‘Investigating Zeb1-mediated Drug Resistance in Solid Tumors’

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Abstract

The epithelial-to-mesenchymal transition (EMT) is a cellular program, through which epithelial cells lose their normal, epithelial phenotype, and become mesenchymal. This process is found to play a critical role in enhanced therapy resistance and metastatic capacities in many solid tumors. One of the most important players that activate EMT is Zeb1, which belongs to the family of ZEB transcription factors, and it alone was shown to affect the poor clinical outcomes of cancer patients due to elevating the radio resistance and multidrug resistance in a variety of tumors. Here, we examined the effect of a combination of active compounds that are FDA approved drugs and inhibitors targeting protein kinase C (PKC) on the Zeb1 expressing breast cancer cells at distinct stages of the EMT, and concluded that the resistance of Zeb1 can be affected by kinase inhibitors, by counteracting its effect on cell cycle arrest at G1 proliferation phase. These results open new opportunities for manipulating the Zeb1-mediated therapy-resistance pathway in breast cancer, and consequently other solid tumors.

Introduction

The epithelial to mesenchymal transition (EMT) is a cellular program by which epithelial cells become mesenchymal. Importantly, this mechanism is hijacked by cancer cells and is used by solid tumors to become metastatic and resistant to certain anticancer drugs. Essentially, epithelial cells lose their contacts due to the loss of E-cadherin which is the main component of adherent junctions (Manshouri et al., 2019) and acquire mesenchymal traits, resulting in loss of cell polarity and cell-cell adhesions, along with acquisition of migratory and invasive properties of tumor cells (Ren et al., 2013, Marcucci et al., 2016). Metastasis itself is a complex phenomenon that is characterized by a series of molecular and cellular events including intravasation of tumor cells into blood vessels, their accumulation near metastatic niches followed by their extravasation and the restoration of the epithelial phenotype (Housman et al., 2014). One of the key players that induce EMT is the Zeb1 transcription factor, which is one of the hallmarks of mesenchymal cells. Hence, studying the exact role of Zeb1 in cancer drug resistance becomes an important topic for the research focusing on breast cancer cell lines, in which EMT machinery was shown to be one of the main drivers of enhanced tumor progression and invasion, resistance to radiotherapy and chemotherapy, and overall aggressive phenotype.

Zeb1 belongs to the family of ZEB transcription factors that contain two zinc finger clusters responsible for DNA binding; more specifically, DNA sequences called E-boxes (Zhang et al., 2015). The role of Zeb1 has been already studied in drug screening studies with different solid tumors, and the knockdown of Zeb1 in MDA-MB-231 human breast cancer cells was shown to affect the process of EMT (Zhang et al., 2015). Zhang et al. (2015) also outline that abnormal expression of Zeb1 is observed in a variety of other human cancers including colon cancer, lung cancer, osteosarcoma, etc. Zeb1 was also shown to enhance resistance of MCF-7 and Hs578T breast cancer cells to DNA-damaging agents by a process of autophagy (Fedorova et al., 2022). Overall, expression of Zeb1 is linked to a variety of malignancies and augmented resistance of cancer cells to different genotoxic drugs, as well as higher metastatic capacity of cancer cells (Parfenyev et al., 2021). As a transcription factor and master-regulator of EMT, Zeb1 suppresses the expression of E-cadherin, and is linked to mesenchymal phenotype, by inducing expression of mesenchymal biomarkers, such as vimentin and N-cadherin (Parfenyev et al., 2021). Fedorova et al. (2022) have tested the effect of drugs such as doxorubicin and etoposide on the MCF-7 and Hs578T breast cancer cell lines with different levels of Zeb1 expression, showing that cancer cells with induced Zeb1 expression conferred resistance to the drugs, while the knockdown of Zeb1, conversely, increased sensitivity of Hs578T cells to doxorubicin.

EMT is also involved in the acquisition of cancer stem cell (CSC) properties by proliferating cancer cells, which also explains enhanced resistance of the latter to therapeutic regimens and de-differentiation (Dongre & Weinberg, 2019; Parfenyev et al., 2023). CSCs are characterized by elevated tumor-initiating potential and ability to self-renew, and the overall properties of CSCs are found to be similar to those cells that have undergone EMT (Dongre & Weinberg, 2019). According to Dongre & Weinberg (2019), although the specific molecular mechanisms that govern cancer stemness during EMT are not fully understood yet, it is evident that EMT-TFs, including Zeb1, directly affect this process by repressing miR-200 family miRNAs in pancreatic, lung, and breast

cancers. EMT was also shown to play a crucial role in breast, lung, and colorectal cancers, as there is a wide range of evidence showing the importance of specific EMT inducers, such as TGF-β, Snail, Twist and Zeb1 in progression of lung cancer, breast cancer and colorectal cancer (Argast et al., 2011; Lu et al., 2023; Wang et al., 2017).

Cancer cells are also found to confer resistance to chemotherapy and radiotherapy due to the action of Zeb1 in particular, which might be independent of EMT process (Zhang et al., 2014). Zhang et al. (2014) have identified that only Zeb1 conferred radioresistance to breast cancer cells (MCF-7), while overexpression of other EMT-TFs, Snail and Twist, did not result in EMT in MCF-7 cells. Therefore, it means that tumor radiosensitivity may be EMT-independent, but rather regulated by Zeb1, suggesting that Zeb1 in particular should become a specific target in cancer therapy.

There is also a wide range of evidence showing that Zeb1 determines resistance to a variety of chemotherapeutic drugs; in mantle cell lymphoma, Zeb1 expression resulted in resistance to doxorubicin, cytarabine and gemcitabine, but not to vincristine (Sanchez-Tillo et al., 2014). Another evidence for the role of Zeb1 in enhanced tumor radiosensitivity suggests that the knockdown of Zeb1 in SUM159-P2, U2OS, H460, H1299 cancer lines resulted in increased radiosensitivity, while ectopic expression of Zeb1 in radiosensitive epithelial breast cancer cells, HMLE and MCF-7, enhanced radioresistance (Zhang et al., 2014, Zhang et al., 2015). Similarly, ectopic expression of Zeb1 in SPC-A1 cell line increased chemoresistance to docetaxel, while Zeb1 inhibition enhanced chemosensitivity (Ren et al., 2013). Importantly, the radioresistance conferred by Zeb1 is regulated through ATM-Zeb1-CHK1 signaling pathway (Zhang et al., 2015). Thus, another effective approach to sensitize the proliferating tumor cells to the therapy is to target DNA damage response and checkpoint kinases, such as Chk1 in particular, since the activity of chemotherapeutic agents would be more effective if they were combined with the specific players that control cell cycle (Dent et al., 2011). Specifically, one of the most widely employed Chk1 and Protein kinase C (PKC) inhibitors is 7-Hydroxystaurosporine (UCN-01), which has been proven to eradicate tumor cells by promoting the activation of Cdk1 and Cdk2 and overall resulted in enhanced cytotoxicity when used with cisplatin, another genotoxic anticancer drug. However, despite the promising chemosensitizing and cytotoxic effects, UCN-01 is not widely employed, due to poor results in clinical trials (Dent et al., 2011). Therefore, there is a need in drug-screening of FDA-approved compounds that could be efficient in eliminating tumors along with specific checkpoint kinase or PKC inhibitors, considering that the EMT-driven metastasis and drug resistance is an emerging area of cancer treatment, and the exact molecular machinery of these processes are not fully understood.

Overall, these findings highlight the intricate web of Zeb1-mediated cancer drug resistance, emphasizing the need to seek for combinational approaches in a more targeted therapy of solid tumors.

In order to better understand the molecular machinery of EMT driven tumor metastasis and the role of Zeb1 in particular, it is possible to perform Tet-inducible expression of EMT markers, including Zeb1, upon treatment with doxycycline, an analogue of tetracycline (Argast et al., 2011; Parfenyev et al., 2021). This reporter cell system will contain a CDH1-luciferase reporter, expression of which will indicate the presence of E-cadherin, a prominent biomarker of epithelial cells. Argast et al. (2011) have generated three engineered models of EMT in non-small cell lung carcinoma in which EMT inducers are activated by a tetracycline-responsive promoter, and these models possess a well characterized molecular and phenotypic description of EMT that is

maintained in the *in vitro* and *in vivo* settings. Similarly, Parfenyev et al. (2021) have used MCF-7 human breast cancer cell line with Zeb1 inducible expression that was generated via lentiviral insertion of Zeb1 gene that was fused with GFP gene under tetracycline-activated promoter, and induced Zeb1 expression by introducing doxycycline.

There is a library of FDA approved drugs that can be tested on human colon cancer and breast cancer cell lines forced to undergo EMT due to inducible expression of Zeb1 (Parfenyev et al., 2023). By performing the drug screening of the given active compounds, which constitute for a variety of kinase inhibitors, it will be possible to identify the effect of specific FDA-approved active compounds on cancer cells that have wild-type (WT) expression of Zeb1 and knockdown of Zeb1 and to assess their efficacy in combination with various kinase inhibitors, such as Chk1 or PKC inhibitors, since the latter ones can induce sensitivity of cancer cells to DNA-damaging agents by directly interfering with the cell cycle progression of tumor cells at various stages. Thus, it will be possible to better understand the Zeb1 mediated pathway in cancer drug resistance and shed light on a more targeted therapeutic approach in treating tumors.

