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Processing of anthracycline-DNA adducts via DNA replication and interstrand crosslink repair pathways

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ABSTRACT

Anthracycline chemotherapeutics are well characterised as poisons of topoisomerase II, however many anthracyclines, including doxorubicin, are also capable of forming drug-DNA adducts. Anthracycline-DNA adducts present an unusual obstacle for cells as they are covalently attached to one DNA strand and stabilised by hydrogen bonding to the other strand. We now show that in cycling cells processing of anthracycline adducts through DNA replication appears dominant compared to processing via transcription-coupled pathways, and that the processing of these adducts into DNA breaks is independent of topoisomerase II. It has previously been shown that cells deficient in homologous recombination (HR) are hypersensitive to adduct forming treatments. Given that anthracycline-DNA adducts, whilst not true crosslinks, are associated with both DNA strands, the role of ICL repair pathways was investigated. Mus81 is a structure specific nuclease implicated in Holliday junction resolution and the resolution of branched DNA formed by stalled replication forks. We now show that ICL repair deficient cells ($Mus81^{-/-}$) are hypersensitive to anthracycline-DNA adducts and ET-743, a compound which causes a chemically similar type of DNA damage. Further analysis of this mechanism showed that Mus81 does not appear to cause DNA breaks resulting from either anthracycline- or ET743-DNA adducts. This suggests Mus81 processes these novel forms of DNA damage in a fundamentally different way compared to the processing of classical covalent crosslinks. Improved understanding of the role of DNA repair in response to such adducts may lead to more effective chemotherapy for patients with BRCA1/2 mutations and other HR deficiencies.

1. Introduction

Anthracyclines, such as doxorubicin and daunorubicin, are amongst the most common clinically used anti-cancer agents and are well documented to function via poisoning of topoisomerase II. However, the mechanism of action of doxorubicin can be modified by formaldehyde to form an adduct on the DNA which consists of an aminal (N–C–N) linkage between the N3′ of doxorubicin and the N2 of guanine on one strand of DNA, with formaldehyde contributing the central carbon atom of the link [1–3] (Fig. 1A). The other strand of DNA interacts with doxorubicin via hydrogen bonds that stabilise the complex but as this is not a covalent linkage to both DNA strands, the adduct is not a true interstrand crosslink (ICL). Therefore, the cellular processing of this type of damage may be different to the mechanisms involved for the repair of true ICLs which are lethal at the level of 200–500 ICL per genome [4].

Combination therapy using doxorubicin and formaldehyde-releasing drugs has been shown to have a synergistic effect on the growth inhibition of tumour cells in culture [5], in particular against cells resistant to doxorubicin as a single agent, such as the MCF-7/Dx cell line which expresses P-glycoprotein [6,7]. The activation of doxorubicin by formaldehyde-releasing drugs has been shown to increase its therapeutic potential in mice bearing human breast tumour xenografts (Cutts et al., unpublished results). Furthermore, a carboxylesterase 2-activated prodrug of formaldehyde-modified doxorubicin is in preclinical development, and has shown efficacy against human non-small cell lung cancer and hepatocellular carcinoma xenografts [8]. Adduct formation causes cells to undergo apoptosis independently of topoisomerase II [9]. Barminomycin (Fig. 1B) is a model adduct forming anthracycline that does not require formaldehyde to form adducts [10]. Barminomycin does not appear to exert any cytotoxicity through topoisomerase II poisoning, making it an

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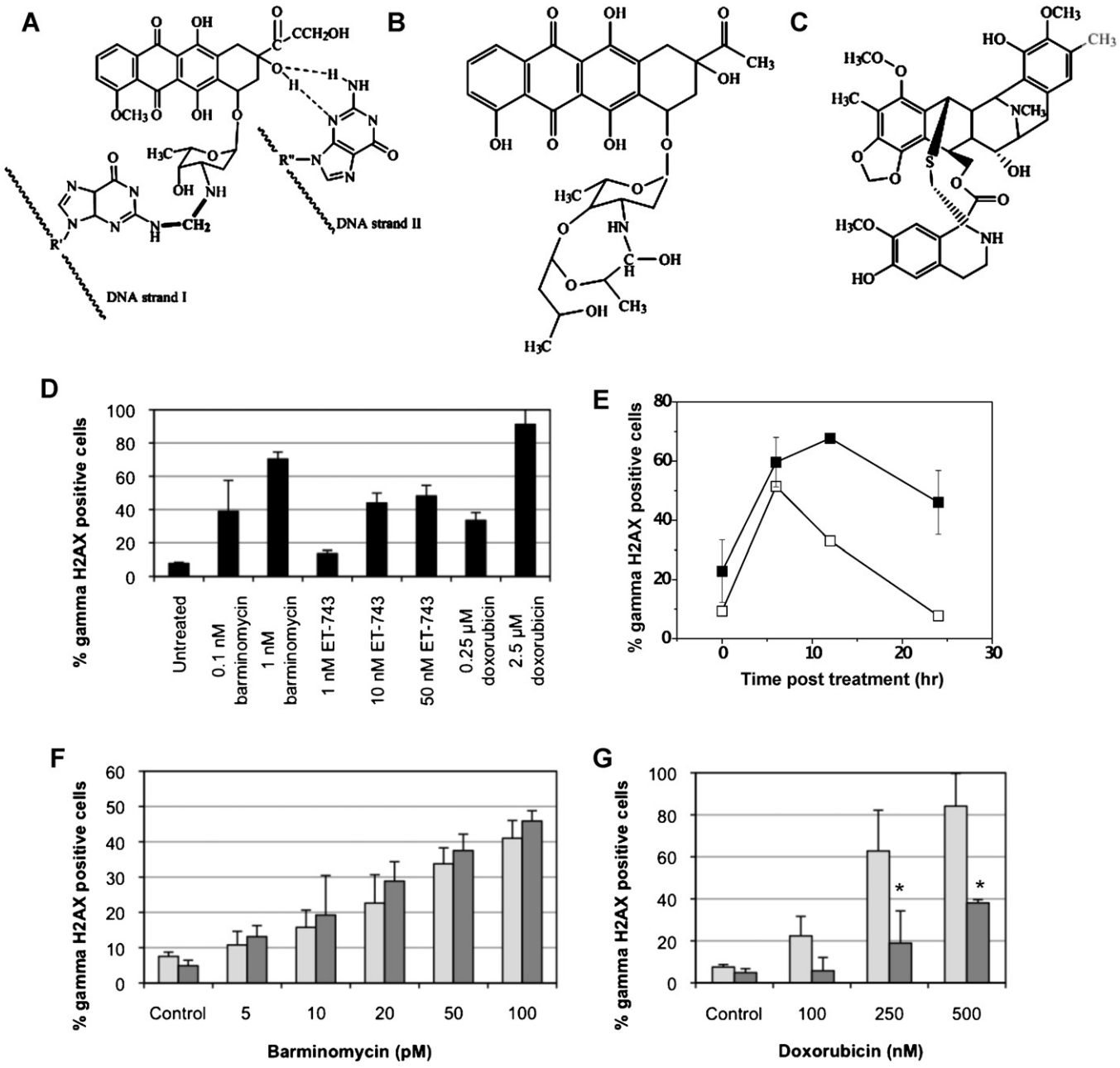

