5-aza-2′-deoxycytidine induces telomere dysfunction in breast cancer cells

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ABSTRACT

Aims: Azacitidine, a drug that epigenetically modifies DNA, is widely used to treat haematological malignancies. However, at low doses, it demethylates DNA, and as a result, can alter gene expression. In our previous publication, we showed that low doses of azacitidine induce telomere length elongation in breast cancer cells. In this study, we aim to identify the mechanisms which lead to telomere length increases.
Methods: Breast cancer cell lines representing different molecular sub-types were exposed to 5-aza-2′-deoxy-cytidine (5-aza) in 2 and 3D cultures, followed by DNA, RNA, and protein extractions. Samples were then analysed for telomere length, DNA damage, telomerase, and ALT activity.
Results: We show that treatment of the cell lines with 5-aza for 72 h induced DNA damage at the telomeres and increased ALT activity 3-fold. We also identified a gene, *POLD3*, which may be involved in the ALT activity seen after treatment.
Conclusion: Our results indicate that while 5-aza is a useful drug for treating haematological cancers, surviving cancer cells that have been exposed to lower doses of the drug may activate mechanisms such as ALT. This could lead to cancer cell survival and possible resistance to 5-aza clinically.

1. Introduction

Breast cancer is the most common malignancy affecting women globally. In the UK, around 69,900 new cases will be diagnosed by 2040 (Cancer Research UK, 2023). The main causes of breast cancer are not fully understood. However, factors such as age, family history, and the consumption of alcohol may increase the risk of developing the disease [36]. Telomeres are specialised structures, which are present at the ends of eukaryotic chromosomes to prevent DNA damage and cellular senescence [15]. Telomeres consists of a simple repeat sequence TTAGGG, which extends for up to 15 kb in humans [45], 50 kb in mice [6] and 150 kb in plants [17]. Due to the end replication problem, telomeric DNA shortens after each round of replication in normal cells until they reach a critical length which triggers cellular senescence and growth arrest [28]. Telomerase, the enzyme responsible for synthesising telomeric repeats, is expressed at very low levels in normal human somatic cells [3]. However, cancer cells have developed two mechanisms to maintain telomere length, the first is mediated by telomerase which synthesises G- rich repetitive sequences onto the telomeres to prevent them from shortening [35]. The second and less frequently used mechanism known as Alternative Lengthening of Telomeres (ALT) is active in around 10 % of cancer cells and maintains telomeres via homologous recombination [38,49,54]a.

Azanucleosides (AZN) are pyrimidine analogues that were first synthesised in 1964 [52]. They were approved as a treatment for patients with acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) [26]. There are two types of AZN that are currently used in clinics, 5-azacytidine and 5-aza-2′-deoxycytidine (5-aza). Both drugs are mechanistically similar in that they are incorporated into DNA during DNA replication, the main difference between them is the dose administered [11]. AZN's are analogues of the nucleotide cytidine and once incorporated into DNA they act as potent inhibitors of the methyltransferase enzymes. Studies have shown that 5-aza-2′-deoxycytidine is more potent in *vivo* [27].

At high doses, these drugs are cytotoxic to cancer cells, however at lower concentrations, they decrease DNA methylation and reactivate the expression of target genes [43]. Motevalli et al., [39], found that 5-aza-2′-deoxycytidine (decitabine, 5-aza) caused rapid telomere elongation in breast cancer cells. The effect was not specific to cancer cells as elongated telomeres were also found in a normal mammary epithelial

cell strain (HMEC) after treatment. Bull et al., [4] reported that the lower doses of 5-aza were associated with increased telomere length and DNA damage in human lymphoblastoid WIL2-NS cells. Moreover, [5] tested the effect of low concentration (1 μM) of 5-aza in AGS gastric epithelial cells infected with H. pylori. 5-aza treatment caused telomere shortening and DNA hypermethylation in the epithelial cells followed by downregulation of *hTERT* expression during the infection. Therefore, the overall effects 5-aza has on telomeres and telomere length are not fully understood and require further investigation. In addition, the actual mechanisms that lead to telomere length elongation need to be elucidated. AZNs are part of a new type of anti-cancer treatment known as epigenetic therapy [1]. This type of therapy targets modifications that have taken place to the DNA within cancer cells such as DNA methylation and histone modification. Both events are usually targeted together to increase the effectiveness of epigenetic therapy. 5-aza is commonly used with the histone deacetylase (HDAC) inhibitor trichostatin A to inhibit DNA methylation and histone modification respectively [23].

In this article, we investigated the mechanisms through which 5-aza-2′-deoxycytidine (5-aza) could trigger telomere dysfunction in different subtypes of breast cancer cell lines. We analysed the effect of 5-aza on telomere length, telomeric DNA damage, *hTERT* mRNA expression ALT and telomerase enzyme activity. Our findings here demonstrate that 5-aza increased telomere length and DNA damage at the telomeres. *hTERT*, but not telomerase activity was upregulated suggesting that another mechanism is at play. Finally, ALT activity was shown to be increased in cells treated with 5-aza.

2. Materials and methods

2.1. Cell lines, culture conditions

The human breast cancer cell line 21NT was grown as monolayers in 1x Modified Eagle's medium alpha (alpha MEM) (Gibco), 2.8 μM hydrocortisone, 1 μg/ml insulin, 10 % foetal calf serum (FCS), 1 % glutamax, 10 mM HEPES, 0.1 mM NEAA and 12.5 ng/ml Epithelial Growth Factor (EGF), [9]. MCF7, BT474, and HS78T cell lines were grown in DMEM/F12 (Gibco) with 10 % FCS and 1 % glutamax, 0.5 μg/ml hydrocortisone [39]. Cells were maintained in T75 flasks at 37^0 C in a humidified environment with 5 % CO_2.

2.2. 3D culture using GrowDex

BT474 cells were cultured in 0.5 % GrowDex hydrogel (UPM Bioforbeyond fossils). 50000 cells /well (6-well plate) were embedded in a total volume of 1200 μl of Growdex diluted in DMEM/F12 growth medium. 800 μl of growth media/ well was added on top of the Growdex to keep the gel hydrated. Cells were incubated at 37^0 C and monitored daily for 3 days. The culture medium above the gel was then removed and replaced with fresh medium containing 5uM of 5-aza twice with a four hours gap between treatments and left for 72 h. After that, Grow-Dase (UPM Bioforbeyond fossils) mixed with growth medium was added to the wells and left for 10 h. Finally, the DNA was extracted from the cells using the Promega kit as described below.

2.3. Reagents and treatment of cells

5-aza-2′-deoxycytidine (5-aza) and trichostatin A (TSA) were both purchased from Merck, UK. Stock solutions of 5 mM 5-aza and TSA were prepared by dissolving the lyophilised powder in DMSO, aliquots were stored at -80^0 C. 21NT cells were plated at a density of $1x10^5$ into six well plates. Cells were treated with 10 μM concentrations of 5-aza twice with a four hours gap between treatments. During the last 16 h of incubation, 50 ng/ml of TSA was added to modify gene expression [39].

2.4. Genomic DNA extraction

DNA was isolated from the breast cancer cell lines using the Wizard genomic DNA purification kit and protocol from Promega (A1120) and quantified using the NanoDrop.

2.5. RNA extraction, cDNA conversion, and qPCR

Total RNA was extracted from all breast cancer cell lines using the RNeasy Mini Kit from QIAGEN (ID:74104) according to the manufacturer`s protocol. 1 μg of total RNA was reverse transcribed to complementary DNA (cDNA) using a reverse transcription kit from ThermoFisher (4368814). qPCR master mixes were prepared from 2x SYBR® (ThermoFisher), 1 μl of the forward primer (10 mM), 1uL of the reverse primers (10 mM) (Table 1), 1 μL of prepared cDNA and nuclease free water to make the final volume up to 10 μl. The qPCR reactions were performed in triplicate for *hTERT, POLD,* and *GAPDH* in 96 well plates sealed with an optical adhesive film (MicroAmp® Optical Adhesive Film, ThermoFisher). The default PCR cycle was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The Relative Quantity (RQ) of gene expression was obtained using the QuanStudio V1.3 software.