Specific aims:

- To test the effect of FDA approved drugs in combination with kinase inhibitors on cancer cells expressing Zeb1 and assess whether the drugs can overcome drug resistance conferred by Zeb1
- To assess the role of Zeb1 in drug resistance of cancer cells
- To identify the drugs that will be efficient in eliminating Zeb1 expressing cancer cells

Hypothesis:

It is hypothesized that through the screening of FDA-approved drugs several molecules will be identified that either as solo or in combination will specifically inhibit Zeb1 in cancer cells. This combination of drugs that are kinase inhibitors is anticipated to enhance sensitivity of cancer cells to DNA-damaging agents and make cancer cells less resistant to the therapy.

Materials and Methods

For complete microscopy images of all magnifications and any supplementary materials refer to Appendix Section.

Cell Culture

An epithelial, human breast adenocarcinoma cell line MCF-7 with Tet-inducible expression of Zeb1 (kindly provided by Dr. Eugene M. Tulchinsky) was cultured in Dulbecco's Modified Eagle Medium (DMEM) medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated at 37℃ with 5% CO_2.

Induction with Doxycycline

The induction media for Zeb1 activation was prepared by diluting doxycycline in DMEM (0.5 ug/mL), and added to the cells that were seeded at 70-80% confluence. The cells were incubated under regular conditions, and collected after 48, 72, 96 hours of induction (the non-induced control samples correspond to 0 h).

Treatment with kinase inhibitors

The three randomly selected kinase inhibitors (See Table 1) from the drug library (Stock concentration of each - 100 mM) were added to the culture medium in the final concentration of 10 uM, to the cells before induction (0 h) and after 72 hours of Zeb1-induction with doxycycline, with prior aspiration of the doxycycline-medium after 24 hours of administration of doxycycline. Then, the cells were harvested for fixation after 48 hours. As a placebo control, DMSO was added similarly, in the final concentration of 100mM.

Transfection of reporter plasmid for luciferase assay

The reporter plasmid (Stock concentration - 384 ng/ul) for luciferase assay and GFP plasmid (used as a control; stock concentration – 1 mg/ul) were transfected into the MCF-7/Zeb1 cells using Lipofectamine-LTX with Plus reagent (Invitrogen, 15338-030), and incubated under standard conditions (37℃ with 5% CO_2) in Opti-MEM medium for four hours. The ratio of GFP plasmid to Luciferase reporter plasmid was 1:1, and the final DNA mix (GFP + reporter) contained 7.8 ul of the reporter plasmid, 3 ul of GFP plasmid and 18ul of Lipofectamine (DNA to Lipofectamine ratio – 1:3). The control sample (GFP only) contained 6 ul of GFP plasmid and 18 ul of Lipofectamine. The medium was then replaced with regular DMEM with 15% FBS after 4 hours. The fluorescence (emitted by GFP) of the transfected cells was observed under FM.

Luciferase Reporter Assay

In order to assess the levels of expression of E-cadherin and Zeb1, the cells, transfected with luciferase reporter, were induced with doxycycline (0.5 ug/ml) to activate Zeb1 expression, as described above. The cell lysates at 0, 48, 72 and 96 hours of induction were collected by detaching the cells and treating them with the Cell Lysis Buffer from the Luciferase Kit (Abcam, ab287865). The control samples (not transfected) at 0 hours and 72 hours after doxycycline induction were also collected in the same manner. All samples were stored at -80℃ prior to the assay. 50uL of lysates were then placed into the special assay microplate reader, and the substrates were added, according

to the manufacturer's protocol. The remaining 50 uL were stored for future Western Blotting. The BioTek Cytation 5 Cell Imaging Multi-Mode Reader was used to record the luminescence. The readings were repeated in triplicates to get statistically significant results.

Western Blot

The cell lysates of control (before induction, 0 hrs) samples, and samples after induction with doxycycline at 48, 72 and 96 hours were prepared by treating with RIPA buffer for Western blotting. The primary antibodies that were used include ZEB1 (ABClonal, Cat. No. A1500), β-actin (Cat. No. 7074P2), E-cadherin (BD Biosciences, Cat. No. 610181). The membranes were then incubated in a blocking buffer (5% non-fat milk solution), followed by secondary antibodies: anti-rabbit (Cell-Signaling, Cat. No.7074P2), anti-mouse (Cell Signaling, Cat. No.7076). The visualization was performed using enhanced chemoluminiscence (ECL) via Bio-Rad ChemiDoc Imaging System. The range of the protein ladder that was used is 10-180 kDa (Thermo Scientific™, Cat. No. 26616).

Flow Cytomerty

The flow cytometry analysis of the cell cycle using propidium iodide (PI) of doxycycline induced samples at four different time points (0, 48, 72, 96 h) and samples treated with inhibitors (0h and 72h post-doxycycline-induction) and DMSO was performed using Attune NxT Flow Cytometer. Briefly, the cells were fixed in 70% Ethanol (dissolved in PBS 1x) solution, following the washing and incubation (at r.t.) in PI staining solution containing RNAse A: PI (10 ug/ml), RNAse A (10 ug/ml), dissolved in PBS 1x. The samples were then placed to the cytometer and the necessary measurements were recorded.

Statistical Analysis

The data were provided as Mean ± Standard deviation. For the full datasets, please, refer to the Appendix section.

Results and Discussion

Zeb1 activates EMT upon induction with doxycycline

We confirmed that Zeb1 mediates EMT upon treatment of MCF-7/Zeb-1 cells with doxycycline. After 48 hours, the cells started to lose their normal epithelial phenotype, reaching the most vivid mesenchymal phenotype at 72 hours. It is worth to notice, that the cells still continued to divide after Zeb1 induction, which was counterintuitive, since Zeb1 causes cell cycle arrest at G1 phase. That might happen due to fact that some population of cells did not express Zeb1, so they were not affected by the incorporation of doxycycline. Since Zeb1 was fused with GFP, we could observe the glowing of the cells under FM (the glowing intensity of the cell nuclei increased at later time points), which also confirms that Zeb1/GFP was induced. To further prove and substantiate the presence of Zeb1 we performed Western Blot analysis of the samples at each time point. The results of the Western blot analysis (Fig.2) demonstrate the increased expression of Zeb1 with time points, which corresponds to the literature results (Parfenyev et al., 2021; Fedorova et al., 2022). The peak expression of Zeb1 was observed to take place at 72 hours after induction. Conversely, E-cadherin expression is only seen in 0 h sample (not-induced), which confirms central role of Zeb1 as a transcriptional repressor of E-cadherin. In order to correctly assess the trend in expression of the protein of interest, the loading amounts of the samples were normalized based on the amount of β-actin.

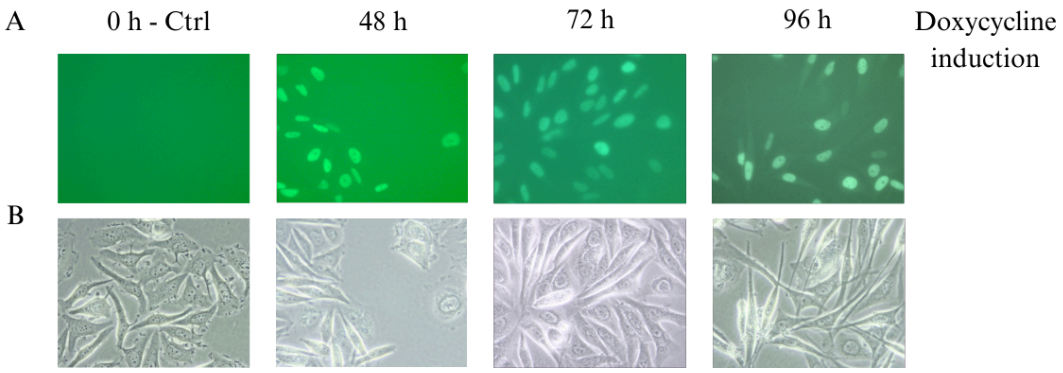


Figure 1. Microscopy images (40x magnification) of the MCF-7/Zeb1 cells after treatment with doxycycline. The most vivid mesenchymal phenotype is observed at 72 and 96 hours. Row (A) demonstrates fluorescent images of the cells, and row (B) shows respective brightfield images of the cells. Bright, green-glowing cell nuclei correspond to the increased activation of Zeb1/GFP fusion protein. The mesenchymal phenotype (spindle-shaped, fibroblast-like morphology) predominates with the increased exposure to doxycycline.