Fig. 1. Barminomycin and ET-743 form topoisomerase II independent DNA breaks. Structures of (A) a doxorubicin-DNA adduct, (B) barminomycin and (C) ET-743. (D) V79 cells were treated with the indicated drugs for 2 h and analysed via flow cytometry for γH2AX levels. (E) V79 cells were treated with 50 pM barminomycin (■) or 1 nM ET-743 (□) for 2 h and allowed to recover in drug-free media for the indicated times. γH2AX was analysed via flow cytometry. Wild type HL60 cells (▒) and topoisomerase II deficient HL60/MX2 cells (■) were treated for 6 h with (F) barminomycin or (G) doxorubicin and analysed via flow cytometry for γH2AX formation. All values represent the average of three independent experiments ± standard deviation. *p value <0.05 when comparing HL-60/MX2 cells with wild type HL-60 cells. There was no significant difference between the cells lines following barminomycin treatment.

ideal drug to understand the cellular responses that are specific to anthracycline-DNA adducts, although barminomycin is considered too toxic for clinical development [11]. Anthracycline-DNA adducts are of interest as they pose more cytotoxic lesions to cells than poisoned topoisomerase II complexes [9]. Therefore, if subjected to appropriate activation conditions, lower doses of anthracyclines could potentially be used to effectively treat various tumours, and this would allow treatment to continue for longer before the maximum lifetime accumulated dose of anthracycline was reached. There is also an accumulating body of evidence that DNA-adduct forming anthracyclines have less potential to induce cardiotoxicity [8,12,13].

Both nucleotide excision repair (NER) and homologous recombination (HR) are involved in the processing of anthracycline adduct damage [14]. This indicates that adducts may be processed in a similar way to ICLs. However, since NER has been shown to be important for inducing apoptosis following anthracycline adduct damage [14], this rules out a traditional ICL repair pathway, as drugs that induce ICLs (such as MMC) are more cytotoxic in NER deficient cells [15] where the repair of the damage is impaired [16]. The effect of ecteinascidin 743 (ET-743; Fig. 1C) on repair deficient cells is similar to that observed for anthracycline adducts. ET-743 is a DNA alkylating drug that binds via an aminal linkage to the N2 of guanine similarly to anthracycline adducts [17]. This damage

causes stalling of RNA polymerase and initiation of repair by the transcription coupled NER (TC-NER) pathway [18]. NER is unable to repair the damage and it is hypothesised that this leads to a trapped NER complex at the damage site, which initiates apoptosis [18]. HR deficient cells are more sensitive to ET-743 than wild type cells [19], suggesting that HR may also play a role in repair of the damage through a cell survival pathway. More recently, an anthracycline derivative known as nemorubicin has also been shown to be less effective in NER deficient cells [20]. This drug is currently in Phase II clinical trials for hepatocellular carcinoma and the structure of its daunosamine-modified activation product PNU-159682 suggests it may function as an activated anthracycline to form covalent DNA adducts.

Previous work has established that HR deficient cells, specifically cells with mutations in BRCA2 and XRCC3, are more sensitive than wild type cells when treated with anthracycline-DNA adduct forming drugs [14]. This is a potentially very important niche for adduct forming therapy as tumours with such mutations may be more sensitive to the treatment than the surrounding normal tissue, giving the treatment a degree of tumour specificity. Therefore, the molecular events involved in cell responses to anthracycline adduct forming treatments in HR proficient and deficient cells needs to be explored further. Specifically, we determined the extent of DNA strand breaks formed in cells following treatment with anthracycline adduct-forming drugs, as well as the comparative levels between HR proficient and deficient cells. To determine a mechanism for break formation, the dependence of breaks on replication and transcription was also assessed. Finally, the role of Fanconi Anaemia (FA) pathway proteins and the crosslink unhooking nuclease, Mus81, vital components of the ICL repair process, were investigated.

2. Materials and methods

2.1. Drugs and reagents

Doxorubicin was provided by FarmItalia Carlo Erba (now Pfizer) and was solubilised in MilliQ H_2O and stored at −20 °C. Barminomycin was isolated as previously described [21] and was dissolved in methanol and stored at −20 °C. The concentration of these compounds was determined spectrophotometrically at 480 nm using a molar extinction coefficient of 11,500 M^{-1} cm^{-1}. Ecteinascidin 743 (ET-743) was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (MD, USA) and was dissolved in DMSO and stored at −20 °C. All other chemicals were purchased from Sigma–Aldrich (MO, USA) unless otherwise stated. Anti-H2AX (pSer139) rabbit polyclonal antibody was purchased from Merck Biosciences (Darmstadt, Germany) and goat anti-rabbit IgG (H+L) Alexa Fluor 488 conjugate was purchased from Invitrogen (CA, USA).

2.2. Cell lines

Chinese hamster ovary cells AA8 and irs1SF (wild type and XRCC3 deficient respectively) were provided by Prof J Hartley (University College, UK). V79 and V-C8, Chinese hamster lung cells (wild type and BRCA2 deficient respectively) were provided by Prof M Zdzienicka (Leiden University, Amsterdam, The Netherlands). All hamster cells were maintained in Hams F-12 media (Invitrogen) supplemented with 10% FBS (Thermo Fisher Scientific, Melbourne, Australia) in a humidified incubator at 37 °C, 5% CO_2. Wild type and Mus81$^{-/-}$ mouse embryonic fibroblasts (MEFs) were provided by Dr. L. Niedernhofer (University of Pittsburgh, USA). MEFs were maintained in RPMI 1640 media (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Human promyelocytic leukaemia HL-60 cells and their mitoxantrone resistant derivative HL-60/MX2 (catalogue numbers CCL-240 and CRL-2257, respectively) were obtained from the American Type Culture Collection (VA, USA). The HL-60/MX2 cell line has decreased expression of topoisomerase IIα and no detectable levels of topoisomerase IIβ [22]. HL-60 cells were maintained in RPMI 1640 media supplemented with 10% FBS. Human FA deficient cells, PD20 RV: Empty (ΔFANCD2 carrying empty vector), PD20 RV:D2 puro (corrected with wildtype FANCD2) and PD352 were provided by Dr Fang Zhang, Fanconi Anaemia Cell Repository, Oregon Health & Science University (USA) and were maintained in alpha modified minimal essential media (Invitrogen) supplemented with 10% FBS.