2.6. Telomere length measurement by qPCR

To determine telomere length, the qPCR technique was used [42]. qPCR was performed in 96- well plates using 20 ng of DNA per 20 μl reaction. A telomere standard curve was established by serial dilutions of the telomere standard (1018400 kb through 10184 kb dilution) (Table 1) to calculate the telomeric sequence in kb. A single copy gene 36B4, was used as a genomic DNA control, this was done through a serial dilution of the 46B3 standard (6125000 kb through 6.125 kb dilution). The copy number values generated from the qPCR and the serial dilutions were used to calculate the total telomere length in kb. (As described by [42]).

2.7. Quantification of telomere repeat amplification protocol (TRAP)

To quantify telomerase activity, the TRAP assay was used. Protein from the breast cancer cell lines was extracted using the TRAPeze 1 x CHAPS lysis buffer (S7705, Millipore) and quantified using the CB-X protein assay kit (G- Bioscience). For estimation of the telomerase activity from the samples, the procedure outlined in [53] was followed. A serial dilution of the prostate cancer cell line PC-3 (*hTERT* telomerase positive) protein was used to construct a standard curve ranging from 50 ng – 500 ng of protein. A sample of PC3 protein was heated at 95^0 C to inactivate the telomerase enzyme, this served as a negative control for enzyme activity. A non-template control was also included. The master mix was prepared by adding 12.5 μl of the 2x Universal SYBR (ThermoFisher) 5.5 μl of RNAse free water, 1 μl ACX primer (0.05 ug/ul), and 1 μl TS primer (0.1 ug/ul) (Table 1). The reactions were incubated at 25^0 C for 20 min to allow telomerase to synthesise the TRAP ladders, then the qPCR was carried out at 95^0 C for 10 min and 35 cycles of 95^0 C for 30 s, and 60^0 C for 90 s. Telomerase activity was quantified using the PC-3 *hTERT* standard curve and QuanStudio V1.3 software.

2.8. Immunofluorescence detection of γ-H2AX foci

Breast cancer cells were plated onto glass microscope slides and grown in an incubator at 37^0 C/5 % CO_2 (Thermo Scientific). The slides containing the cells were treated with 10 μM 5-aza either alone or in combination with TSA for 72 h. Cells were fixed in 2 % formaldehyde (Fisher Scientific) for 10 min. Following this, cells were washed three times for 5 min each with PBS and permeabilized with 0.3 % (v/v) Triton X-100 (Sigma-Aldrich) solution for 10 min. Cells were washed three times for 5 min each with PBS and blocked with 5 % BSA blocking buffer

Table 1
Primer sequences used for qPCR quantification.

Oligomer name	Oligomer sequence	Product size (bp)
36B4 standard	CAGCAAGTGGGAAGGTGTAATCCGTCTCCACAGACAAGGCCAGGACTCGTTTGTACCCGTTGATGATAGAATGGG	75
Telomere standard	(TTAGGG)$_{14}$	84
Telo (F)	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	>76
Telo (R)	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	>76
36B4 (F)	CAGCAAGTGGGAAGGTGTAATCC	75
36B4 (R)	CCCATTCTATCATCAACGGGTACAA	75
hTERT(F)	CGGAAGAGTGTCTGGAGCAA	200
hTERT(R)	GGATGAAGCGGAGTCTGGA	200
gapdh(F)	GAAGGTGAAGGTCGGAGT	226
gapdh(R)	GAAGATGGTGATGGGATTTC	226
Telomerase Substrate (TS)	AATCCGTCGAGCAGAGTT	
Anchored Return Primer(ACX)	GCGCGG(CTTACC)3CTAACC	
POLD3 (F)	AAAAAGCAGAGCCTGTTAAG	110
POLD3 (R)	GTCTGGAAAGACTTCATCTTC	110

(5 % BSA in Tween before incubation with primary monoclonal anti-phospho histone H2AX (γ-H2AX, Millipore) antibody for one hour at 37^0 C. After incubation, the cells were washed three times before the addition of the anti-mouse IgG FITC conjugated secondary antibody (Invitrogen). After a one-hour incubation at 37^0 C, the cells were washed four times, for 5 min each in PBS. Then, 15 µl DAPI was added. Finally, cells were analysed using an Axioskop Zeiss fluorescence microscope. For each slide, 100 interphase cells were scored to determine the DNA damage repair kinetics [2].

2.9. Telomere dysfunction-induced foci (TIF) assay

This method is based on the co-localization detection of DNA damage by γ-H2AX with the telomeric probe (cy3) to detect DNA damage at telomeres [2]. Firstly, the slides from the γ-H2AX foci procedure above were fixed with 2 % formaldehyde for 10 min. Then, they were washed three times in PBS for five minutes on a shaker in a jar. After that, they were hybridized with the cy3 PNA probe TTAGGG [2] and incubated for two hours at room temperature. Lastly, 15 µl of DAPI was added and a coverslip was added. Cells were analysed using an Axioskop Zeiss fluorescence microscope for each sample 100 interphase cells were scored and TIF foci were counted.

2.10. Telomeric C-circle assay

The C-circle assay was performed using qPCR as outlined in [21]. The genomic DNA of breast cancer cell lines treated with 5-aza was extracted using a genomic DNA isolation kit (Promega). Overall, 30 ng of DNA was used and diluted in 10 mM TRIS (pH 7.6) buffer and made up to a final volume of 10 µl. Then the diluted genomic DNA was added to a 10 µl reaction mix containing 0.2 mg/ml BSA, 4 mM DTT, 0.10 % Tween, 0.1 mM dTTTp, 1X phi29 buffer, and 15 U phi 29 DNA polymerase. Reactions without the phi 29 polymerase enzyme were included as a negative control. Amplification was performed at 30°C for 8 h followed by 65°C for 20 min. The C-circle reactions were diluted with 80 µl of 10 mM Tris, pH 7.6. qPCR was performed in 96-well plates using 5 µl of diluted C-circle product per 25 µl reaction. A single copy gene 36B4 was used as a reference and telomere and 36B4 primers were used according to Table 1. qPCR was carried out at 95 °C for 15 min, 30 cycles of 95 °C for 7 s, and 58 °C for 10 s, followed by 95 °C for 5 min, 40 cycles of 95 °C for 15 s and, 58 °C for 30 s

2.11. Statistical analysis

All statistical analyses were carried out using GraphPad Prism (GraphPad Software). Statistical analysis was performed using unpaired student's test (*P<0.05 **P<0.01, ***P<0.001). Three biological repeats under the same conditions were performed for all experiments.

3. Results

### 3.1. 5-aza-2′-deoxycytidine increased telomere length in 2D cultures

A preliminary experiment was performed to determine which concentration of 5-aza would induce the highest telomere length elongation without adversely affecting cell viability. From this experiment (supplementary figure Fig. 3) we found that the low dose of 10 µM was optimal. Breast cancer cell lines were then treated with 10 µM of 5-aza for 72 h, and telomere length was measured using qPCR. Fig. 1 revealed that treatment with 5-Aza increased telomere length in 21NT, BT474, MCF7, and HS578T respectively. The combined treatment with 5-aza and TSA resulted in an insignificant increase in telomere length compared to 5-aza alone. Therefore, the addition of TSA did not enhance the effect, thereby suggesting that 5-aza was responsible for the observations obtained. This is in line with the results we previously obtained [39]. Cells treated with DMSO showed no significant increase in telomere length and were similar to the untreated cells.

3.2. 5-aza-2′-deoxycytidine increased telomere length in 3D cultures using Growdex

Although we observed a telomere length increase in 2D cultures, this does not fully represent the way cancer cells grow *in vivo* [19] Therefore, we repeated our treatments with 5-aza using a 3D cell culture system called Growdex. BT474 cells were cultured in Growdex for five days and then treated with 10 µM of 5-aza twice with a 4-hour gap between treatments. The 10 µM treatments resulted in a high degree of cell death therefore we reduced the concentration of 5-aza to 5 µM. BT474 cells grew well at the lower concentration producing acini structures in the Growdex (Fig. 2A and B). After 72 h of treatment DNA was extracted from the acini and telomere length was measured using qPCR. Here, we demonstrated for the first time the effects of 5-azacitidine on telomere length in 3D cultures (Fig. 2C). As shown in Figs. 2C, 5 uM concentration of 5-aza increased telomere length in BT474 cells grown in 3D cell cultures. The telomere length of BT474 increased 3-fold in 3D cultures, compared to two-fold in 2D cultures. Due to the BT474 cell lines being grown in a 3D culture, spheroids were formed, which were stimulated from the outer zone that is associated with highly proliferating cells. Moreover, the drug sensitivity in 3D could potentially be impacted by the shape or size of spheroids [16,40]a.