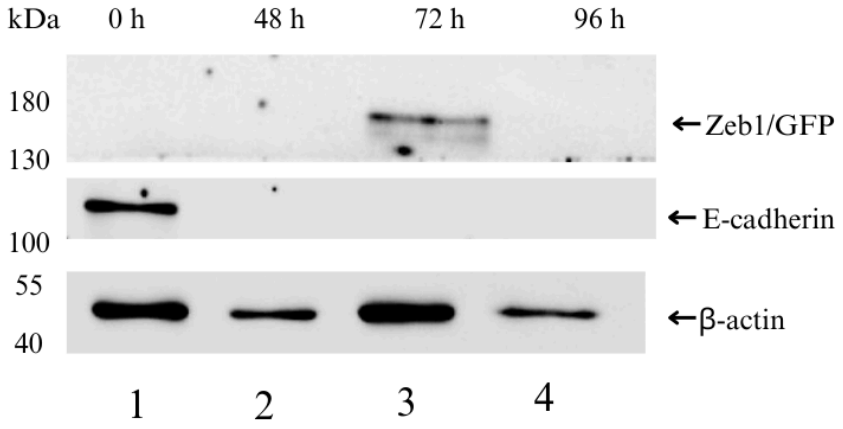


Figure 2. Western blot results of MCF-7/Zeb1 cell lysates, collected at 0 (Ctrl), 48, 72 and 96 hours after Zeb1 induction with doxycycline; the blots obtained after staining with the primary antibodies for the indicated proteins, after cutting the membranes at specific band ranges to prevent non-specific bands. The numbering of the lanes is provided in the bottom row. Here, Zeb1/GFP is only observed at 72 hours, when the expression of Zeb1 is known to be highest (Lane 3). Conversely, E-cadherin is only expressed in control, epithelial cells with no induction of Zeb1 (Lane 1). Since Zeb1 was fused with GFP, the molecular weight was observed to be at ~160-180 kDa. The protein molecular weight marker in kDas is on the left panel of the blot images; the loading amounts of the samples were normalized to the content of β-actin (MW: ~44 kDa).

Transfection for Luciferase reporter assay

After successfully inducing the MCF-7/Zeb1 cells with doxycycline and confirming the expression of a mesenchymal marker - Zeb1, we proceeded with transfection of luciferase reporter plasmid to the cells in order to assess the levels of expression of Zeb1 and E-cadherin, an epithelial marker, using luciferase reporter assay. In order to ensure that the plasmid of interest was transfected, the control cell population (non-Zeb1-induced) was transfected with GFP plasmid, and that was used as a positive control for transfection. After several days of culturing the transfected cells they were seeded on the 6-well plate for subsequent induction with doxycycline. Fig. 3 demonstrates microscopy images of the cells at 0, 48 and 72 hours of induction with doxycycline. There, the predominating mesenchymal phenotype was observed at 72 hours, and the increasing glowing intensity also confirms that Zeb1/GFP was induced, as stated above. The collected cell lysates (prepared in triplicates) were then placed to the 96 well black/clear bottom plates, and the luminescence was recorded right after the addition of the substrates, to initiate the chemiluminiscent reaction triggered by association of luciferase with its substrate, luciferin. The resulting bar chart (Fig.4) illustrates the decrease in the signal with the increase of Zeb-1 expression level that, conversely, repressed E-cadherin. These results also confirm the role of Zeb1 as the direct transcriptional repressor of E-cadherin. In further trials, in order to completely track the stages of EMT it might be possible to investigate the expression of other important mesenchymal biomarkers,

such as vimentin, N-cadherin and TGF-β.

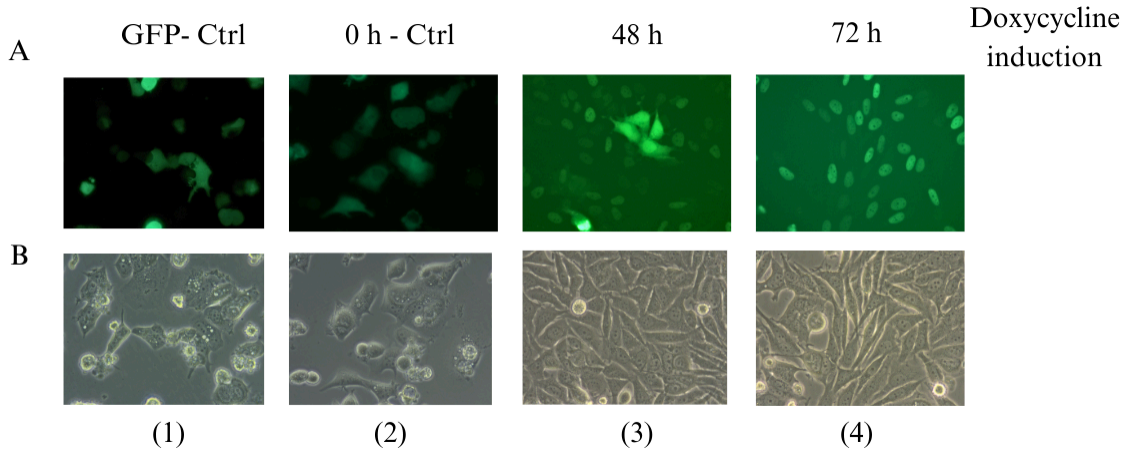


Figure 3. Microscopy images of the cells (40x magnification) after transfection with luciferase reporter and GFP plasmids. Row (A) shows the fluorescent images, where bright, green-glowing nuclei correspond to GFP; (B) shows brightfield microscopy images. (1) As a positive control for transfection, the non-induced control cells were transfected only with GFP plasmid. (2-3) demonstrate the cells at 0, 48 and 72 hours after induction with doxycycline, respectively, that were transfected with both luciferase reporter plasmid and GFP plasmid. As with the non-transfected cells in Fig.1, the characteristic mesenchymal phenotype (spindle-shaped, fibroblast-like features) was observed in the cells after transfecting them with the luciferase reporter plasmid.

Despite of the presented results of successful transfection, and subsequently, Zeb1 - induction of the transfected cells, initially, the transfected cells were observed to possess very poor growth rate, and subsequently, the number of non-viable cells kept increasing. This was an expected outcome, since transfection poses a certain stress for cells, however, the growth of the cells, expressing luciferase, should have been restored. Therefore, the increase in serum concentration up to 15% was a relatively effective strategy to restore the viability of transfected cells. Nevertheless, due to the anticipated toxicity of the Lipofectamine reagent used for transfection, its dosage should be adjusted in future trials.

Apart from that, due to the overall low signal of luminescence recorded by the luminometer and the presence of signals in the empty wells, we decided to proceed with the cell cycle flow cytometry of samples, to assess the effect of the drugs on MCF-7/Zeb1 cells.

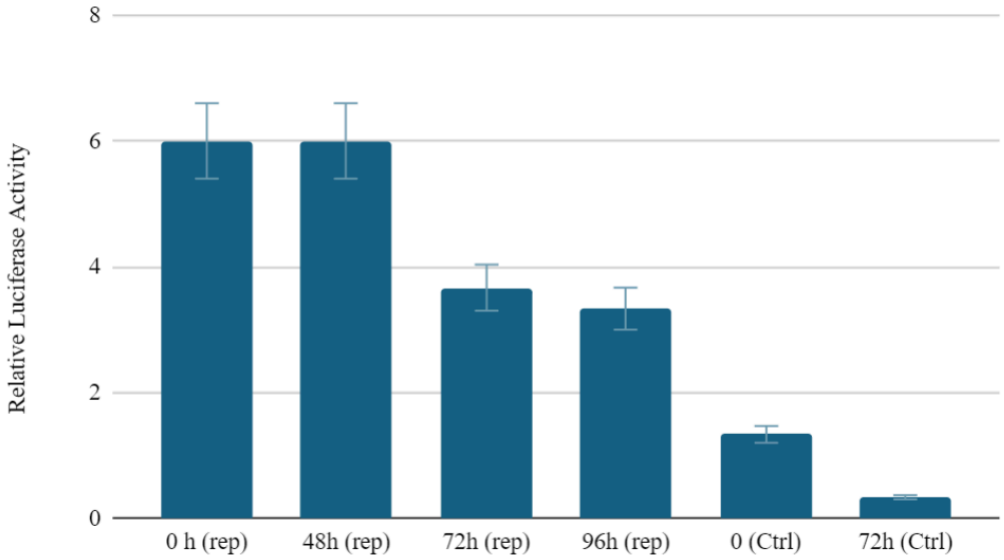


Figure 4. The chart of the relative luciferase activity recorded at 0, 48, 72 and 96 hours after Zeb1-induction of cells transfected with luciferase reporter and at 0 and 72 hours after Zeb1-induction of cells transfected with GFP plasmid only. The relative expression of CDH1 luciferase declined with the increased induction of Zeb1, suggesting that the expression of E-cadherin was repressed. The presence of the signals in last control samples at 0 and 72 hours might be due to the possible optical activity exerted by GFP-glowing, given that luciferase absent samples should yield no signal. (See Fig.A7 for the full chart with the measurements of the triplicates)

Screening of the PKC inhibitors library

In order to test the drugs on their potency in inhibiting Zeb1 mediated drug resistance in MCF-7 cells, the three protein kinase C (PKC) inhibitors (selected randomly) were tested.

#	Plate layout	Inhibitor	Specific target
1	L1021-08 A5	Sotrastaurin	PKC
2	L1021-07 H8	Riluzol	Na-channels, PKC, glutamate
3	L1021-01 C6	Enzastaurin	PKC (PKCβ)

Table 1. The three indicated inhibitors (Sotrastaurin, Riluzol, Enzastaurin) were selected randomly from the library of available PKC inhibitors. The '#' numbering indicates the order of the drugs to be tested via flow cytometry.

