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Myeloperoxidase enhances etoposide and mitoxantrone mediated DNA damage: a target for myeloprotection in cancer chemotherapy

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Running title: MPO increases TOP2 poison-mediated DNA damage

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Non standard abbreviations
AML, Acute myeloid leukemia; APL, Acute promyleocytic leukemia; ATM, ataxia-telangiectasia mutated;BSO, Buthionine sulfoximine; CML, Chronic myeloid leukemia; DMSO, Dimethyl sulfoxide; DSB, DNA double-strand break; GSH, Reduced glutathione; H2AX, histone H2A.X; γH2AX, S-139 phospho histone H2A.X; MPO, Myeloperoxidase, SA, Succinylacetone; TARDIS, Trapped in Agarose DNA Immunostaining assay, t-AML, therapy-related acute myeloid leukemia; TOP2, Topoisomerase II; TOP2A, DNA topoisomerase IIα; TOP2B, DNA topoisomerase IIβ; TOP2-CC, Topoisomerase II covalent complex

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Abstract

Myeloperoxidase is expressed exclusively in granulocytes and immature myeloid cells and transforms the topoisomerase II poisons etoposide and mitoxantrone to chemical forms that have altered DNA damaging properties. TOP2 poisons are valuable and widely used anti-cancer drugs, but they are associated with the occurrence of secondary acute myeloid leukemias. These factors have led to the hypothesis that myeloperoxidase inhibition could protect hematopoietic cells from TOP2 poison-mediated genotoxic damage, and therefore reduce the rate of therapy-related leukemia. We show here that myeloperoxidase activity leads to elevated accumulation of etoposide- and mitoxantrone-induced TOP2A and TOP2B-DNA covalent complexes in cells, which are converted to DNA double-strand breaks. For both drugs, the effect of myeloperoxidase activity was greater for TOP2B than for TOP2A. This is a significant finding as TOP2B has been linked to genetic damage associated with leukemic transformation, including etoposide-induced chromosomal breaks at the *MLL* and *RUNX1* loci. Glutathione depletion, mimicking *in vivo* conditions experienced during chemotherapy treatment, elicited further MPO-dependent increase in TOP2A and especially TOP2B-DNA complexes and DSB formation. Together these results support targeting MPO activity to reduce genetic damage leading to therapy-related leukemia, a possibility that is enhanced by the recent development of novel specific MPO inhibitors for use in inflammatory diseases involving neutrophil infiltration.

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Introduction

Drugs targeting DNA topoisomerase II (TOP2 poisons) are important, effective and widely used anti-cancer agents, but they are associated with short and long-term toxic side effects including neutropenia and rare but life-threatening therapy-related acute myeloid leukemia (t-AML) (Allan and Travis, 2005; Leone *et al.*, 2010; Cowell and Austin, 2012). As cancer survival rates have increased t-AML has become a more important clinical problem, and it is estimated that up to 15% of all acute myeloid leukemia cases can be classified as t-AML (Mauritzson *et al.*, 2002). Therapy-related acute leukemias occur after a wide range of primary neoplasias, but prior treatment for breast cancer accounts for about 50% of cases, while haematological malignancies account for approximately 30% (Kayser *et al.*, 2011). In its normal cellular role TOP2 facilitates changes to DNA topology by allowing one double stranded segment to pass through another via an enzyme-bridged DNA DSB (Austin and Marsh, 1998; Vos *et al.*, 2011; Cowell and Austin, 2012). In this configuration, each protomer of the homodimeric TOP2 enzyme is covalently coupled to a cleaved DNA strand via a 5'-phosphotyrosine linkage. TOP2 poisons such as etoposide and mitoxantrone exert their tumoricidal effect by stabilising this normally transient enzyme-bridged break, resulting in the accumulation of cytotoxic covalently linked TOP2 protein-DNA complexes, which can be processed in the cell to DNA double-strand breaks (Burma *et al.*, 2001; Cowell and Austin, 2012; Lee *et al.*, 2012, 2016). Therapy related leukemias, especially those appearing after exposure to TOP2 poisons often contain recurrent chromosome translocations, including *t(15,17)(PML-RARA)*, *t(8,21)(AML-ETO)* and *11q23* rearrangements involving the *MLL* gene (Rowley and Olney, 2002; Cowell and Austin, 2012). These genetic lesions disrupt blood cell development and play a pivotal role in the development of the disease. Such t-AML cases arise as a result of TOP2 poison mediated DNA damage in bone marrow blood precursor cells. There are two vertebrate TOP2 paralogues, TOP2A and TOP2B; TOP2 poisons such as etoposide affect both paralogues, but recent evidence points to a greater role for TOP2B in generating the genotoxic damage associated with TOP2 poisons (Azarova *et al.*, 2007; Cowell *et al.*, 2012; Smith *et al.*, 2013).

We are interested in why cells of the myeloid hematopoietic lineage are sensitive to TOP2 poison mediated genotoxic damage which leads to t-AML, and how this sensitivity could be reduced. Myeloperoxidase is expressed exclusively in cells of the myeloid lineage, it is present at high levels in neutrophils where it exerts its anti-microbial action, but is also expressed in myeloid precursor/progenitor cells, including human and mouse common myeloid progenitor (CMP) and

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granulocyte / macrophage progenitor (GMP) cells (Strobl *et al.*, 1993; Mori *et al.*, 2009; Goardon *et al.*, 2011) and is readily detectable in *ex vivo* normal human bone marrow $CD34^+$ cells (Supplemental Fig. 1) (Strobl *et al.*, 1993; Vlasova *et al.*, 2011). Thus, MPO is likely to be present in the cells in which t-AML arises. In its physiological role MPO generates hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions to kill pathogenic microorganisms. However, MPO activity also leads to the oxidative activation of etoposide. This occurs by one-electron oxidation of the etoposide E-ring, yielding a phenoxy radical species and by O-demethylation to the reactive orthoquinone (Supplemental Fig. 2A) (Haim *et al.*, 1986; Kalyanaraman *et al.*, 1989; Kagan *et al.*, 2001; Fan *et al.*, 2006; Jacob *et al.*, 2011; Vlasova *et al.*, 2011). Additionally, CYP3A4 and/or CYP3A5 can oxidise etoposide to etoposide catechol, a metabolite found in patient plasma (Zheng *et al.*, 2004; Zhuo *et al.*, 2004) and oxidation of etoposide by CYP3A4 has been implicated in the incidence of t-AMLs (Felix *et al.*, 1998) . Etoposide catechol can be oxidised to etoposide quinone by MPO via a semi-quinone species (Supplemental Fig. 2A). Etoposide quinone is more effective at inducing TOP2-mediated DNA breaks than etoposide *in vitro* (Jacob *et al.*, 2011) and this is particularly true for TOP2B (Smith *et al.*, 2014). This increased potency of etoposide quinone as a TOP2 poison has been attributed to covalent modification of the enzyme by the quinone (Jacob *et al.*, 2011; Smith *et al.*, 2014). