PSA*, *TMPPSS2*, *PARP1*, *MRE11* and *DNA-PK* gene expression in LNCaP cells treated with DIM or DMSO (control). *PSA* was assessed as a positive control (well known AR-target gene) and *SAFB1* (AR-independent gene) was assessed as a negative control. Gene expression (mRNA) levels were normalized to *GAPDH*. C) AR ChIPs were performed after DIM or DMSO treatment in LNCaP cells and the *ARE-PSA*, *PARP1*, *MRE11* promoter regions were amplified to determine AR occupancy. *CCR4* promoter (AR-independent gene) was assessed as a negative control and *ARE-PSA* as a positive control. D) *DNA-PK* promoter and AREs-*DNA-PK* regions were amplified from AR-ChIPs. The lower diagram shows a schematic of ARE regions of *DNA-PK* gene. C-D) IgG (DMSO+DIM) is an average of two ChIP assays from each treatment, using Normal-IgG as negative control. Grey bars indicate positions relative to transcriptional start site of amplicons analyzed. ChIP-data are expressed as % input relative to DMSO vehicle control. In all experiments LNCaP cells were treated with 15 μ M DIM or DMSO as a vehicle control for 48h. Data represents mean of three biological replicates. Graphs depict mean + standard deviation. Significance was determined by Student's t-Test with * $p < 0.05$, ** $p < 0.01$.

Figure 2.

DIM stimulates a repressive chromatin code in *PSA* regulatory regions. A) Schematic of AREs in the promoter and enhancer regions of *PSA*. Grey bars indicate positions of amplicons analyzed relative to transcriptional start site. ChIPs and qPCR assays were used to evaluate H3K4me3, H3K9me3 and H3K27me3 levels at *PSA* regulatory regions in LNCaP cells treated with 15 μ M DIM for 48h. B) promoter (-120 bp), C) ARE-promoter (-170 bp) and D) ARE-enhancer regions of *PSA* gene. ChIP-data are expressed as % input compared to DMSO vehicle control. IgG (DMSO+DIM) is an average of two ChIP assays from each treatment, using Normal-IgG as negative control. Data represents three biological replicates. Graphs depict mean + standard deviation. Significance was determined by Student's t-Test with * $p < 0.05$, ** $p < 0.01$.

Figure 3.

Increase in repressive chromatin mark H3K27me3 at the promoter region of DNA repair AR-target genes in response to DIM. LNCaP cells were treated with 15 μ M DIM or DMSO as a vehicle control for 48h. H3K27me3 and H3K4me3 levels were assessed by ChIP in the promoters of A) *PARP1*, B) *MRE11* and C) *DNA-PK*. H3K9me3 levels following DIM treatment were also assessed in the *DNA-PK* promoter. IgG (DMSO+DIM) is an average of two ChIP assays from each treatment, using Normal-IgG as negative control ChIP-data are expressed as % input relative to DMSO vehicle control. Data represents three biological replicates. Graphs depict mean + standard deviation. Significance was determined by Student's t-Test with *p<0.05, **p<0.01.

Figure 4.

Increased EZH2 at the promoter region of *PSA* and DNA repair AR-target genes following DIM treatment. LNCaP cells were treated with 15 μ M DIM or DMSO as a vehicle control for 48h. EZH2 ChIPs were performed after treatment and the ARE-promoter *PSA* (*PSA.ARE* promoter), ARE-enhancer *PSA* (*PSA.ARE* enhancer), *DNA-PK*, *MRE11* and *PARP1* promoter regions were amplified by qPCR. ChIP-data are expressed as % input relative to DMSO vehicle control. *PSA.ARE* promoter was evaluated as a positive control; *GAPDH* and *CYR61* (non AR targets genes) were evaluated as negative controls. IgG (DMSO+DIM) is an average of two ChIP assays from each treatment, using Normal-IgG as negative control. Data represents three biological replicates. Graphs depict mean + standard deviation. Significance was determined by Student's t-Test with * $p < 0.05$.

Figure 5.

Decreased gene expression of AR-target DNA repair genes after DIM treatment is associated with an increase in DNA double strand breaks (DSBs). A) Western blot assays were used to analyze the global levels of γ H2Ax in LNCaP cells treated with the indicated concentrations of DIM for 48h. DMSO was used as vehicle control. Fibrillarin was probed as a loading control. Graph depicts mean + standard deviation for five independent experiments. Significance was determined by Student's t-Test with *p<0.05. B) LNCaP cells were treated with 15 μ M DIM or DMSO as a vehicle control for 48h. The number of γ H2Ax foci per cell were counted for 115 cells per condition in three independent biological experiments. Red: γ H2Ax, Blue: DAPI. Graph depicts mean + standard deviation. Significance was determined by Student's t-Test with *p<0.05, **p<0.01. C) Relative expression of transposable element *LINE1* and pericentromeric α -satellite repeat normalized to *GAPDH* expression in LNCaP cells treated with the indicated concentrations of DIM or DMSO for 48h. All Graphs depict mean + standard deviation for three to five independent experiments. Significance was determined by Student's t-Test with *p<0.05, **p<0.01.