PKC inhibitors are able to counteract the effect of Zeb1 in MCF-7/Zeb1 cells

The Flow Cytometry analysis of the cell cycle profiles was initially performed on untreated cells at 0, 48, 72 and 96 hours post-induction with doxycycline, in order to confirm that the system operates properly, focusing primarily on the DNA content at G1 phase (Fig. 5). To this end, we have observed that the population of cells at G1 increased with the time points of exposure to doxycycline, meaning that the expression of Zeb1 corresponds to the cell cycle arrest at G1 phase.

The highest number of cells was recorded to be at 72 hours, the peak expression of Zeb1, as it was confirmed by Western Blot (Fig. 2). This is consistent with the fact that Zeb1 augments the resistance of cancer cells towards the genotoxic drugs by halting their growth specifically at G1 phase, where they can evade the cytotoxic effects of the drugs. Therefore, the decrease in the population of cells with activated Zeb1 at G1 phase would mean that the drug was able to eliminate the effect of Zeb1.

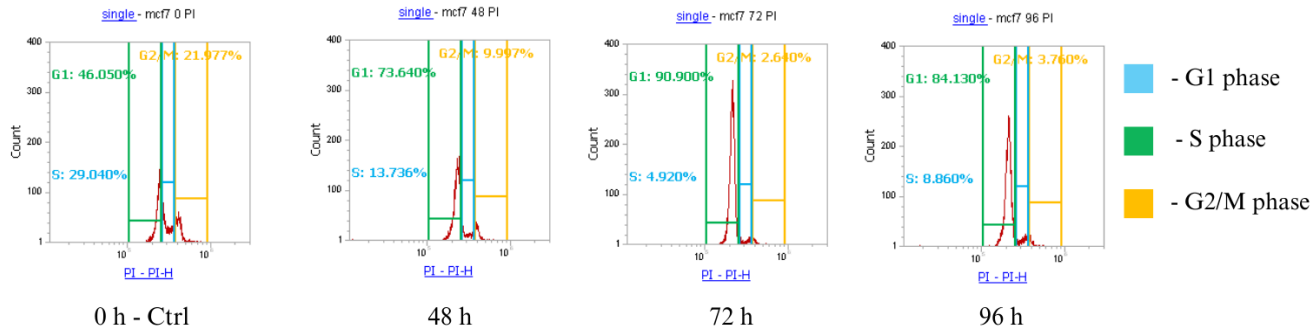


Figure 5. The histograms of cell cycle profiles (with the gating) of samples at 0, 48, 72 and 96 hours after Zeb1-induction, stained with propidium iodide (PI). (See Fig.A3 in Appendix section for cell cycle profiles of unstained samples used as controls for staining and the scatter plots illustrating the population of singlets at each measurement.)

Finally, to investigate how the administration of the PKC inhibitors will affect the cells expressing Zeb1, we performed the flow cytometry analysis of cells at 0 hours and 72 hours of Zeb1 induction, treated with the kinase inhibitors (See Table 1). Overall, we could observe that the G1-population of cells treated with the inhibitors was lower than in the untreated control samples, both for 0 hours (no activity of Zeb1) and 72 hours (peak expression of Zeb1). The decrease in G1 peak in Zeb1-expressing samples treated with the inhibitors demonstrates that the inhibitors were able to overcome the effect of Zeb1. The most significant decrease in G1 was observed in samples treated with riluzol, that is known to target the voltage-gated Na-channels and glutamate signaling, also affecting PKC signaling (Blyufer et al., 2021).

The 10% difference in the population of cells at G1 recorded for non-induced samples treated with enzastaurin (Fig.6C, (1-2)) might be due to the possible human error, since this kind of difference of ~10% was observed when comparing specifically inhibitor- treated and non-treated cells, and between the doxycycline-induced and non-induced cells for all sets of measurements, suggesting that Zeb1 and PKC inhibitors indeed do have direct effect on the cell cycle arrest at G1.

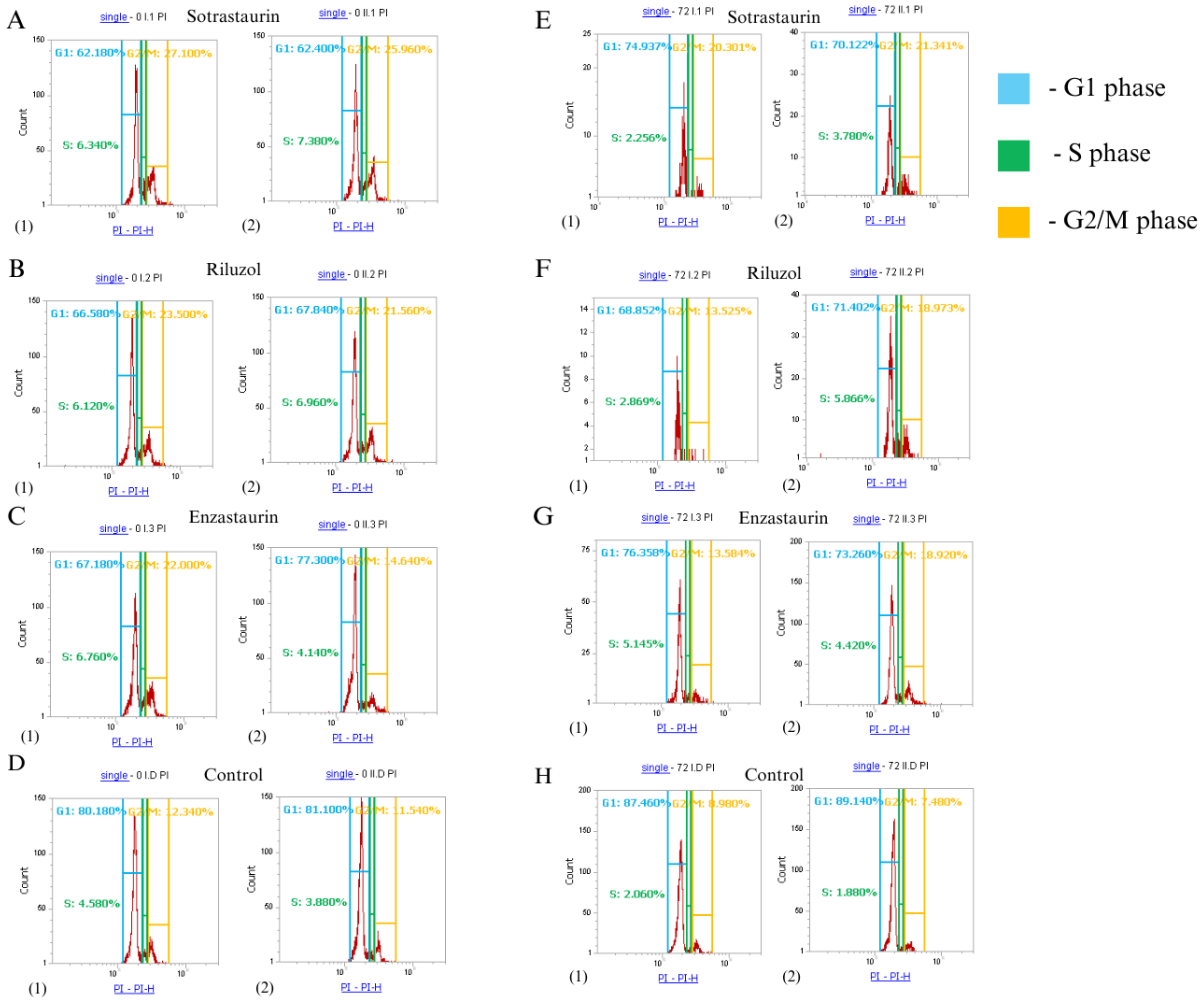


Figure 6. The histograms of cell cycle profiles (with the gating) of samples stained with propidium iodide (PI) after administration of the inhibitors. A-D indicate the cell cycle profiles at 0 hours (without Zeb1 induction), with the addition of sotrastaurin (A), riluzol (B), enzastaurin (C) and no treatment DMSO control (D). Similarly, E-H indicate the cell cycle profiles at 72 hours after induction of Zeb1 by doxycycline, with the addition of sotrastaurin (E), riluzol (F), enzastaurin (G) and no treatment DMSO control (H). (1) – (2) indicate the respective duplicates of the samples, for a statistically significant result.

	Sotrastaurin		Riluzol		Enzastaurin		Non-treated	
	0 h	**72 h**	**0 h**	**72 h**	**0 h**	**72 h**	**0 h**	**72 h**
Population of cells at G1	62.29 ± 2.888	72.5295 ± 2.621	67.21 ± 5.076	70.127 ± 3.161	72.24 ± 6.382	74.809 ± 7.921	80.64 ± 0.651	88.3 ± 1.188

Table 2. The comparison of percentage population of cells (72 h Zeb-1 induced and non-induced) at G1 phase treated with the inhibitors and non-treated, obtained from the histograms of the cell cycle profiles (Fig.5). The data is presented as Mean ± Standard Deviation.

In general, we have identified that the drugs eliminated G1 cell cycle arrest at around the same pattern in non-Zeb1-induced control cells and cells with activated Zeb1, which also suggests that the selectivity of the kinases might be specifically studied in cells at various stages of EMT, where cells imply partly-mesenchymal or absolute mesenchymal phenotype.

In this part of the experiment, we have tested the combination of the effects of kinase inhibitors on cells, induced for full 24 hours with doxycycline, and harvested after 48 more hours, when the cells were already exposed to inhibitors. Since doxycycline is known to be stable for more than 48-72 hours, we introduced the drugs to the cells with prior aspiration of doxycycline media. The cells were supposed to retain some of the doxycycline and reach the status of mesenchymal cells at 72 hours post-induction, as it was demonstrated earlier. However, after monitoring the cells under microscope after 72 hours, we observed that there was significantly less visible Zeb-1 phenotype (Fig.7). Given these circumstances, we predict that might be the reason of why the difference in the G1 peaks within the 0 hours treated and non-treated and 72 hours treated and non-treated was recorded to be in relatively the same range of ~10% difference. Therefore, we concluded that the induction of Zeb1 at this stage was not enough, and in future trials, the drugs should be administered only after full 72 hours of exposure to doxycycline (without the removal of doxycycline media), which might require longer time of exposure to doxycycline and inhibitors, but that would ensure that Zeb1 was activated and reached its peak expression.

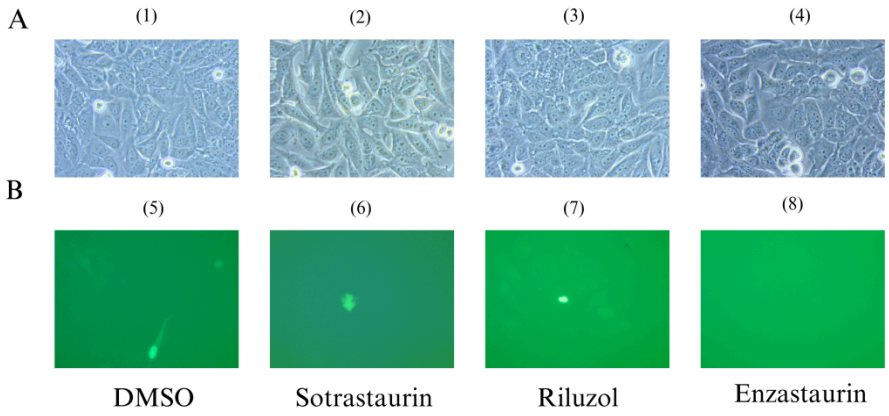


Figure 7. Microscopy images (40x magnification) of the MCF-7/Zeb1 at 72 hours of induction with doxycycline, treated the PKC inhibitors (2-8) and non-treated (1-2). Row (A) demonstrates brightfield images of the cells, and row (B) shows respective fluorescent images. Here, the mesenchymal phenotype is not as vivid as it was observed with earlier Zeb1-induction trials (See Fig.1). There was no glowing observed in the cells treated with enzastaurin (4&8).

It is also worth to notice, that in order to further substantiate the effect of inhibitors on Zeb1 expressing cancer cells, and ensure that the drugs possess selective toxicity only to Zeb1-expressing cancer cells, the cytotoxicity profiles need to be obtained, and the effect of the inhibitors should be tested at various concentrations.

The similar study investigating effect of PKC inhibitors, specifically staurosporine derivatives, UCN-01 and Midostaurin, demonstrated that these kinase inhibitors selectively eliminated chemoresistant mesenchymal hepatocellular carcinoma cells (M-HCC) (Sreekumar et al.,

2019). In their study, Sreekumar et al (2019) have also substantiated the role of PKC pathway in augmenting the M-HCC survival, which also suggests that this pathway in particular can be selectively targeted by available kinase inhibitors specifically in EMT cells, that are implied in this study as well, with possibility to translate it to animal models and further clinical trials.

Here, we also employed other derivatives of staurosporine, sotrastaurin and enzastaurin that both demonstrated the abrogation of cell cycle arrest at G1, suggesting their potential role in eliminating Zeb1 expressing cancer cells. However, these results do not explicitly show at which exact stage of EMT inhibitors are most effective in eliminating cells, and whether they are selective in killing the cells based on the EMT status. Therefore, further trials should be performed with different concentrations of the inhibitors (here all inhibitors were administered at a final concentration of 10uM), on cancer cells, expressing other mesenchymal markers, such as vimentin, N-cadherin and TGF-β.

Moreover, another evidence shows that there is a direct correlation between the PKCα activity and Zeb1, also suggesting that specific sites of Zeb1 are direct targets of PKC inhibitors, which also validates the role of Zeb1 in drug-screening studies, aimed to differentially eradicate metastatic cancer cells, with mesenchymal characteristics, that also have CSC properties (Llorens et al, 2019).

Zeb1, as a master regulator of EMT, is shown to have a very dynamic interplay between different players of cell signaling that are responsible for enhanced metastatic capacities of tumors, their ability to renew and acquire stem cell properties. However, there is still little known about the exact molecular machineries of how Zeb1 might interact with various molecules that affect the cell proliferation. To this end, our results suggest that kinase inhibitors such as Riluzol and staurosporine derivatives, Enzastaurin and Sotrastaurin, might be further employed in investigating the Zeb1-mediated resistance and survival pathway in cancer cells and how this molecular network can be manipulated with minimal invasion and more selective eradication of tumors.

Conclusion

Breast cancer is the most common type of cancer diagnosed in women worldwide, and it is known to have high recurrence risk, as in many other solid tumors. The poor clinical outcomes of cancer patients is dependent upon various factors that have to do with the complex interplay of molecular machineries that cancer cells imply to get more aggressive and resistant to therapies. Zeb1, as a master regulator of one such machinery, epithelial-to-mesenchymal transition, has a variety of important downstream targets that affect the clinical outcomes of cancer patients. However, the functional consequences of Zeb1 activity was not studied completely within the context of drug screening, specifically, kinase inhibitors. In this research, we have shown that Zeb1 is relatively easy to study and observe within the context of our aims, since MCF-7/Zeb1 cell line was demonstrated to have a definite phenotypic expression of mesenchymal markers upon induction of Zeb1 by doxycycline. Thus, the same methodology can be applied in cell lines of other solid tumors, to study the Zeb1 mediated drug resistance in a broader sense.

References

Argast, G. M., Krueger, J. S., Thomson, S., Sujka-Kwok, I., Carey, K., Silva, S., ... & Epstein, D. M. (2011). Inducible expression of TGFβ, snail and Zeb1 recapitulates EMT in vitro and in vivo in a NSCLC model. *Clinical & experimental metastasis*, *28*, 593-614. https://doi.org/10.1007/s10585-011-9394-8

Blyufer, A., Lhamo, S., Tam, C., Tariq, I., Thavornwatanayong, T., & Mahajan, S. S. (2021). Riluzole: A neuroprotective drug with potential as a novel anti-cancer agent. *International journal of oncology*, *59*(5), 1-11. https://doi.org/10.3892/ijo.2021.5275

Dent, P., Tang, Y., Yacoub, A., Dai, Y., Fisher, P. B., & Grant, S. (2011). CHK1 inhibitors in combination chemotherapy: thinking beyond the cell cycle. *Molecular interventions*, *11*(2), 133. https://doi.org/10.1124%2Fmi.11.2.11

Dongre, A., & Weinberg, R. A. (2019). New insights into the mechanisms of epithelial–mesenchymal transition and implications for cancer. *Nature reviews Molecular cell biology*, *20*(2), 69-84. https://doi.org/10.1038/s41580-018-0080-4

Fedorova, O., Daks, A., Parfenyev, S., Shuvalov, O., Netsvetay, S., Vasileva, J., ... & Barlev, N. A. (2022). Zeb1-mediated autophagy enhances resistance of breast cancer cells to genotoxic drugs. *Biochemical and Biophysical Research Communications*, *589*, 29-34. https://doi.org/10.1016/j.bbrc.2021.11.088

Housman, G., Byler, S., Heerboth, S., Lapińska, K., Longacre, M., Snyder, N. A., & Sarkar, S. (2014). Drug Resistance in Cancer: an overview. *Cancers*, *6*(3), 1769–1792. https://doi.org/10.3390/cancers6031769

Llorens, M. C., Rossi, F. A., García, I. A., Cooke, M., Abba, M. C., Lopez-Haber, C., ... & Soria, G. (2019). PKCα modulates epithelial-to-mesenchymal transition and invasiveness of breast cancer cells through ZEB1. *Frontiers in oncology*, *9*, 1323. https://doi.org/10.3389/fonc.2019.01323

Lu, J., Kornmann, M., & Traub, B. (2023). Role of epithelial to mesenchymal transition in colorectal cancer. *International Journal of Molecular Sciences*, *24*(19), 14815. https://doi.org/10.3390/ijms241914815

Manshouri, R., Coyaud, E., Kundu, S. T., Peng, D. H., Stratton, S. A., Alton, K., ... & Gibbons, D. L. (2019). ZEB1/NuRD complex suppresses TBC1D2b to stimulate E-cadherin internalization and promote metastasis in lung cancer. *Nature communications*, *10*(1), 1-15. https://doi.org/10.1038/s41467-019-12832-z