Lymphocytes were isolated from fresh, pooled buffy coat of healthy human donors, which was provided by the Australian Red Cross Blood Service (South Melbourne, Victoria). Lymphocytes were isolated using Ficoll-Paque™ PLUS (GE Life Sciences, Buckinghamshire, UK) according to the manufacturer's instructions. A further purification step as described previously [23], was performed to remove excess platelets. The purified lymphocytes were resuspended in RPMI 1640 media with 10% FBS and placed in a tissue culture flask and incubated overnight at 37 °C, 5% CO_2. All cells in suspension were then removed for subsequent use.

2.3. Growth inhibition

The MTT growth inhibition assay was performed as previously described [14]. Cells were seeded at a density of 2000 cells/well (MEFs) or 5000 cells/well (FA cells) in 96-well plates and allowed to attach for 24 h prior to 4 h treatment with concentrations of the various compounds spanning a 4 log range. Cells were then incubated in drug-free media for 68 h. The IC_{50} value was determined as the drug concentration inhibiting MTT metabolism in 50% of cells.

2.4. Mus81 knockdown

HeLa cells were transfected with 5 nM Silencer Select siRNA, targeted to either Mus81, GAPDH or a non-targeted control (Applied Biosystems, CA, USA), using Lipofectamine2000 (Invitrogen) and allowed to incubate for 48 h prior to drug treatments. Samples were harvested at 48 and 72 h post transfection and the levels of Mus81 and GAPDH were analysed by western blotting to confirm protein knockdown.

2.5. Sub G1 DNA fragmentation

Cells were seeded at a density of 1.5×10^5 cells per well in 6 well plates and allowed to attach overnight before being treated with compounds for 4 h and allowed to incubate for 36 h in drug free media. Cells were harvested via trypsinisation and centrifuged for 5 min at 130 × *g*. Cell pellets were then resuspended in 1 ml 70% ethanol and allowed to fix overnight at 4 °C. Cells were washed with cold PBS, resuspended in staining solution (25 μg/ml propidium iodide and 100 μg/ml RNase A in cold PBS) and incubated at 37 °C for 30 min. Samples were analysed on a FACSCanto II flow cytometer (BD Biosciences, NY, USA) and 10,000 events were recorded for each sample. Data was analysed using FACS Diva (BD Biosciences) and FCS Express (De Novo Software, CA, USA) software programs and gated to omit doublets and cellular debris.

2.6. Caspase activation assay

Caspase activity was determined using the ApoOne Caspase 3/7 assay according to the manufacturers instructions (Promega, WI, USA).

2.7. γH2AX foci detection

γH2AX detection was performed essentially as described [24] with minor modifications being that cells were seeded at a density of 1.5×10^5 cells per well in 6 well plates and allowed to attach overnight before treatment with drugs. Cells in suspension were seeded at a density of 3×10^5 (HL-60 and HL-60/MX2 cells) or 5×10^5 (lymphocytes) per well, 30 min prior to treatment. Cells were stained in 100 μl anti-H2AX (pSer139) antibody (Merck Biosciences, Darmstadt, Germany), washed, and resuspended in 100 μl anti-rabbit Alexa Fluor 488 conjugate diluted 1:200. The cells were resuspended in 300 μl TBS containing 5 μg/ml propidium iodide for flow cytometry analysis. Cells were analysed on a FACS Canto II flow cytometer and gated to omit doublets. When analysing the cells via fluorescence microscopy, cells were resuspended in 75 μl TBS with 4% FBS and 0.1% Triton-X100 with 50 ng/ml Hoechst dye to DNA. 25 μl of this was applied to a glass slide using a Cytospin centrifuge at 10% speed for 5 min. Mountant (1% (w/v) Dabco in 90% (v/v) glycerol in PBS, pH 7.4) was added and the sample sealed with a coverslip. Foci were viewed using an Olympus IX81 fluorescence microscope and images captured using AnalySIS LS Research software (Soft Imaging Solutions, Olympus).

2.8. Comet assay (single cell gel electrophoresis)

The alkaline comet assay was performed as previously described [9,25] with minor modifications. Cells were seeded at a density of 1.5×10^5 cells per well in 6 well plates and allowed to attach overnight before treatment with drugs and trypsinisation. Before centrifugation, 200 μl of cell suspension was set aside for each sample to be processed for the comet assay. The remaining portion of cells was analysed for sub G1 fragmentation to ensure that no DNA damage due to apoptosis was present. Slides were viewed on an Olympus BX40 fluorescence microscope using a 20× objective lens. Images were captured using a CoolSNAP-Pro monochrome CCD camera (Media Cybernetics, MD, USA) and 50 cells per slide analysed using Komet 5.5 software (Andor Technology, Belfast, UK).

2.9. FANCD2 foci formation

Wild type MEF cells were treated for 4 h with the indicated drugs and incubated in drug-free media for a further 12 h. Cells were harvested via trypsinisation and fixed in 4% (w/v) formaldehyde in PBS for 15 min. The cells were rinsed in PBS and permeabilised in 0.3% Triton X-100 for 15 min, rinsed with PBS again and blocked in 5% (w/v) BSA in PBS for 30 min. Primary antibody, α-FANCD2 (Novus Biologicals, CO, USA) was diluted 1:500 in 5% BSA and incubated with the cells for 90 min with gentle shaking. The primary antibody was removed and the cells washed twice with 2.5% BSA in PBS before addition of a 1:500 dilution of α-rabbit IgG conjugated to AlexaFluor 488 in 5% BSA. This was incubated for 60 min with gentle shaking. The cells were washed twice in 2.5% BSA and resuspended in 30 μl PBS containing 10 ng/ml Hoechst dye. This solution was applied to a glass slide using a Cytospin centrifuge at 10% speed for 5 min. Mountant (1% Dabco in 90% glycerol and 10% PBS, pH 7.4) was added and the sample sealed with a coverslip. Foci were viewed using an Olympus IX81 fluorescence microscope and images captured using AnalySIS LS Research software (Soft Imaging Solutions, Olympus). At least 7 fields of view were counted for each sample.