3.3. 5-aza-2′-deoxycytidine up-regulated hTERT expression HER2 positive cells

The two mechanisms that are responsible for telomere length maintenance in humans, telomerase expression and ALT activity were further investigated to see if they are responsible for the rapid telomere elongation observed in our study. Cell lines were incubated with 5-aza
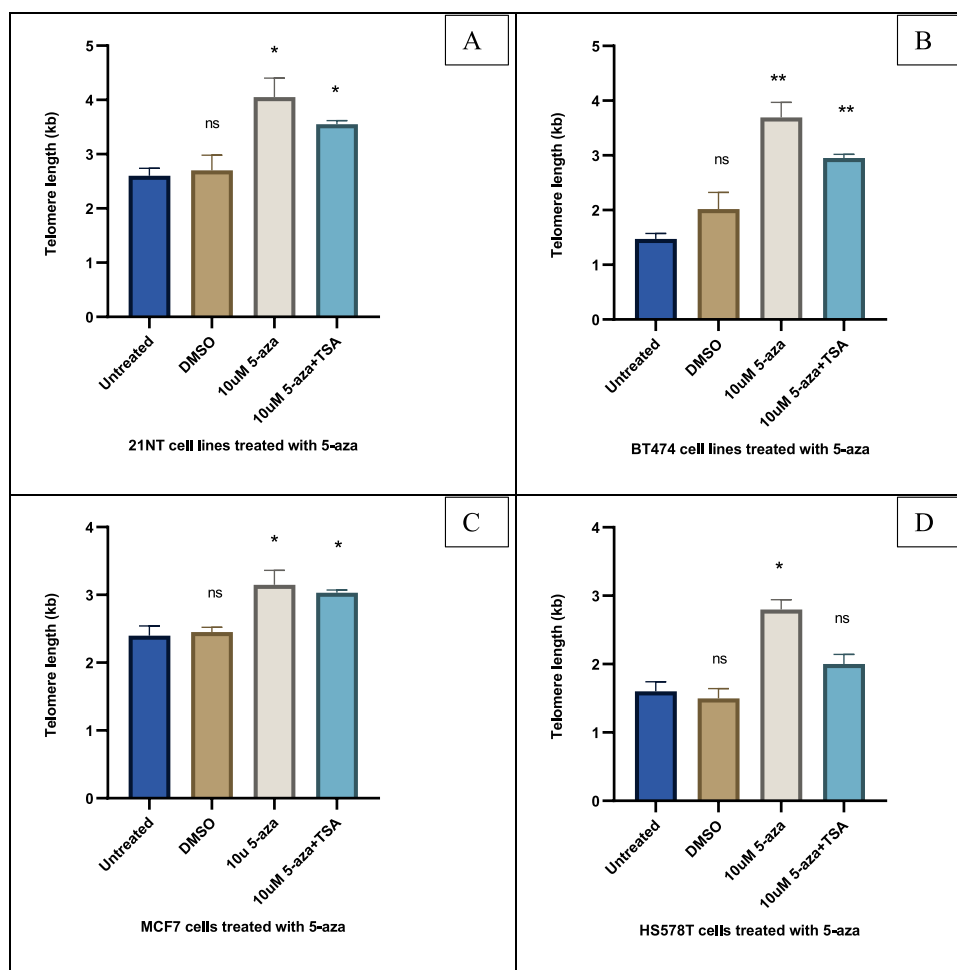
Fig. 1. 5-aza induced telomere length elongation in breast cancer cell lines after exposure for 72 h. DMSO and untreated cells were used as control. A) 21NT treated with 10 μM of 5-aza either alone or in combination with 50 ng/ml of TSA. B) BT474 cell lines treated as above. C) MCF7 cell lines treated as above. D) HS578T cell lines treated as above. All cell lines showed an increase in telomere length when treated with 10 μM of 5-aza. Statistical analysis between untreated and treated cells evaluated by unpaired student's test *P<0.05, **P<0.01, ***P<0.001.

and TSA for 72 h then total RNA was extracted. *hTERT* mRNA expression was then quantified using qPCR. The mean Ct value of each triplet was used for further analysis using the $2^{-\Delta\Delta Ct}$ method to obtain the Relative Quantification (RQ) values. The amplification was then repeated 3 times. As shown in Fig. 3, *hTERT* mRNA expression was significantly upregulated by the treatments in the HER2-positive breast cancer cells 21NT and BT474 [22]. However, in MCF7 and HS578T, the changes in gene expression were not significant, suggesting that HER2 may influence the regulation of *hTERT* in 5-aza treated cells.

3.4. Marginal increase in telomerase activity after a 72-h exposure to 5-aza-2′-deoxycytidine

5-aza and TSA increased *hTERT* expression in the HER2-positive cell lines therefore, we wanted to see if this would increase telomerase activity. To investigate this, 21NT and BT474 cells were exposed to 5-aza for 72 h then a TRAP assay was performed to quantify telomerase activity (Fig. 3E and F). We used protein extracts from the prostate cancer cell line PC-3 as telomerase positive control. Breast cancer cells treated with 5-aza alone or in combination with TSA showed a marginal increase in telomerase activity.

3.5. DNA damage induced by 5-aza-2′-deoxycytidine in breast cancer cell lines

γ-H2AX was used to investigate DNA damage in 5-aza treated cells. Double strand breaks (DSB) can be detected using γ-H2AX and the quantity of damage is proportional to the number of DSB observed. Treatment of breast cancer cell lines with 10 μM of 5-aza either alone or with TSA for 72 h increased γ-H2AX staining over that seen in the DMSO control (Fig. 4 A-D and supplementary Fig. 1). The 21NT, BT474, and MCF7 cell lines revealed a 3-fold increase in γ-H2AX at 10 μM of 5-aza (15/cell) in comparison to the DMSO control (Fig. 4A, B, and C). The combination treatment of 10 μM 5-aza-and TSA presents the highest frequency of γ-H2AX in 21NT (17.8/cell) and BT474 (17.5/cell). The triple negative cell line HS578T, exhibited less γ-H2AX foci after treatment when compared to the other breast cancer cell lines (9.8/cell) suggesting that it is more resistant to DNA damage by 5-aza (Fig. 4 D). These results indicated that 5-aza induces significant DNA damage at low doses, furthermore, triple negative breast cancers are more resistant to DNA damage by this drug.

3.6. 5-aza-2′-deoxycytidine induces DNA damage at the telomeres

Although it is well known that 5-aza induces DNA damage at therapeutic concentrations [33], there have not been any reports suggesting that this drug causes DNA damage at the telomeres. To confirm the
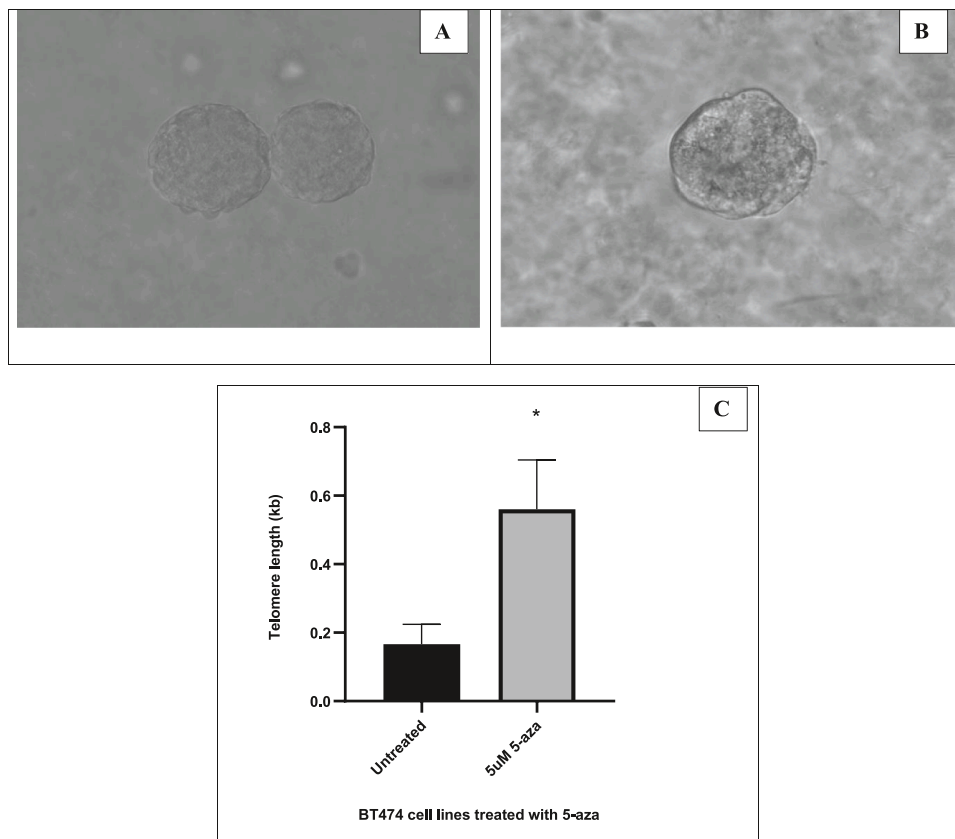
Fig. 2. Telomere length of BT474 cells grown in 3D culture and treated with 5 μM of 5-aza for 72 h. A and B. The typical 3D cellular morphology of BT474 under bright field microscopy. Cells grew as packed spherical aggregates. A represents untreated cells and B. treated with 5 μM 5-aza for 72 h. C. 5 μM concentration of 5-aza was associated with an increase in telomere length in comparison to the untreated control. Statistical analysis between untreated and treated cells evaluated by unpaired student's test *$P<0.05$.

presence of telomeric DNA damage post treatment with 5-aza, the telomere dysfunction induced foci (TIF) assay was performed using an antibody against the DNA damage marker γ-H2AX with a PNA telomere probe [2]. From the results obtained in Fig. 4 E and F (supplementary Fig. 2), a 3-fold increase in TIF was seen for 21NT and H5S78T following treatment with 5-aza, either alone or in combination with TSA. This indicates that 5-aza induces telomeric DNA damage which could lead to telomere dysfunction in breast cancer cell lines. One of the possible consequences of double stranded telomeric DNA damage is the activation of repair mechanisms. Homologous recombination-mediated telomere synthesis, similar to ALT is initiated to repair the damaged DNA occurring at telomeres. This may then lead to a telomere length increase [7,37]. To test for the activation of a homologous repair mechanism, we performed an ALT assay on cell lines treated with 5-aza for 72 h.

3.7. 5-aza-2′-deoxycytidine elevated ALT levels

Fig. 5A-C shows the results of an ALT assay after exposure of the cell lines to 5-aza for 72 h. U2OS was used as an ALT positive control and HELA, an ALT negative, telomerase positive control. From the results obtained, it can be seen that 5-aza induced an increase in ALT activity in all cell lines suggesting that the DNA damage obtained at the telomeres may have activated genes involved in the ALT process. It is noteworthy to mention that the telomerase positive HELA showed some ALT activity along with the telomerase positive breast cancer cell lines. Frank et al. [20] detected low levels of ALT within HELA and other telomerase positive cells, this is in line with our data and shows that telomerase positive cancers could also show the presence of ALT.

3.8. Upregulation of POLD3 following exposure to 5-aza-2′-deoxycytidine

The *POLD3* gene plays an essential role in the maintenance of genome stability and telomere length [8]. *POLD3* has also been shown to be involved in alternative lengthening of telomeres (ALT) through a mechanism of break induced telomere synthesis [12]. Based on the role of *POLD3* in maintaining genome stability and telomere length, we hypothesized that this gene may be involved in telomere length elongation via ALT activation in breast cancer cells treated with 5-aza. We quantified the mRNA expression of *POLD3* in the different subtypes of breast cancer after treatment with 5-aza either alone or in combination with TSA. As shown in Fig. 5 D-F, breast cancer cells exposed to 5-aza dramatically up-regulated *POLD3* mRNA expression when compared to untreated cells. It is interesting to note that when 5-aza is used in combination with TSA, *POLD3* expression is downregulated in 21NT and BT474.

4. Discussion

Previously [39], we found that the chemotherapeutic drug 5-aza-2′-deoxycytidine (5-aza) induced rapid telomere elongation in breast cancer cell lines after a 72 h exposure. In this paper, we aim to uncover the possible mechanisms that lead to telomere length increase in cells treated with 5-aza.

Cell lines representing different molecular sub-types of breast cancer (Luminal A/B, HER2 positive, and triple negative), grown in 2D cell cultures increased their telomere lengths (Fig. 1A-D) after 72 h of treatment. This is in line with what has been shown by others (Motevalli et al., 1014, [4]) and serves as a starting point for the following investigation. As 2D cultures are not a true representation of how cells would
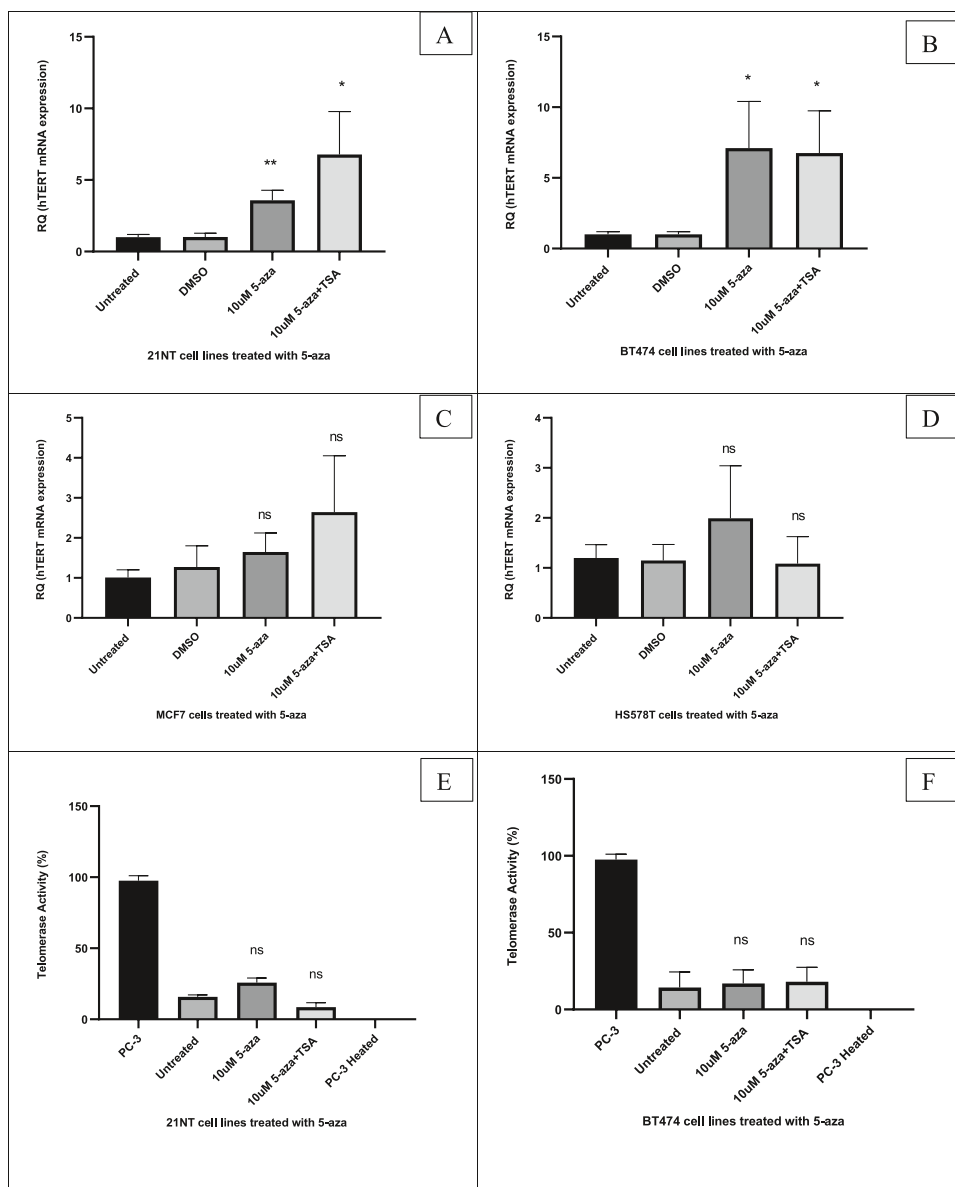
Fig. 3. *hTERT* mRNA expression (RQ) and telomerase activity in breast cancer cell lines after treatment with 5-aza for 72 h, untreated cells were used as controls. *hTERT* mRNA expression for 21NT (A), BT474 (B), MCF7 (C) and HS578T (D). E and F. telomerase activity in the HER2 positive cell lines 21NT and BT474 after exposure to 5-aza. Only a marginal increase in telomerase activity was seen in cells treated with 5-aza when compared to the untreated control. Statistical analysis between untreated and treated cells evaluated by unpaired student's test *P<0.05, **P<0.01.