Marcucci, F., Stassi, G., & De Maria, R. (2016). Epithelial–mesenchymal transition: a new target in anticancer drug discovery. *Nature reviews Drug discovery*, *15*(5), 311-325. https://doi.org/10.1038/nrd.2015.13

Parfenyev, S. E., Shabelnikov, S. V., Pozdnyakov, D. Y., Gnedina, O. O., Adonin, L. S., Barlev, N. A., & Mittenberg, A. G. (2021). Proteomic analysis of Zeb1 interactome in breast carcinoma cells. *Molecules*, *26*(11), 3143. https://doi.org/10.3390/molecules26113143

Parfenyev, S. E., Shabelnikov, S. V., Tolkunova, E. N., Barlev, N. A., & Mittenberg, A. G. (2023). p53 Affects Zeb1 Interactome of Breast Cancer Stem Cells. International Journal of Molecular Sciences, 24(12), 9806. https://doi.org/10.3390/ijms24129806

Ren, J., Chen, Y., Song, H., Chen, L., & Wang, R. (2013). Inhibition of ZEB1 reverses EMT and chemoresistance in docetaxel‐resistant human lung adenocarcinoma cell line. *Journal of cellular biochemistry*, *114*(6), 1395-1403. https://doi.org/10.1002/jcb.24481

Sanchez-Tillo, E., Fanlo, L., Siles, L., Montes-Moreno, S., Moros, A., Chiva-Blanch, G., ... & Postigo, A. (2014). The EMT activator ZEB1 promotes tumor growth and determines differential response to chemotherapy in mantle cell lymphoma. *Cell Death & Differentiation*, *21*(2), 247-257. https://doi.org/10.1038/cdd.2013.123

Sreekumar, R., Emaduddin, M., Al-Saihati, H., Moutasim, K., Chan, J., Spampinato, M., ... & Sayan, A. E. (2019). Protein kinase C inhibitors override ZEB1-induced chemoresistance in HCC. *Cell death & disease*, *10*(10), 703.

Wang, H., An, X., Yu, H., Zhang, S., Tang, B., Zhang, X., & Li, Z. (2017). MiR-29b/TET1/ZEB2 signaling axis regulates metastatic properties and epithelial-mesenchymal transition in breast cancer cells. *Oncotarget*, *8*(60), 102119. https://doi.org/10.18632%2Foncotarget.22183

Zhang, P., Sun, Y., & Ma, L. (2015). ZEB1: at the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. *Cell cycle*, *14*(4), 481-487. https://doi.org/10.1080/15384101.2015.1006048

Zhang, P., Wei, Y., Wang, L. I., Debeb, B. G., Yuan, Y., Zhang, J., ... & Ma, L. (2014). ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1. *Nature cell biology*, *16*(9), 864-875. https://doi.org/10.1038/ncb3013

Appendix

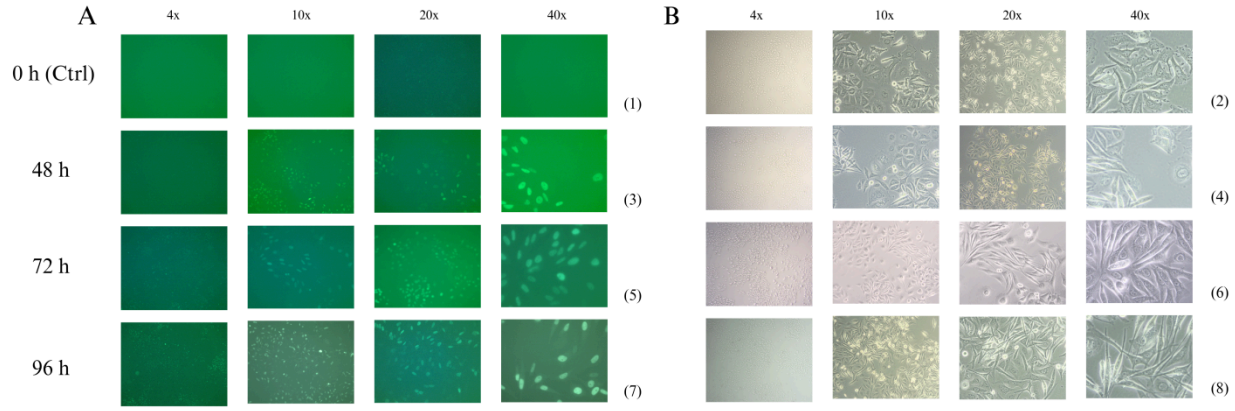


Fig A1. Microscopy images of MCF-7/Zeb1 cells at four different magnifications at 0, 48, 72 and 96 hours after Zeb1 induction mediated by doxycycline. Block A shows fluorescent microscopy images, block B demonstates the respective images under brightfield microscopy.

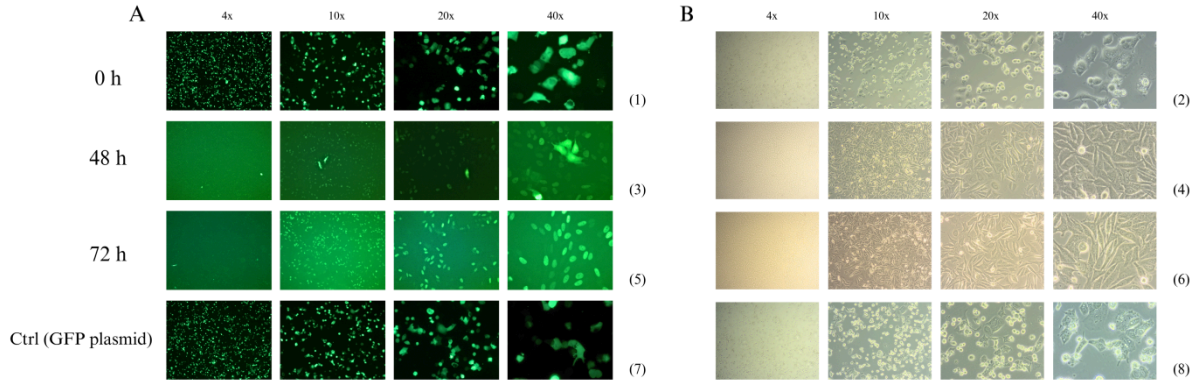


Fig A2. Microscopy images of MCF-7/Zeb1 cells after transfection with luciferase reporter plasmid and GFP plasmid at four different magnifications at 0, 48, 72 and 96 hours after Zeb1 induction with doxycycline (1-6), and not-induced control cells transfected solely with GFP plasmid (7-8). Block A demonstrates fluorescent microscopy images, while block B shows the respective images under bright field microscopy.

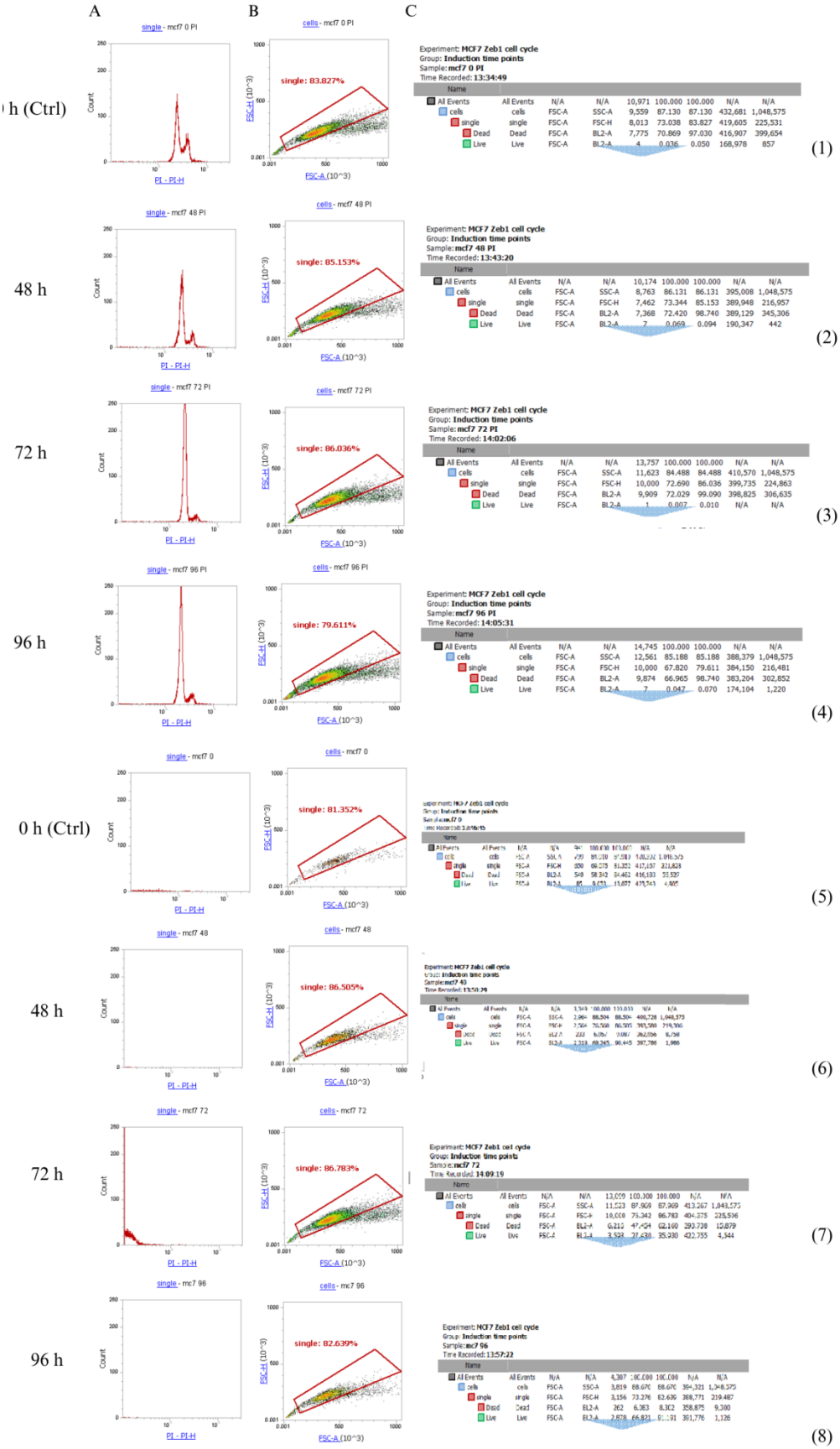

A
B
C
h (Ctrl)
single - mcf7 0 PI
cells - mcf7 0 PI
single: 83.827%
Count
PI - PI-H
FSC-H (10^3)
FSC-A (10^3)
Experiment: MCF7 Zeb1 cell cycle
Group: Induction time points
Sample: mcf7 0 PI
Time Recorded: 13:34:49
(1)
48 h
single - mcf7 48 PI
cells - mcf7 48 PI
single: 85.153%
Experiment: MCF7 Zeb1 cell cycle
Group: Induction time points
Sample: mcf7 48 PI
Time Recorded: 13:43:20
(2)
72 h
single - mcf7 72 PI
cells - mcf7 72 PI
single: 86.036%
Experiment: MCF7 Zeb1 cell cycle
Group: Induction time points
Sample: mcf7 72 PI
Time Recorded: 14:02:06
(3)
96 h
single - mcf7 96 PI
cells - mcf7 96 PI
single: 79.611%
Experiment: MCF7 Zeb1 cell cycle
Group: Induction time points
Sample: mcf7 96 PI
Time Recorded: 14:05:31
(4)
0 h (Ctrl)
single - mcf7 0
cells - mcf7 0
single: 81.352%
(5)
48 h
single - mcf7 48
cells - mcf7 48
single: 86.505%
(6)
72 h
single - mcf7 72
cells - mcf7 72
single: 86.783%
(7)
96 h
single - mcf7 96
cells - mcf7 96
single: 82.639%
(8)

Fig A3. The cell cycle profiles of cells after 0, 48, 72 and 96 hours of Zeb1-induction mediated by doxycycline after PI staining (1-4), and with no staining (5-8), without gating. Column A shows the histograms with the distribution of DNA content with the highest peak corresponding to the G1 phase, the smaller peak in the right – G2/M and S-phase are in between the peaks. Column B shows the scatter plots of the distribution of cells. Column C corresponds to additional numerical parameters recorded for each measurement.

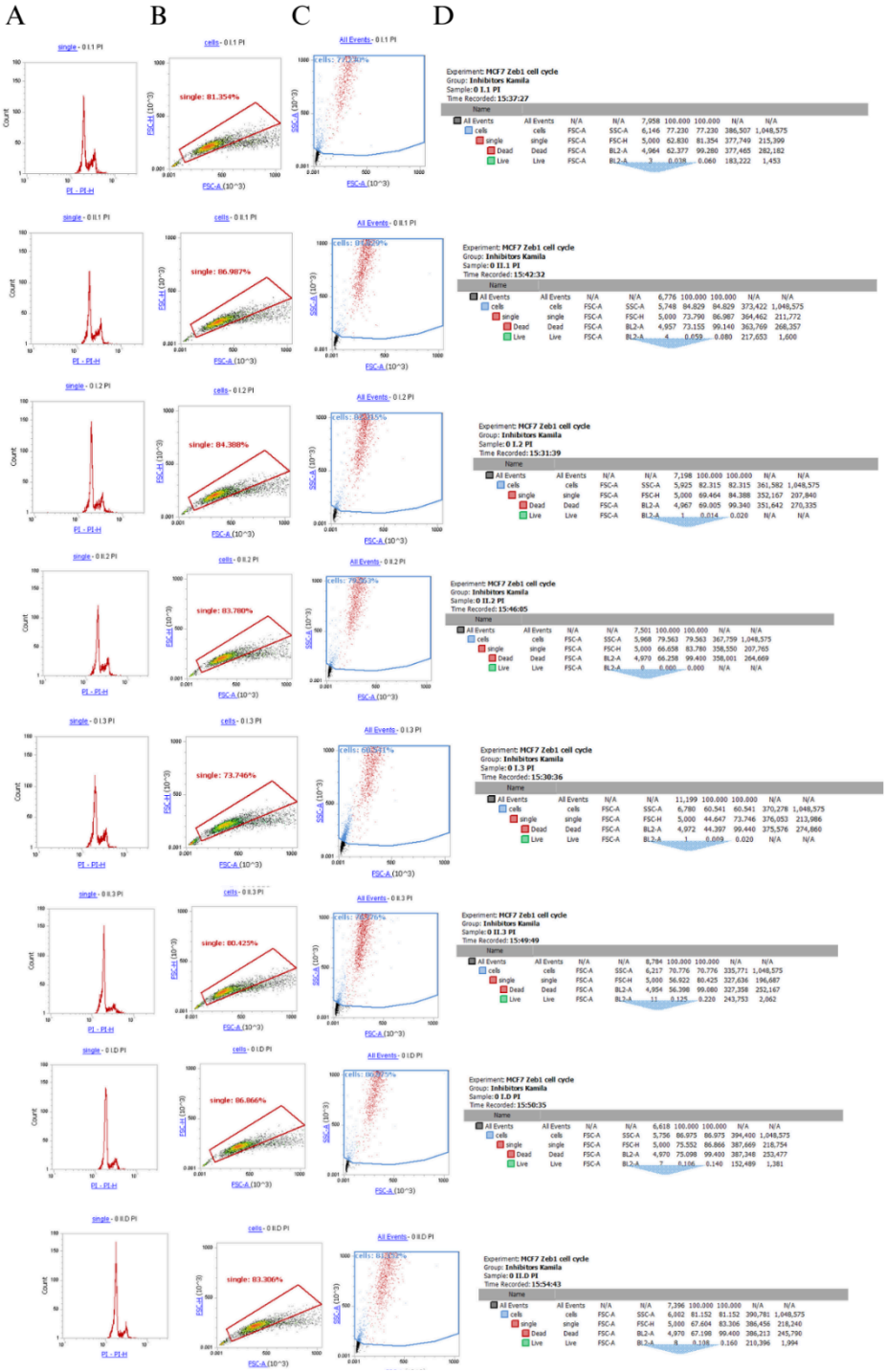


Fig A4. The cell cycle profiles of control (0 h) cells after PI staining, without gating. Column A shows the histograms with the distribution of DNA content. Column B and C show the scatter plots of the distribution of cells. Column D corresponds to additional numerical parameters recorded for each measurement.