3. Results

3.1. Barminomycin forms topoisomerase II independent DNA breaks

Whilst it has been known for some time that anthracyclines can covalently attach to DNA, it was not known whether these adducts would be processed into DSBs inside a cell. ET-743 covalent adducts have been shown to cause DNA breaks and this results in the formation of γH2AX foci, therefore, initially cells were tested for their ability to form DNA breaks following anthracycline adduct-forming treatments and then the difference in HR deficient cells was assessed. Fig. 1D shows that there was a dose dependent increase in γH2AX positive V79 cells following a 2 h treatment with barminomycin with a significant number of breaks apparent at concentrations as low as 0.1 nM. Doxorubicin proved to induce the most breaks, which was expected considering that topoisomerase II poisoning (leading to DSBs) is its primary mechanism of action when used as a single agent. ET-743 treatment resulted in an increase in γH2AX formation with concentration but this appears to plateau at a much lower level than for barminomycin, suggesting that it is not as efficient at forming DNA breaks. Both barminomycin and ET-743 induced γH2AX levels decreased over time, falling to background at 24 h after ET-743 treatment (Fig. 1E) and whilst barminomycin-induced γH2AX did not return to background levels after 24 h, the levels were beginning to decrease. As both doxorubicin and barminomycin have been shown to form DNA breaks it was important to determine if barminomycin was forming topoisomerase II dependent breaks in a similar manner to that known for doxorubicin, or if they were actually a consequence of adduct mediated damage. This was accomplished using HL-60 cells compared to the mitoxantrone-resistant variant sub-line HL-60/MX2. These drug resistant cells exhibit a reduced topoisomerase II expression profile but no upregulation of P-glycoprotein [22]. Doxorubicin fails to form topoisomerase II associated breaks in HL-60/MX2 cells [9]. Fig. 1F shows that barminomycin causes equal levels of γH2AX in both cell lines whilst doxorubicin mediated γH2AX formation is severely limited in HL-60/MX2 cells (Fig. 1G). This suggests that the two different anthracycline treatments are associated with entirely different mechanisms of DNA break formation.

3.2. Homologous recombination deficient cells do not show increased DNA break accumulation

DNA breaks detected by the comet assay in HR deficient cells, which have previously been shown to be hyper-sensitive to barminomycin [14], shows that these cells form breaks to a lesser extent than HR proficient cells in response to barminomycin treatment (Fig. 2A). Treatment with doxorubicin (Fig. 2B) also shows that the V-C8 (ΔBRCA2) cells formed less breaks than the V79 cells, contrary to expectations.

The γH2AX assay was also used to determine the extent of breaks in both V79 and V-C8 cells as well as AA8 and irs1SF (ΔXRCC3) cells. Fig. 2C shows that both HR deficient cell lines show a significant lack of induction of γH2AX induction following a 6 h treatment with either barminomycin or ET-743. The cells were also treated with DRB for 1 h prior to addition of barminomycin or ET-743 in order to inhibit transcription. This was investigated because it had previously been shown that ET-743 induced DSBs were partially dependent on transcription [26], however the effect of blocking transcription in HR deficient cells had not been shown and the effect of transcription inhibition on the formation of barminomycin induced breaks had not been tested. Fig. 2C shows a slight decrease in the amount of barminomycin induced breaks formed when transcription was inhibited in V-C8 and AA8 cells. There was a significant decrease in breaks formed by ET-743 when transcription was inhibited in V79, V-C8 and AA8 cells.

V79 and V-C8 cells were also analysed by fluorescence microscopy to determine the location of the increased γH2AX signal. Fig. 2D shows that the γH2AX fluorescence was organised into discrete foci, which are characteristic of DSBs rather than a general upregulation or pan-nuclear fluorescence. This validates

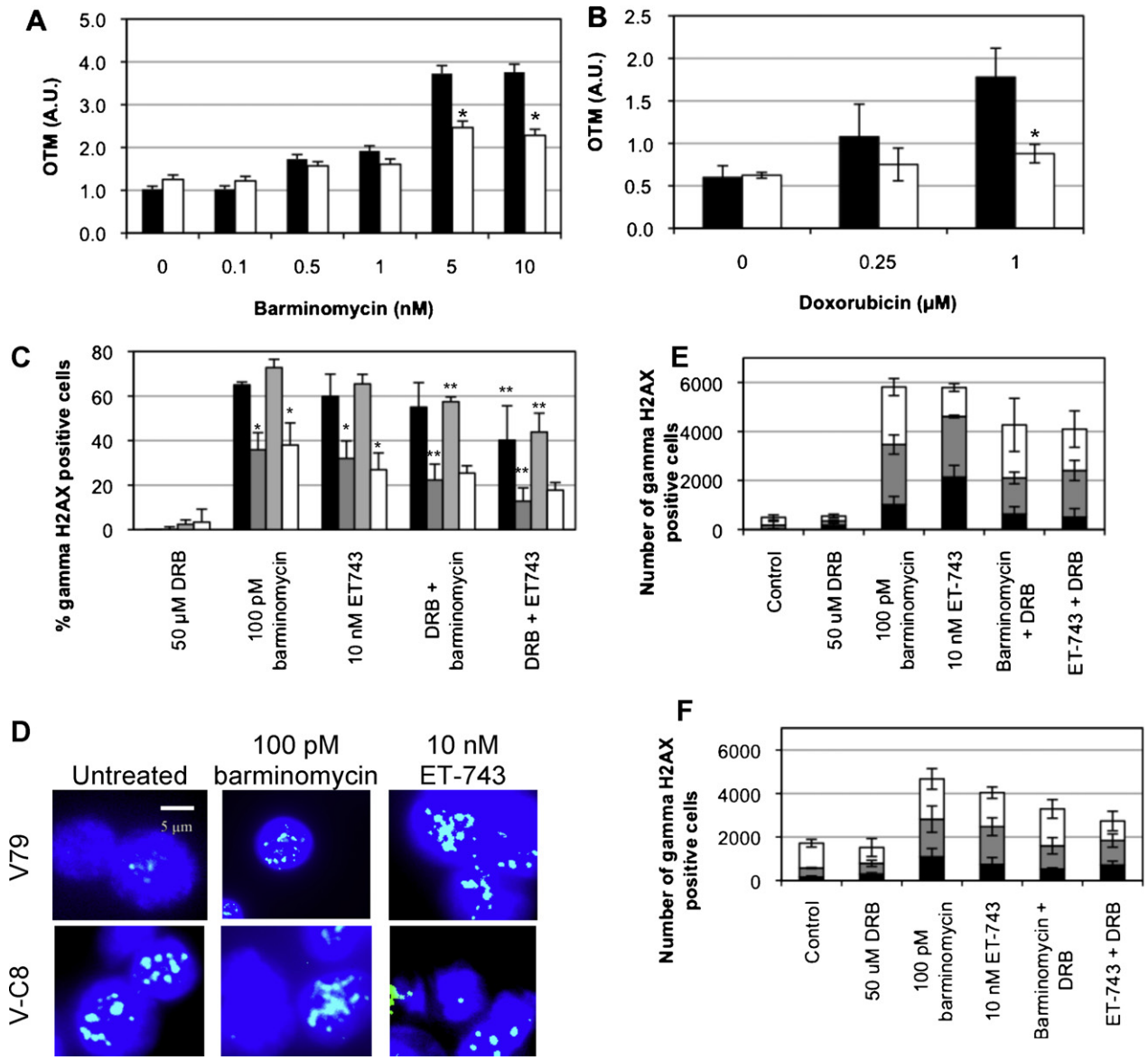

Fig. 2. Homologous recombination deficient cells do not show an increase in γH2AX following treatment with anthracyclines or ET-743. Alkaline comet analysis of V79 (■) and V-C8 (□) cells shows that HR deficient V-C8 cells do not develop DNA breaks to the same level as wild type V79 cells following 2 h treatment with (A) barminomycin or (B) doxorubicin. (C) V79 (■), V-C8 (■), AA8 (■) and irs1SF (□) cells were treated for 6 h with and without a 1 h pre-treatment with DRB and analysed for γH2AX formation. $^{*}p$ value <0.05 when comparing wild type cells with mutant cells receiving the same treatment. $^{**}p$ value <0.05 when comparing these cell lines treated with DRB and barminomycin or ET-743 with the same cell line treated with barminomycin or ET-743 alone (p values were determined using a students' unpaired t test). (D) V79 and V-C8 cells were also viewed microscopically to show γH2AX is organised into discrete foci following treatment with 100 pM barminomycin or 10 nM ET-743. γH2AX positive (E) V79 and (F) V-C8 cells were analysed for cell cycle stage, G_1 (■), S (■) and G_2 (□). All values represent the average of three independent experiments ± standard deviation.