grow in a human body, we repeated our experiments in a 3D culture system using Growdex. Some cell lines did not grow well in this 3D system (21NT, MCF7, data not shown) however we did get good growth with BT474 which allowed us to carry out our analysis. The results (Fig. 2A-C) indicate that even in a 3D cell culture system, rapid telomere length increase occurs after treatment with 5-aza for 72 h. Interestingly, BT474 showed a 2-fold increase in telomere length for the 2D cultures and a 4-fold increase in 3D cultures. The cell line also required a lower dose of 5 μM in 3D cultures as opposed to 10 μM in 2D cultures. This could be a result of the drug having access to the whole of the cell's surface area and not just one surface as you would get in 2D cultures. The results presented in Fig. 2 validate our findings that cells grown in any situation could induce telomere length increase when exposed to 5-aza. Others have found that cancer cell lines grown in 3D cultures tend to be more resistant to chemotherapeutic drugs depending on the type of system used [19,40]. Our findings suggest that Growdex 3D cultures are more sensitive to added drugs. The two known mechanisms that can use

to maintain telomeres are telomerase activity and ALT [50], therefore we investigated both pathways to see if they can explain what we have observed in our cell lines treated with 5-aza.

Although *hTERT* mRNA levels increased after exposure to 5-aza in the HER2 positive cell lines 21NT and BT474 (Fig. 3), the HER2 negative cells showed non-significant changes. In addition, only a marginal increase in telomerase activity occurred in the HER2 positive cells (Fig. 3E-F) suggesting that this may not be the main mechanism responsible for the rapid telomere increase obtained in Fig. 1 but could contribute to it. 22 splice variants of *hTERT* are known to exist [24,47], however only a few variants code for the fully active telomerase enzyme. Therefore, even though we obtained significant increases in the *hTERT* mRNA expression for 21NT and BT474, lower levels of the fully functional variant may have been produced hence no correlation between mRNA expression and telomerase enzyme activity. Papanikolaou et al. [44] Found a direct positive correlation between hTERT expression (and telomerase activity) and HER2, and our results indicate that HER2
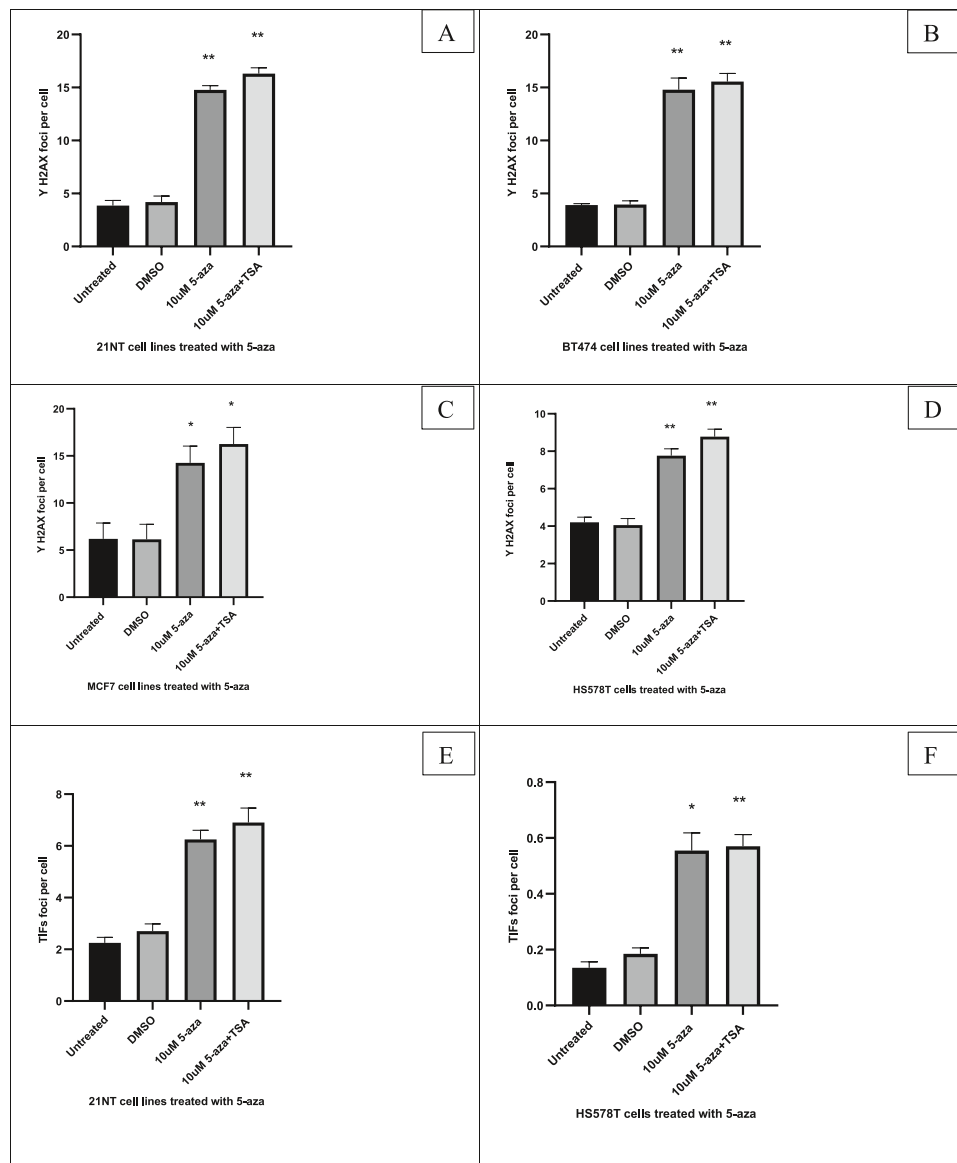
Fig. 4. Frequencies of DNA DSBs and TIF in breast cancer cell lines following treatment with 5-aza or a combination of 5-aza and TSA. 21NT (A), BT474 (B) and MCF7 (C) showed the highest frequencies of γ-H2AX foci. HS78T (C) showed less sensitivity to DSB compared to 21NT, BT474 and MCF7 cell lines. TIF analysis for 21NT (E) and BT474 (F). Untreated and DMSO was used as the controls for the 5-aza treated cells. Statistical analysis between untreated and treated cells evaluated by unpaired student's test *P<0.05, **P<0.01, ***P<0.001.

positive cells enhance their expression of *hTERT* after exposure to 5-aza.

We next looked at the effect 5-aza has on telomeric DNA. Previously it was shown that 5-aza triggers DNA double strand breaks in cells treated with therapeutic concentrations of the drug and this is part of its anti-cancer effects [30] (Laranjeira et al., 2023). However, to our knowledge, 5-aza induced DNA damage at the telomeres has not been reported for solid tumours. We show that 5-aza caused significant DNA damage in all cell lines, on average 2-to-3-fold increase in γ-H2AX foci was obtained (Fig. 4A-D). More importantly, damage was also seen at the telomeres using the TIF assay (Fig. 4E-F). Double stranded telomeric DNA damage may induce DSBR (double strand break repair) and ALT like activity to repair and elongate telomeric DNA [18]. Indeed, telomere damage can induce ALT activity in telomerase positive cells by triggering homologous recombination mediated telomere synthesis [7, 37].

We next investigated ALT activity in cells treated with 5-aza (Fig. 5A-C). ALT activity was significantly increased in the 21NT and BT474 cell lines when compared to the controls HELA (*hTERT* positive) and U2OS

(ALT positive). This provides evidence that the damage seen at the telomeres in Fig. 4 may have been repaired by an HR driven mechanism leading to a telomere length increase [7,14]. We found that the *hTERT* positive cell line HELA had detectable Levels of ALT. This is not unusual as the co-existence of telomerase and ALT in cancer cell lines has been reported in the literature [10,25,46]. From this, we can conclude that 5-aza induced DNA damage at the telomeres activated DSBR and ALT like activity which could have been responsible for the increase in telomere length we observed in our breast cancer cell lines.