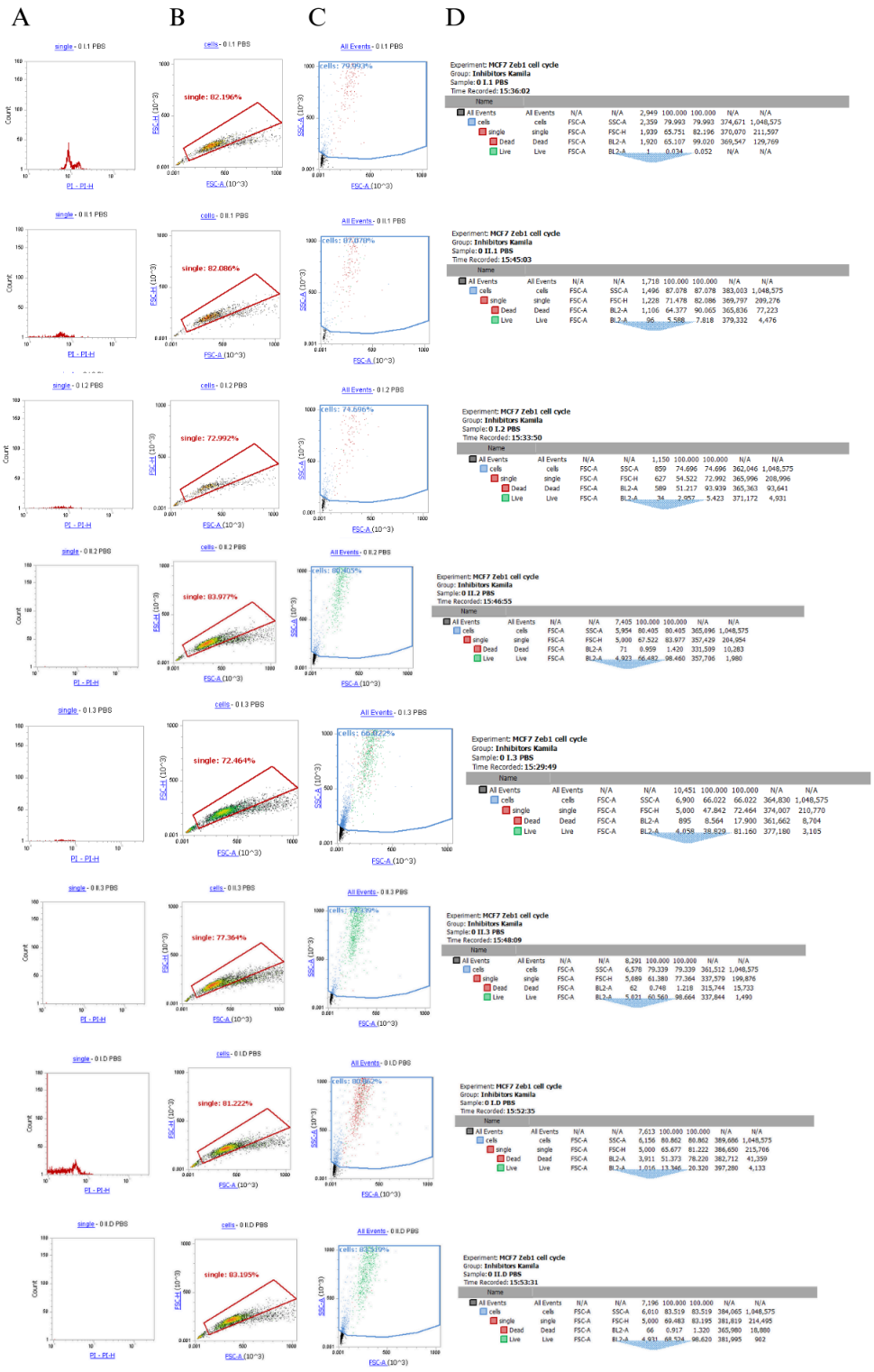


Fig A5. The cell cycle profiles of control kinase-treated and non-treated cells (0 h), with no staining, without gating. Column A shows the histograms with the distribution of DNA content. Column B and C show the scatter plots of the distribution of cells. Column D corresponds to additional numerical parameters recorded for each measurement.

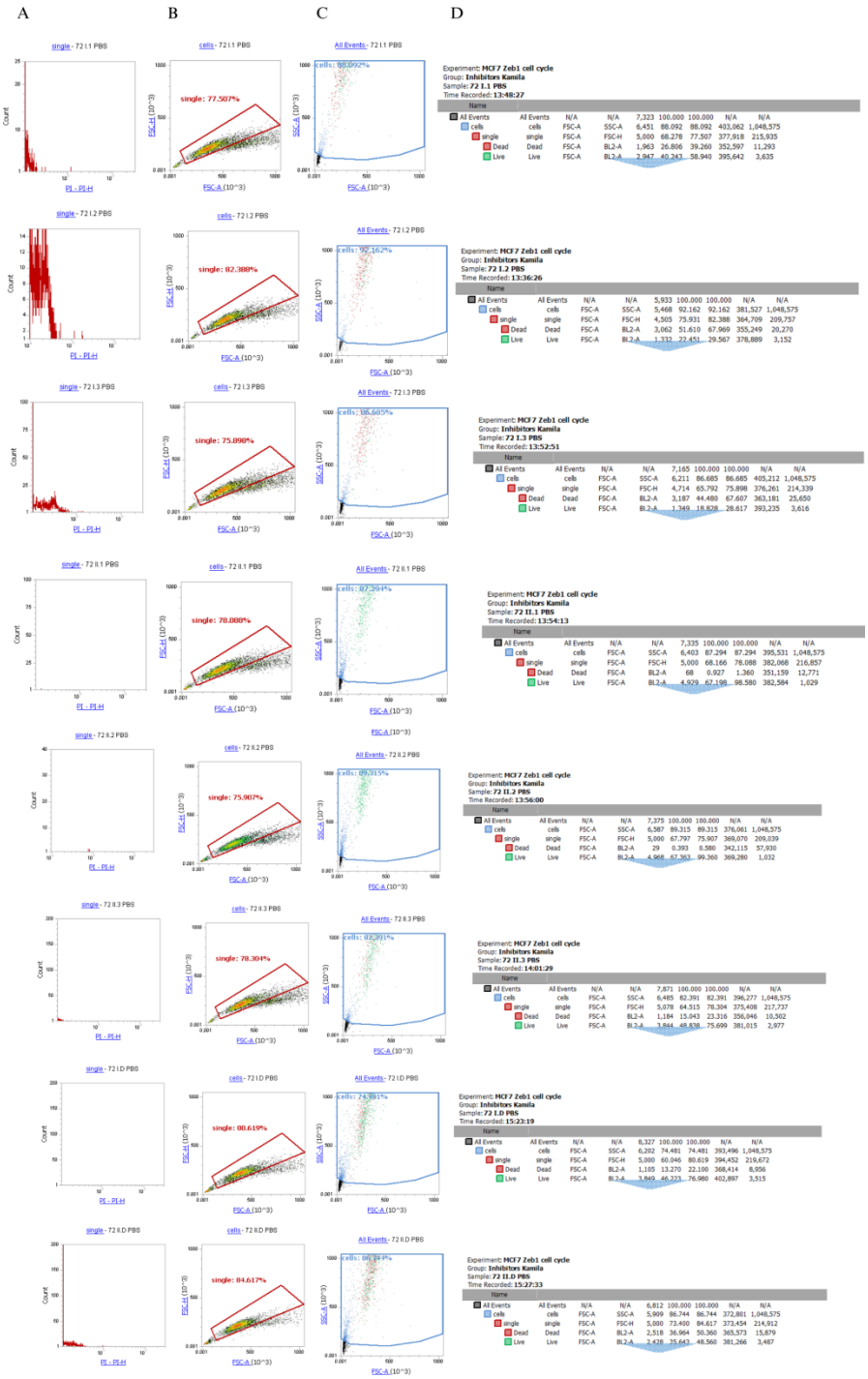
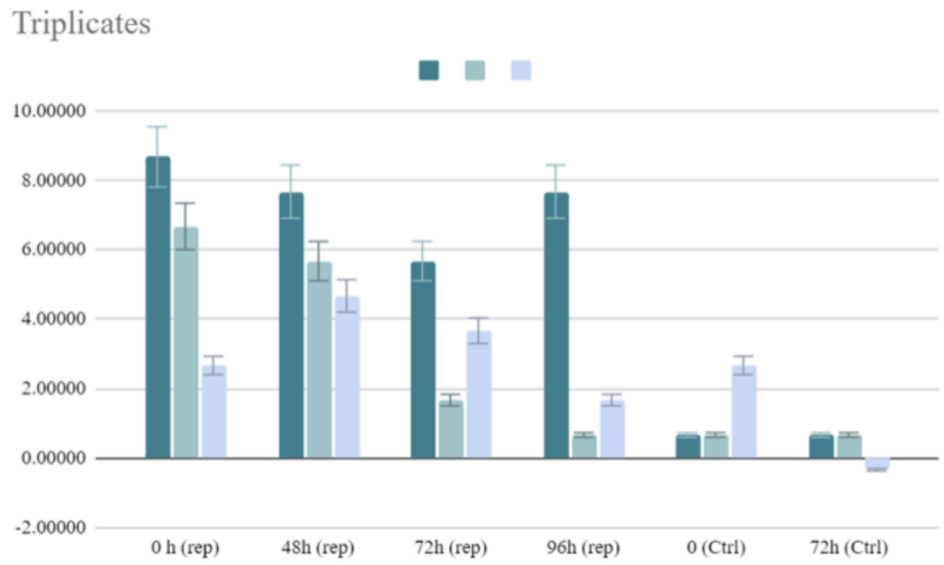


Fig A6. The cell cycle profiles of kinase-treated and non-treated cells after 72 hours of Zeb1-indcution with doxycycline, with no staining, without gating. Column A shows the histograms with the distribution of DNA content. Column B and C show the scatter plots of the distribution of cells. Column D corresponds to additional numerical parameters recorded for each measurement.

A



B

	0 h (rep)	48h (rep)	72h (rep)	96h (rep)	0 (Ctrl)	72h (Ctrl)	Bckgrnd Lysate
	1	2	3	4	5	6	7
A	11	10	8	10	3	3	4
B	9	8	4	3	3	3	2
C	5	7	6	4	5	2	1
							2.333333333

	0 h (rep)	48h (rep)	72h (rep)	96h (rep)	0 (Ctrl)	72h (Ctrl)	
A	8.66667	7.66667	5.66667	7.66667	0.66667	0.66667	
B	6.66667	5.66667	1.66667	0.66667	0.66667	0.66667	
C	2.66667	4.66667	3.66667	1.66667	2.66667	-0.33333	

Figure A7. (A) The chart of the luminescence signal recorded at 0, 48, 72 and 96 hours after Zeb1-induction of cells transfected with luciferase reporter and at 0 and 72 hours after Zeb1-induction of cells transfected with GFP plasmid only. Each sample was prepared in triplicates. (B) the recordings registered by the luminometer.