the use of the flow cytometric assay to generate quantitative data. The V79 cells show a substantial increase in green γH2AX foci following barminomycin or ET-743 drug treatment whilst the V-C8 cells did not show a difference between the treated and untreated samples.

3.3. Transcription dependent DNA breaks occur in G_1 though most breaks are replication dependent

The cell cycle stage of the γH2AX positive V79 and V-C8 cells was also analysed (Fig. 2E and F). This shows that the vast majority of γH2AX positive cells were in either the S or G_2 phase of the cell cycle. This corresponds to the time of replication and also to times when HR can occur (i.e. when there are two copies of the chromosomes). The number of G_1 phase γH2AX positive cells is higher in ET-743 treated cells than in barminomycin treated cells but it appears that the reduction in the total number of ET-743 induced breaks when DRB was added is almost entirely from the G_1 population. This suggests that transcription coupled breaks occur predominantly in G_1.

Due to the finding that most γH2AX positive cells were in the S or G_2 phase of the cell cycle, it was necessary to determine the effect of replication on break formation. To achieve this, peripheral blood lymphocytes were isolated from human buffy coat and treated with barminomycin with or without the addition of DRB. Fig. 3A is a representative PI stained cell cycle distribution plot showing that the lymphocytes were not replicating, as evidenced by the cells forming a single population rather than the traditional elongated populations characteristic of cycling cells. Fig. 3B shows that there was a dose dependent increase in γH2AX, which plateaued at 400 pM barminomycin. However, when DRB was added to the samples, none of the treatments resulted in an increase in γH2AX above background, indicating that in the absence of replication all breaks were transcription dependent.

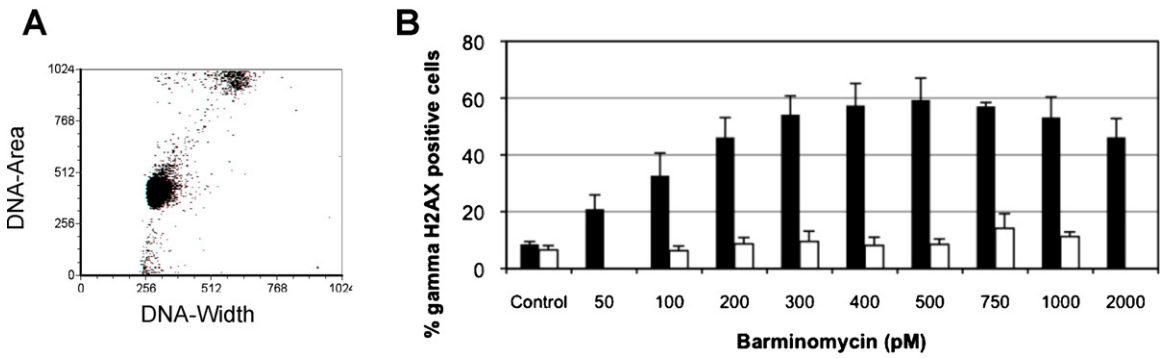

Fig. 3. γH2AX foci form in non-proliferating lymphocytes following barminomycin treatment. Lymphocytes were isolated via Ficoll gradient density centrifugation. (A) DNA content analysis shows a single cell population that is not cycling. Cells were treated for 6 h with various concentrations of barminomycin alone (■) or in combination with 50 μM DRB (□) and analysed for γH2AX formation via flow cytometry. All values represent the average of three independent experiments ± standard deviation. p value <0.05 between DRB ± samples treated with barminomycin 100–1000 pM.

3.4. The Fanconi Anemia pathway plays a role in adduct processing but is not crucial

Fanconi Anemia cells deficient in either FANCD2 (PD20 RV: Empty [retrovirally transformed with an empty vector]) or FANCG (PD352), proteins that form part of the FANC ID and core complex respectively, and a corrected cell line, PD20 RV:D2 (PD20 cells which have been retrovirally transformed with a wild type FANCD2 protein), were tested for their sensitivity to barminomycin, ET-743, doxorubicin, cisplatin and MMC in growth inhibition assays. Table 1 shows that all cell lines were equally sensitive to barminomycin, ET-743 and doxorubicin. The FA deficient cells were more sensitive to both cisplatin and MMC than the corrected cells which was as expected [27–29] though this was only statistically significant for MMC. The corrected cell line failed to yield consistent growth inhibition values, as evidenced by the large error associated with the values in Table 1. Due to this, the cells were also tested for apoptosis following drug treatments to further investigate the response of the cells to the various drugs (Fig. 4A). Both FANCD2 and FANCG deficient cells are more sensitive to barminomycin and ET-743 than the corrected cells although there was only a significant increase in apoptosis in the FANCG deficient cells. Both FA cell lines were significantly more sensitive to MMC, which was used as a control. Analysis of γH2AX induction in the FA deficient cell lines showed that there was no difference between the FA deficient and the corrected cell line with any of the drugs tested (Fig. 4B). In order to confirm the activation of this pathway in response to barminomycin and ET-743 induced damage, FANCD2 foci were observed using immunofluorescence (Fig. 4C and D). Fig. 4D shows that FANCD2 foci formed in wild type cells following treatment with either barminomycin, ET-743 or MMC (which was used as a positive control) although there were less foci with barminomycin treatments. All drugs were used at equi-toxic concentrations. Fig. 4C shows the quantitation of several fields of view over multiple experiments and confirmed that there was a dose dependent increase in the number of cells with more than 5 foci when treated with either ET-743 or MMC but not barminomycin.