Recently, the gene *POLD3* was shown to be involved in DSB repair, telomere maintenance, and ALT activity [54,55]. *POLD3* is also considered to be critical for telomere maintenance via ALT [12] and depletion of *POLD3* within cells results in accelerated telomere shortening. This led us to investigate *POLD3*'s expression in 5-aza treated cells. Our data (Fig. 5D-F) demonstrated that 5-aza upregulated *POLD3* mRNA expression in 21NT, BT474, and HS578T. This result provides strong evidence that the *POLD3* gene is involved in the activation of the ALT pathway in cells treated with 5-aza. This will be the focus of our
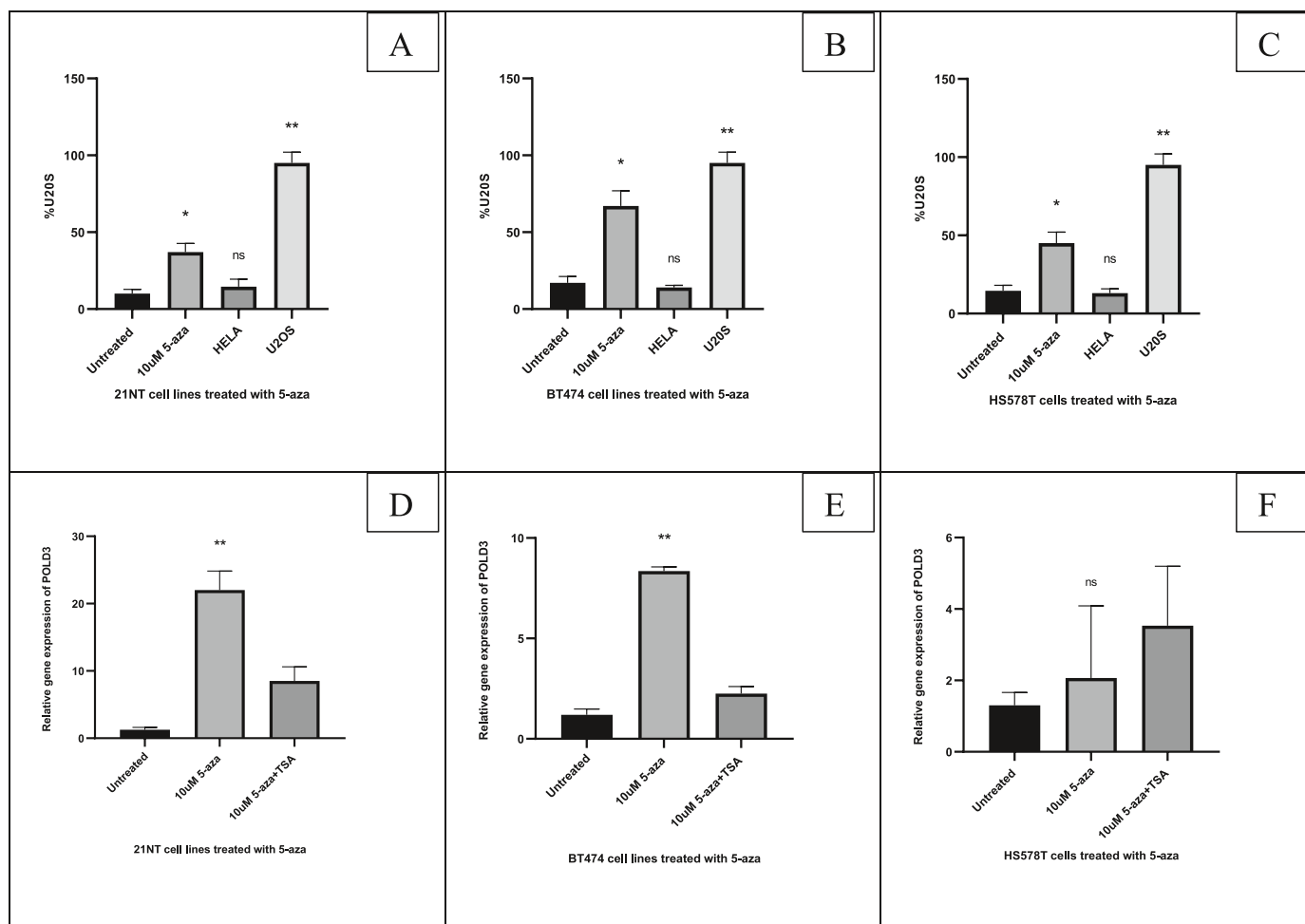
Fig. 5. ALT activity and *POLD3* mRNA expression in breast cancer cell lines after treatment with 5-aza for 72 h, untreated cells were used as controls. A, B and C. ALT activity in the cell lines 21NT, BT474 and MCF7 after exposure to 5-aza. Up-regulation of *POLD3* mRNA expression (RQ) in 21NT (D), BT474 (E) and H5S78T (F) after a 72 h exposure to 5-aza. D, E and F, Statistical analysis between untreated and treated cells evaluated by unpaired student's test *P<0.05, **P<0.01, ***P<0.001.

investigation for future work.

5. Conclusion

We have provided evidence showing that treatment of breast cancer cell lines with the chemotherapeutic drug 5-aza induces telomere length elongation via ALT activation. Furthermore, the gene *POLD3* was found to be up-regulated in cells post treatment with 5-aza suggesting that it could be the driving force behind the ALT activity observed. This is a significant finding as 5-azacitidine is still being used to treat AML and MDS alone or in combination with another chemotherapeutics [13,29]. However, the treatment is associated with a high cytotoxic effect [11, 41]. In addition, preclinical studies in solid tumours are still being performed [34,48]. Elongated telomeres and up-regulation of *hTERT* may lead to the evolution of the tumour into more aggressive forms and resistance to the drugs used to initially treat them. Kuranaga et al., [32] Found that elongated telomeres and telomerase upregulation contributed to multidrug resistance in colorectal cancer cells. Radiation resistance in these cells was associated with *hTERT* upregulation [51]. Ko, Jung [31] found that hepatocellular carcinoma cells with longer telomeres had a greater invasive capacity than those with shorter telomeres. The evidence we provide here suggests that any tumour cells that survive the initial round of treatment with 5-aza could start to clonally grow with the activation of the ALT system within them. Our data also suggests that both telomerase activity and ALT are present in these cells which will give them a robust system to keep telomeres maintained and active. This could result in cells that are highly resistant to further therapeutic intervention.

CRediT authorship contribution statement

Sarah Al-dulaimi: Writing – original draft, Methodology, Investigation, Data curation. **Sheila Matta:** Writing – review & editing, Methodology, Investigation. **Predrag Slijepcevic:** Writing – review & editing, Visualization, Validation, Methodology. **Terry Roberts:** Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117173.

References

[1] N. Ahuja, A.R. Sharma, S.B. Baylin, Epigenetic therapeutics: a new weapon in the war against cancer, Annu. Rev. Med. *67* (1) (2016) 73–89, https://doi.org/10.1146/annurev-med-111314-035900.

[2] S.K. Bains, K. Chapman, S. Bright, A. Senan, M. Kadhim, P. Slijepcevic, Effects of ionizing radiation on telomere length and telomerase activity in cultured human lens epithelium cells, Int. J. Radiat. Biol. *95* (1) (2019) 54–63, https://doi.org/10.1080/09553002.2018.1466066.

[3] T.M. Bryan, A. Englezou, L. Dalla-Pozza, M.A. Dunham, R.R. Reddel, Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines, Nat. Med. *3* (11) (1997) 1271–1274, https://doi.org/10.1038/nm1197-1271.

[4] C. Bull, G. Mayrhofer, M. Fenech, Exposure to hypomethylating 5-aza-2′-deoxycytidine (decitabine) causes rapid, severe DNA damage, telomere elongation and mitotic dysfunction in human WIL2-NS cells, Mutat. Res. Genet. Toxicol. Environ. Mutagen. *868-869* (2021) 503385, https://doi.org/10.1016/j.mrgentox.2021.503385.

[5] F.I. Bussière, V. Michel, J. Fernandes, L. Costa, V. Camilo, G. Nigro, H. De Reuse, L. Fiette, E. Touati, DNA hypermethylation downregulates telomerase reverse transcriptase (TERT) during H. pylori-induced chronic inflammation, 5415761-13, J. Oncol. *2019* (2019), https://doi.org/10.1155/2019/5415761.

[6] D. Cheng, F. Zhang, K.I. Porter, S. Wang, H. Zhang, C.J. Davis, G.P. Robertson, J. Zhu, Humanization of the mouse Tert gene reset telomeres to human length, Res. Sq. (2024). ⟨https://www.ncbi.nlm.nih.gov/pubmed/38260456⟩.

[7] N. Cho, R. Dilley, M. Lampson, R. Greenberg, Interchromosomal homology searches drive directional ALT telomere movement and synapsis, Cell *159* (1) (2014) 108–121, https://doi.org/10.1016/j.cell.2014.08.030.

[8] L. Costantino, S.K. Sotiriou, J.K. Rantala, S. Magin, E. Mladenov, T. Helleday, J.E. Haber, G. Iliakis, O.P. Kallioniemi, T.D. Halazonetis, Break-induced replication repair of damaged forks induces genomic duplications in human cells, Science *343* (6166) (2014) 88–91, https://doi.org/10.1126/science.1243211.

[9] A.P. Cuthbert, J. Bond, D.A. Trott, S. Gill, J. Broni, A. Marriott, G. Khoudoli, E.K. Parkinson, C.S. Cooper, R.F. Newbold, Telomerase repressor sequences on chromosome 3 and induction of permanent growth arrest in human breast Cancer cells, JNCI J. Natl. Cancer Inst. *91* (1) (1999) 37–45, https://doi.org/10.1093/jnci/91.1.37.

[10] M. De Vitis, F. Berardinelli, A. Sgura, Telomere length maintenance in cancer: at the crossroad between telomerase and alternative lengthening of telomeres (ALT), Int. J. Mol. Sci. *19* (2) (2018) 606, https://doi.org/10.3390/ijms19020606.

[11] J. Diesch, A. Zwick, A. Garz, A. Palau, M. Buschbeck, K.S. Götze, A clinical-molecular update on azanucleoside-based therapy for the treatment of hematologic cancers, Clin. Epigenetics *8* (1) (2016) 71, https://doi.org/10.1186/s13148-016-0237-y.

[12] R.L. Dilley, P. Verma, N.W. Cho, H.D. Winters, A.R. Wondisford, R.A. Greenberg, Break-induced telomere synthesis underlies alternative telomere maintenance, Nat. *539* (7627) (2016) 54–58, https://doi.org/10.1038/nature20099.

[13] C.D. DiNardo, K. Pratz, V. Pullarkat, B.A. Jonas, M. Arellano, P.S. Becker, O. Frankfurt, M. Konopleva, A.H. Wei, H.M. Kantarjian, T. Xu, W. Hong, B. Chyla, J. Potluri, D.A. Pollyea, A. Letai, Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia, Blood *133* (1) (2019) 7–17, https://doi.org/10.1182/blood-2018-08-868752.

[14] Y. Doksani, T. de Lange, Telomere-internal double-strand breaks are repaired by homologous recombination and PARP1/Lig3-dependent end-joining, Cell Rep. *17* (6) (2016) 1646–1656, https://doi.org/10.1016/j.celrep.2016.10.008.

[15] A.M. Dworkin, T.H.- Huang, A.E. Toland, Epigenetic alterations in the breast: implications for breast cancer detection, prognosis and treatment, Semin. Cancer Biol. *19* (3) (2009) 165–171, https://doi.org/10.1016/j.semcancer.2009.02.007.

[16] R. Edmondson, J.J. Broglie, A.F. Adcock, L. Yang, Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors, Assay. Drug Dev. Technol. *12* (4) (2014) 207–218, https://doi.org/10.1089/adt.2014.573.

[17] P. Fajkus, V. Peška, M. Závodník, M. Fojtová, J. Fulnečková, Š. Dobias, A. Kilar, M. Dvořáčková, D. Zachová, I. Nečasová, J. Sims, E. Sýkorová, J. Fajkus, Telomerase RNAs in land plants, Nucleic Acids Res. *47* (18) (2019) 9842–9856, https://doi.org/10.1093/nar/gkz695.

[18] C.L. Fasching, A.A. Neumann, A. Muntoni, T.R. Yeager, R.R. Reddel, DNA damage induces alternative lengthening of telomeres (ALT) associated promyelocytic leukemia bodies that preferentially associate with linear telomeric DNA, Cancer Res. *67* (15) (2007) 7072–7077, https://doi.org/10.1158/0008-5472.CAN-07-1556.

[19] J.C. Fontoura, C. Viezzer, F.G. dos Santos, R.A. Ligabue, R. Weinlich, R.D. Puga, D. Antonow, P. Severino, C. Bonorino, Comparison of 2D and 3D cell culture models for cell growth, gene expression and drug resistance, Mater. Sci. Eng. C *107* (2020) 110264, https://doi.org/10.1016/j.msec.2019.110264.

[20] L. Frank, A. Rademacher, N. Mücke, S.M. Tirier, E. Koeleman, C. Knotz, S. Schumacher, S.A. Stainczyk, F. Westermann, S. Fröhling, K. Rippe, ALT-FISH quantifies alternative lengthening of telomeres activity by imaging of single-stranded repeats, Nucleic Acids Res. *50* (11) (2022) e61, https://doi.org/10.1093/nar/gkac113.

[21] J.D. Henson, L.M. Lau, S. Koch, N. Martin La Rotta, R.A. Dagg, R.R. Reddel, The C-Circle Assay for alternative-lengthening-of-telomeres activity, Methods *114* (2017) 74–84, https://doi.org/10.1016/j.ymeth.2016.08.016.

[22] D.L. Holliday, V. Speirs, Choosing the right cell line for breast cancer research, Breast Cancer Res. BCR *13* (4) (2011) 215, https://doi.org/10.1186/bcr2889.

[23] M. Hodjat, P.B. Jourshari, F. Amirinia, N. Asadi, 5-Azacitidine and Trichostatin A induce DNA damage and apoptotic responses in tongue squamous cell carcinoma: an in vitro study, Arch. Oral. Biol. *133* (2022) 105296, https://doi.org/10.1016/j.archoralbio.2021.105296.

[24] R. Hrdličková, J. Nehyba, H.R. Bose, Alternatively spliced telomerase reverse transcriptase variants lacking telomerase activity stimulate cell proliferation, Mol. Cell. Biol. *32* (21) (2012) 4283–4296, https://doi.org/10.1128/MCB.00550-12.

[25] Y. Hu, G. Shi, L. Zhang, F. Li, Y. Jiang, S. Jiang, W. Ma, Y. Zhao, Z. Songyang, J. Huang, Switch telomerase to ALT mechanism by inducing telomeric DNA damages and dysfunction of ATRX and DAXX, 32280-32280, Sci. Rep. *6* (1) (2016), https://doi.org/10.1038/srep32280.

[26] E. Kaminskas, A. Farrell, Y. Wang, R. Pazdur, S. Abraham, A. Baird, L. Hsieh, S. Lee, J.K. Leighton, H. Patel, A. Rahman, R. Sridhara, Approval summary: azacitidine for treatment of myelodysplastic syndrome subtypes, Clin. Cancer Res. *11* (10) (2005) 3604–3608, https://doi.org/10.1158/1078-0432.CCR-04-2135.

[27] H. Kantarjian, Y. Oki, G. Garcia-Manero, X. Huang, S. O'Brien, J. Cortes, S. Faderl, C. Bueso-Ramos, F. Ravandi, Z. Estrov, A. Ferrajoli, W. Wierda, J. Shan, J. Davis, F. Giles, H.I. Saba, J.J. Issa, Results of a randomized study of 3 schedules of low-dose decitabine in higher-risk myelodysplastic syndrome and chronic myelomonocytic leukemia, Blood *109* (1) (2007) 52–57, https://doi.org/10.1182/blood-2006-05-021162.

[28] A. Kar, S. Willcox, J.D. Griffith, Transcription of telomeric DNA leads to high levels of homologous recombination and t-loops, Nucleic Acids Res. *44* (19) (2016) 9369–9380, https://doi.org/10.1093/nar/gkw779.

[29] C. Khan, N. Pathe, S. Fazal, J. Lister, J.M. Rossetti, Azacitidine in the management of patients with myelodysplastic syndromes, SAGE Publ. (2012), https://doi.org/10.1177/2040620712464882.