3.5. Mus81 deficient cells are exquisitely sensitive to barminomycin

The role of Mus81 was investigated using siRNA to knock down protein levels in HeLa cells. This indicated an increase in apoptosis in Mus81 knockdown cells in response to both barminomycin and MMC (Fig. 5A) although the variability was high for these treatments. However, there was no difference in γH2AX levels for any of the drug treatments tested (Fig. 5B). To further elucidate the role of Mus81, knockout cells were used as there would be no residual Mus81 activity in these cells which may have been present following the RNA knockdown. Mus81 knockout cells were tested for sensitivity to barminomycin, ET-743, doxorubicin and MMC to determine if this nuclease was involved in adduct processing. Doxorubicin and MMC were used as controls, where doxorubicin alone does not induce significant levels of ICLs and there should be no difference in sensitivity to it and this was indeed the case (Table 2), whilst MMC causes high levels of ICLs and Mus81-Eme1 deficient cells have been documented to be extremely sensitive to MMC [30], and this was also confirmed (Table 2). The Mus81 knockout cells were shown to be extremely sensitive to barminomycin and ET-743 compared to the wild type cells. The most striking result was that the IC_{50} for barminomycin was so low that it could not be determined in the knockout cells due to the extreme sensitivity of response. The cells were also tested for apoptosis in response to various treatments (Fig. 5C). Mus81$^{-/-}$ cells were sensitive to levels of barminomycin, ET-743 and MMC that did not affect wild type cells. There was no difference in the level of γH2AX between WT and Mus81$^{-/-}$ cells in response to both barminomycin and ET-743. There was however a significantly lower level of γH2AX positive Mus81$^{-/-}$ cells than wild type following treatment with doxorubicin and MMC.

4. Discussion

Many ICL forming drugs have been shown to result in DSBs [31,32] and now anthracycline-DNA adducts have also been shown to result in DNA breaks and γH2AX foci formation (Figs. 1D, F and 2A). Since anthracycline-DNA adducts do not form conventional

Table 1
IC_{50} values for FA deficient and corrected cell lines following 4 h treatment with each of the indicated drugs and 68 h incubation in drug-free media.

Cell line	Mutated gene	Barminomycin (pM)	ET-743 (nM)	Doxorubicin (nM)	Cisplatin (μM)	MMC (ng/ml)
PD20:RV D2 puro	Corrected	312 ± 226	3.0 ± 0.4	131 ± 22	10.9 ± 5.7	1567 ± 757
PD20:RV Empty	FANCD2	176 ± 72	2.0 ± 0.4	96 ± 23	4.8 ± 0.8	285 ± 55*
PD352	FANCG	202 ± 78	2.7 ± 0.3	88 ± 12	4.0 ± 1.2	198 ± 61*

Values represent the average of at least 2 independent experiments performed in quadruplicate ± standard deviation.
* p value as determined by students t test >0.05 when comparing to PD20:RV D2 puro cells.

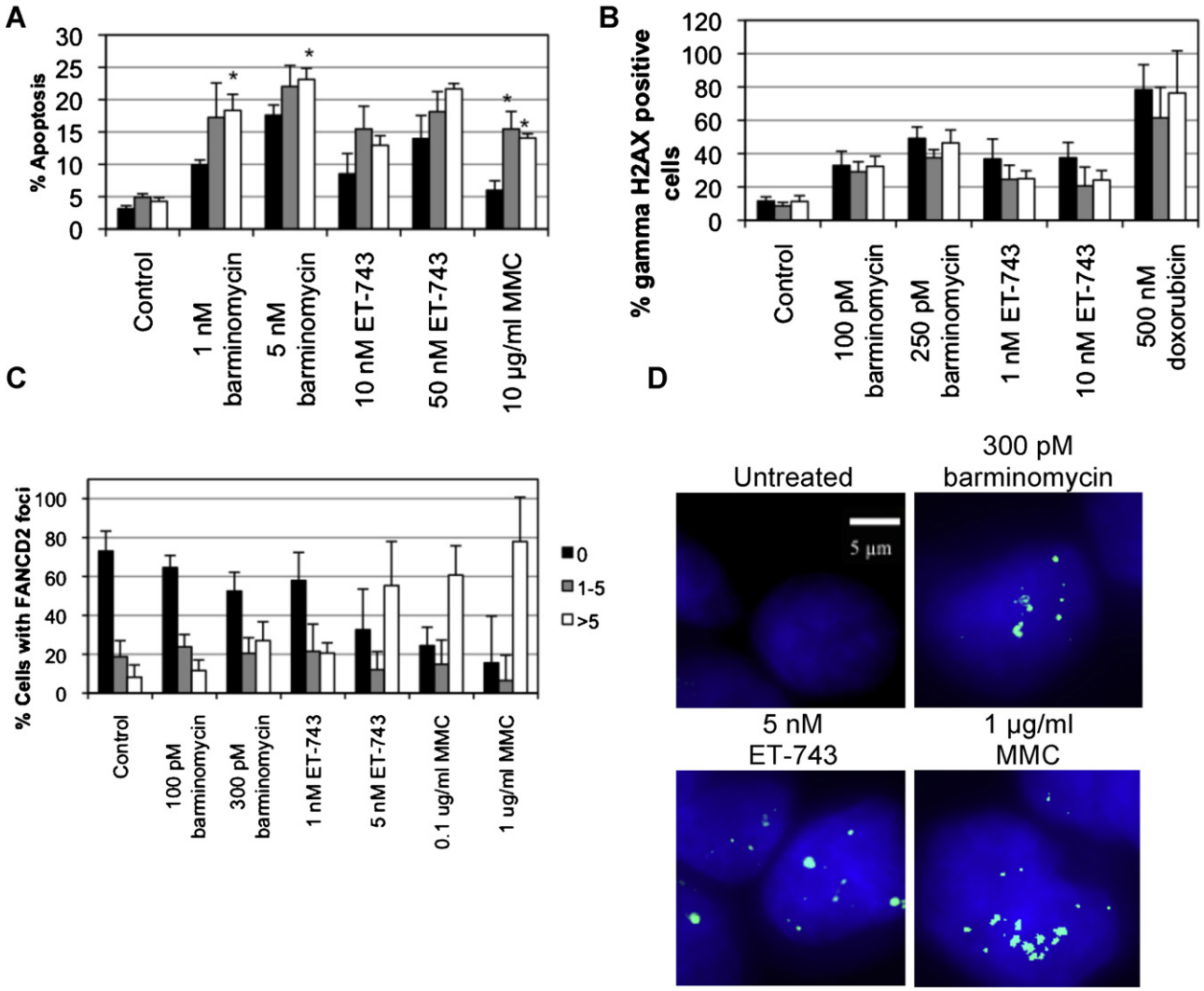

Fig. 4. The Fanconi Anemia pathway is activated but is not necessary for break formation or apoptosis following barminomycin or ET-743 treatment. (A) PD20 RV:D2 (■), PD20 RV: Empty (■) and PD352 (□) cells were treated with various drugs for 4 h and allowed to incubate for 24 h in drug-free media and analysed for apoptosis via sub G_1 DNA fragmentation or (B) treated for 6 h and γH2AX levels measured via flow cytometry. (C and D) Wild type MEF cells were treated with various drugs for 4 h, allowed to recover in drug-free media for 12 h and analysed for FANCD2 foci formation. All values represent the average of at least two independent experiments ± standard deviation. $^*p < 0.05$ as determined by students *t*-test compared to PD20 RV:D2 cells with the same treatment. Data not marked with an asterisk has been analysed and is not statistically significant.