[30] T. Kiziltepe, T. Hideshima, L. Catley, N. Raje, H. Yasui, N. Shiraishi, Y. Okawa, H. Ikeda, S. Vallet, S. Pozzi, K. Ishitsuka, E.M. Ocio, D. Chauhan, K.C. Anderson, 5-Azacytidine, a DNA methyltransferase inhibitor, induces ATR-mediated DNA double-strand break responses, apoptosis, and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells, Mol. Cancer Ther. *6* (6) (2007) 1718–1727, https://doi.org/10.1158/1535-7163.MCT-07-0010.

[31] E. Ko, G. Jung, Positive association of long telomeres with the invasive capacity of hepatocellular carcinoma cells, Biochem. Biophys. Res. Commun. *447* (2) (2014) 358–363, https://doi.org/10.1016/j.bbrc.2014.04.022.

[32] N. Kuranaga, N. Shinomiya, H. Mochizuki, Long-term cultivation of colorectal carcinoma cells with anti-cancer drugs induces drug resistance and telomere elongation: an in vitro study, BMC Cancer *1* (1) (2001) 10, https://doi.org/10.1186/1471-2407-1-10.

[33] A.B.A. Laranjeira, M.G. Hollingshead, D. Nguyen, R.J. Kinders, J.H. Doroshow, S.X. Yang, DNA damage, demethylation and anticancer activity of DNA methyltransferase (DNMT) inhibitors, Sci. Rep. *13* (1) (2023) 5964, https://doi.org/10.1038/s41598-023-32509-4.

[34] J.F. Linnekamp, R. Butter, R. Spijker, J.P. Medema, H.W.M. van Laarhoven, Clinical and biological effects of demethylating agents on solid tumours – A systematic review, Cancer Treat. Rev. *54* (2017) 10–23, https://doi.org/10.1016/j.ctrv.2017.01.004.

[35] Y. Liu, B.E. Snow, M.P. Hande, D. Yeung, N.J. Erdmann, A. Wakeham, A. Itie, D.P. Siderovski, P.M. Lansdorp, M.O. Robinson, L. Harrington, The telomerase reverse transcriptase is limiting and necessary for telomerase function in vivo, Curr. Biol. *10* (22) (2000) 1459–1462, https://doi.org/10.1016/S0960-9822(00)00805-8.

[36] S. Łukasiewicz, M. Czeczelewski, A. Forma, J. Baj, R. Sitarz, A. Stanisławek, Breast cancer—epidemiology, risk factors, classification, prognostic markers, and current treatment strategies—an updated review, Cancers *13* (17) (2021) 4287, https://doi.org/10.3390/cancers13174287.

[37] P. Mao, J. Liu, Z. Zhang, H. Zhang, H. Liu, S. Gao, Y.S. Rong, Y. Zhao, Homologous recombination-dependent repair of telomeric DSBs in proliferating human cells, 12154-12154, Nat. Commun. *7* (1) (2016), https://doi.org/10.1038/ncomms12154.

[38] J. Mergny, J. Riou, P. Mailliet, M. Teulade-Fichou, E. Gilson, Natural and pharmacological regulation of telomerase, Nucleic Acids Res. *30* (4) (2002) 839–865, https://doi.org/10.1093/nar/30.4.839.

[39] A. Motevalli, H. Yasaei, S.A. Virmouni, P. Slijepcevic, T. Roberts, The effect of chemotherapeutic agents on telomere length maintenance in breast cancer cell lines, Breast Cancer Res. Treat. *145* (3) (2014) 581–591, https://doi.org/10.1007/s10549-014-2975-x.

[40] M. Muguruma, S. Teraoka, K. Miyahara, A. Ueda, M. Asaoka, M. Okazaki, T. Kawate, M. Kuroda, Y. Miyagi, T. Ishikawa, Differences in drug sensitivity between two-dimensional and three-dimensional culture systems in triple-negative

breast cancer cell lines, Biochem. Biophys. Res. Commun. *533* (3) (2020) 268–274, https://doi.org/10.1016/j.bbrc.2020.08.075.

[41] C. Nervi, E. De Marinis, G. Codacci-Pisanelli, Epigenetic treatment of solid tumours: a review of clinical trials, Clin. Epigenetics *7* (1) (2015) 127, https://doi.org/10.1186/s13148-015-0157-2.

[42] N.J. O'Callaghan, M. Fenech, A quantitative PCR method for measuring absolute telomere length, Biol. Proced. Online 13(1) 3 10 (2011) 1186/1480–9222-13-3.

[43] S.S. Palii, B.O. Van Emburgh, U.T. Sankpal, K.D. Brown, K.D. Robertson, DNA methylation inhibitor 5-Aza-2′-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B, Mol. Cell. Biol. *28* (2) (2008) 752–771, https://doi.org/10.1128/MCB.01799-07.

[44] V. Papanikolaou, D. Illopoulos, I. Dimou, S. Dubos, I. Tsougos, K. Theodorou, S. Kitsiou-Tzeli, A. Tsezou, The involvement of HER2 and p53 status in the regulation of telomerase in irradiated breast cancer cells, Int. J. Oncol. *35* (5) (2009) 1141–1149, https://doi.org/10.3892/ijo_00000430.

[45] M. Pedram, C.N. Sprung, Q. Gao, A.W.I Lo, G.E. Reynolds, J.P. Murnane, Telomere position effect and silencing of transgenes near telomeres in the mouse, Mol. Cell. Biol. *26* (5) (2006) 1865–1878, https://doi.org/10.1128/MCB.26.5.1865-1878.2006.

[46] K. Perrem, L.M. Colgin, A.A. Neumann, T.R. Yeager, R.R. Reddel, Coexistence of alternative lengthening of telomeres and telomerase in hTERT-transfected GM847 cells, Mol. Cell. Biol. *21* (12) (2001) 3862–3875, https://doi.org/10.1128/MCB.21.12.3862-3875.2001.

[47] A.A. Plyasova, D.D. Zhdanov, Alternative splicing of human telomerase reverse transcriptase (hTERT) and its implications in physiological and pathological

processes, Biomedicines *9* (5) (2021) 526, https://doi.org/10.3390/biomedicines9050526.

[48] Y. Qiu, B.L. Mirkin, R.S. Dwivedi, Inhibition of DNA methyltransferase reverses cisplatin induced drug resistance in murine neuroblastoma cells, Cancer Detect. Prev. *29* (5) (2005) 456–463, https://doi.org/10.1016/j.cdp.2005.05.004.

[49] J.W. Shay, Telomeres and aging, Curr. Opin. Cell Biol. *52* (2018) 1–7, https://doi.org/10.1016/j.ceb.2017.12.001.

[50] J.W. Shay, W.E. Wright, Telomeres and telomerase: three decades of progress, Nat. Rev. Genet. *20* (5) (2019) 299–309, https://doi.org/10.1038/s41576-019-0099-1.

[51] J. Shin, T. Foo, A. Hong, M. Zhang, T. Lum, M.J. Solomon, C.S. Lee, Telomerase expression as a predictive marker of radiotherapy response in rectal cancer, Pathology *44* (3) (2012) 209–215, https://doi.org/10.1097/PAT.0b013e3283511cd5.

[52] F. Sorm, A. Pískala, A. Cihák, J. Veselý, 5-Azacytidine, a new, highly effective cancerostatic, Experientia *20* (4) (1964) 202–203, https://doi.org/10.1007/BF02135399.

[53] H. Wege, M.S. Chui, H.T. Le, J.M. Tran, M.A. Zern, SYBR Green real-time telomeric repeat amplification protocol for the rapid quantification of telomerase activity, Nucleic Acids Res. *31* (2) (2003) e3, https://doi.org/10.1093/nar/gng003.

[54] J. Zhang, L. Zou, Alternative lengthening of telomeres: from molecular mechanisms to therapeutic outlooks, Cell Biosci. *10* (1) (2020) 30, https://doi.org/10.1186/s13578-020-00391-6.

[55] Z. Zhou, L. Wang, F. Ge, P. Gong, H. Wang, F. Wang, L. Chen, L. Liu, Pold3 is required for genomic stability and telomere integrity in embryonic stem cells and meiosis, Nucleic Acids Res. *46* (7) (2018) 3468–3486, https://doi.org/10.1093/nar/gky098.