covalent crosslinks between the DNA strands but strongly stabilise the local region of DNA, it is unknown whether they are processed essentially as monofunctional or bifunctional adducts with respect to DNA DSB formation. Regardless, it was important to rule out topoisomerase II mediated damage as the predominant cause of the DSBs as this is the mechanism of action of anthracyclines such as doxorubicin. This was accomplished using the HL-60 and HL-60/MX2 cell lines which have previously been used to show that doxorubicin-DNA adducts (formed by combining doxorubicin with a formaldehyde-releasing prodrug) exert their cytotoxic effects via a topoisomerase II independent pathway [9]. Barminomycin is a far more potent adduct inducing drug than doxorubicin [10] and exhibits equivalent cytotoxicity against HL60 and HL60/MX2 cells [33]. The present results support the theory that barminomycin induced DSBs are formed independently of topoisomerase II activity (Fig. 1F), suggesting that the adducts result in DSBs via a similar mechanism to other adduct forming agents, in that replication stalls at the damage site and fork collapse results in a DSB. Fig. 1E shows that following treatment with barminomycin or ET-743, γH2AX was lost over time, indicating eventual repair or bypass of the damage.

In order to determine if sensitivity of HR deficient cells to barminomycin is due to higher levels of DSBs due to lack of repair or increased formation, both comet and γH2AX assays were used. The finding that the HR deficient V-C8 cells showed a lower level of breaks in both assays following treatment with barminomycin is contrary to expectations (Fig. 2A and C). However, treatment with doxorubicin produced a similar result (Fig. 2B) so the effect of BRCA2 on adduct mediated break formation remains difficult to establish. AA8 and irs1SF cells were used to further clarify the response to these adducts (Fig. 2C). The γH2AX assay showed a high background level of breaks in the two HR deficient cell lines (data not shown), and this has previously been shown with other HR deficient cells and is not unexpected [26]. Because of this, the difference between control levels and treated levels of γH2AX was examined. The HR deficient cells showed a significantly lower induction of γH2AX in response to barminomycin or ET-743 treatment compared to the HR proficient cells. The cause of this is unknown, although, it has been reported that BRCA2 deficient cells treated with ionising radiation fail to form Rad51 foci [34] and it may be due to a similar mechanism, however, when taken together with the low level of breaks observed using the comet assay, it

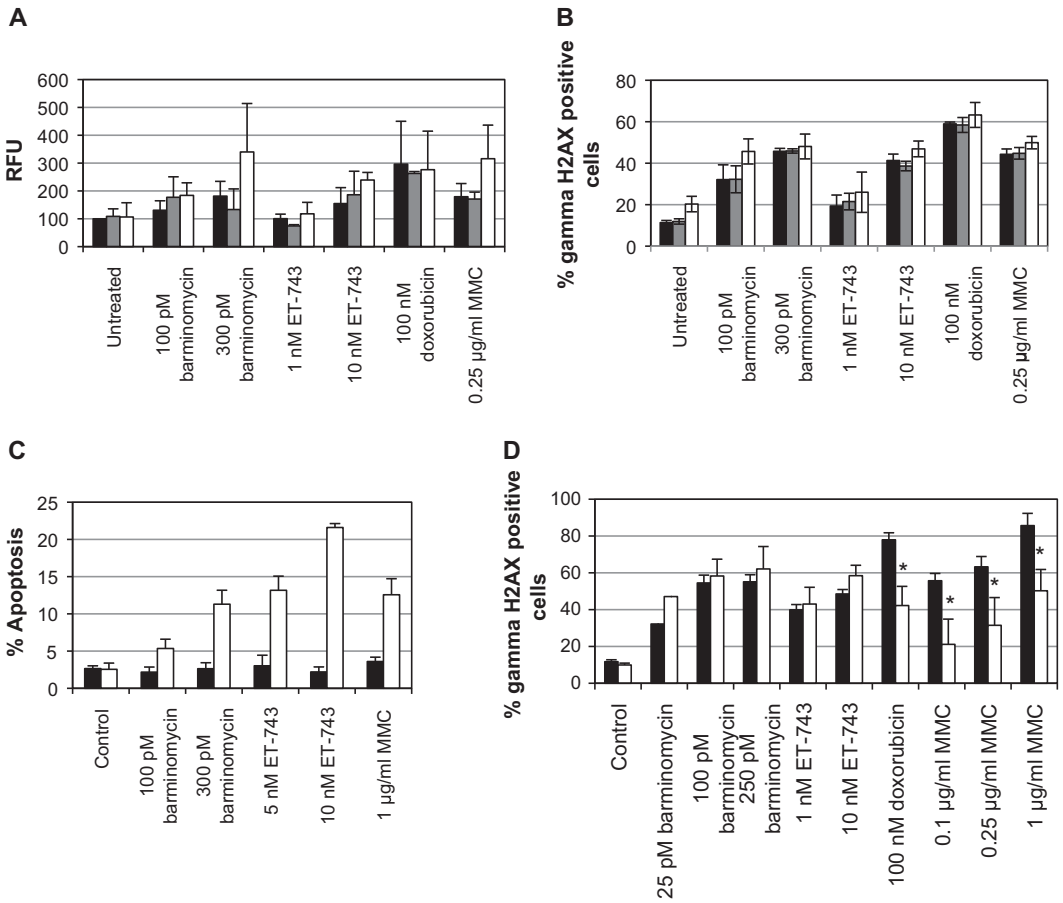

Fig. 5. Loss of Mus81 results in increased anthracycline-DNA adduct induced apoptosis but does not affect γH2AX foci formation. HeLa cells transfected with a non-targeted siRNA scramble control (■), or siRNA targeting GAPDH (■) or Mus81 (□) and were (A) treated for 4 h and allowed to incubate for 24 h in drug-free media and analysed for caspase 3/7 activation. These transfectants were also (B) treated for 6 h and analysed via flow cytometry for γH2AX. MEF wild type (■) and Mus81$^{-/-}$ (□) cells were treated as for the previous experiments and analysed for (C) apoptosis via sub G_1 DNA fragmentation and for (D) γH2AX via flow cytometry. All values represent the average of at least two independent experiments ± standard deviation. $^*p < 0.05$ as determined by students *t*-test when comparing wild type cells to Mus81$^{-/-}$ cells receiving the same treatment. Data not marked with an asterisk has been analysed and is not statistically significant.

seems that fewer breaks were formed in HR deficient cells than in proficient cells. This is an unusual finding, however, considering that less breaks were in V-C8 cells than wild type V79 cells in the comet assay following treatment with doxorubicin (Fig. 2B), it is possible that this is a generalised response that occurs with various types of treatments and is not a specific response to the presence of anthracycline or ET-743 adducts. It should be noted that previous work has shown that Mre11 deficient cells show an increase in γH2AX following ET-743 treatments compared to wild type cells [26], contrary to the findings presented here, but may be due to a different phenotype when Mre11 is mutated compared to BRCA2 or XRCC3, perhaps due to the role that Mre11 also plays in non-homologous recombination [35].

ET-743 has been shown to induce DSBs partially dependent on transcription [26,36] and the present work confirms this finding. When DRB was added to inhibit transcription there was a reduction in the level of γH2AX in both wild type and HR deficient ET-743 treated cells (Fig. 2C) and these were predominantly from the G_1 population (Fig. 2E and F). The decrease in barminomycin treated cells was only seen in AA8 and V-C8 cells and it was minor, suggesting that transcription plays a minimal role in break formation in response to barminomycin. The transcription associated ET-743 induced breaks occur in the G_1 phase and these are linked to the role of TC-NER [26]. In contrast, replication associated breaks occur in S or G_2 phase. Peripheral blood lymphocytes treated with various concentrations of barminomycin (Fig. 3B) show that in cells where replication does not occur, transcription can induce breaks. However, in replicating cells, barminomycin-induced breaks are predominantly induced during replication (Fig. 2).

The FA pathway has been shown to be involved in ET-743 damage processing, with FA deficient cells being more sensitive to the drug than wild type cells in growth inhibition assays. The previous work showed an approximate 8-fold decrease in ET-743 IC_{50} in FA deficient cells, however this required a treatment time of 24 h to observe the effect, and this is a point of difference compared to the current study [37]. There was no significant difference between the FANCD2 corrected and the FANCD2 and FANCG

Table 2
IC_{50} values for WT and Mus81$^{-/-}$ cell lines following 4 h treatment with each of the indicated drugs and 68 h incubation in drug-free media.

Cell line	Barminomycin (pM)	Doxorubicin (nM)	ET-743 (nM)	MMC (µg/ml)
MEF WT	285 ± 98	220 ± 144	27.1 ± 7.3	1.6 ± 1.0
MEF Mus81$^{-/-}$	<1	120 ± 26	0.8 ± 0.5*	0.2 ± 0.0

Values represent the average of at least 2 independent experiments performed in quadruplicate ± standard deviation.
* *p* value as determined by students *t* test >0.05 when comparing to MEF WT.

deficient cells following short term barminomycin or ET-743 treatment using growth inhibition assays (Table 1), however, the FA cells were more sensitive than the corrected cells when measured via apoptosis (Fig. 4A). The FA deficient cells tested in the current work were more sensitive than the corrected cells to cisplatin and MMC, suggesting that they were responding normally as both of these drugs are known to cause ICLs which are processed by the FA pathway leading to hypersensitivity in FA pathway deficient cells [28]. The lack of a difference in γH2AX response between the deficient and corrected cell lines (Fig. 4B) suggests that DNA breaks were forming normally in both cell lines (as expected) and that the FA pathway does not play a role in DSB formation following barminomycin or ET-743 treatment. The FA pathway may be required for repair of damage following removal of the drug. This could be tested via a timecourse of γH2AX loss following drug removal where the FA deficient cells would be expected to show a delayed loss of γH2AX compared to the corrected cells if the FA pathway is involved in repair.

Mus81 knockdown cells were more sensitive than WT cells to MMC or barminomycin treatment (Fig. 5A) however the difference was not statistically significant, possibly due to variability in knockdown efficiency between experiments. However, since there was no difference in γH2AX levels following MMC treatment (Fig. 5B), which was used as a control and should show reduced levels with Mus81 knockdown [38], all further experiments were performed in Mus81 knockout cells to ensure that no residual Mus81 was present and to reduce variability due to knockdown efficiency. Mus81$^{-/-}$ cells were shown to be extremely sensitive to all of the drugs tested except doxorubicin (Table 2). It has previously been shown that cells deficient in Mus81 or Eme1 are more sensitive to MMC and cisplatin [30,39] and this was confirmed. The sensitivity of the Mus81$^{-/-}$ cells was most extreme following treatment with barminomycin where an IC_{50} was too low to be determined. This is similar to the sensitivity of XRCC3 deficient cells to barminomycin [14] and suggests that Mus81 is necessary for cell survival following barminomycin treatment. The knockout cells were also hypersensitive to ET-743. This is the first time that Mus81 has been implicated in cell survival following ET-743 treatment.

Considering the extreme sensitivity of the Mus81$^{-/-}$ cells to anthracycline adducts and ET-743, it was expected that they would show a difference in the level of DSBs induced from the treatments however this was not seen (Fig. 5D). It has previously been reported that Mus81 is involved in DSB formation following ICL damage and when Mus81 is not present, fewer DSBs are induced [38,40,41]. Since the formation of a DSB is a critical step in ICL repair, the inability to create DSBs results in increased sensitivity to ICL inducing drugs. A decrease in γH2AX was seen in this study following MMC treatment but not following barminomycin or ET-743 treatment. This suggests that Mus81 is not required for break formation after damage from either of these drugs but may be required for a different pro-survival mechanism such as a role in HR as a Holliday junction resolvase [42,43]. However we found that repair of barminomycin- and ET-743 induced breaks was equally effective in Mus81 proficient and deficient cells (data not shown).

In summary, this work shows that anthracycline-DNA adducts induce DNA breaks in cells through a mechanism that is completely independent of topoisomerase II. Break formation is largely dependent on replication and involves a complex processing and repair mechanism. DNA breaks are likely to be formed by stalled and collapsed replication forks and then repaired by HR dependent processes. The collective data indicates that Mus81 proficient cells exposed to barminomycin (and also ET-743) process the damage in a fundamentally different way compared to crosslinking agents such as mitomycin C. This suggestion is not unreasonable considering that barminomycin (and ET-743) covalently attach to only one strand of DNA but mimic interstrand crosslinks in terms of duplex stabilisation. Mus81 mediated resolution of toxic intermediates appears crucial for cell survival although the nature of Mus81 involvement is unconventional compared to treatments such as mitomycin C. It is possible that Mus81 is required for Holliday junction resolution following HR repair of the DSB rather than to generate the break. Mus81 has been shown to be downregulated or mutated in various human cancers including hepatocellular and colorectal carcinoma and high grade astrocytoma and is associated with poor prognosis [44–47]. This suggests that treatments that induce anthracycline-DNA adducts may be effective in these highly treatment resistant cancer types. This could be investigated by xenografting human cancer tissue into immuno-compromised mice and treating the mice with barminomycin or other anthracycline adduct inducing therapy. The data contained within this present study may be useful in predicting individual tumour responses to combination therapy regimes involving doxorubicin and a formaldehyde releasing prodrug (which result in high levels of doxorubicin-DNA adducts) and also possibly for nemorubicin.

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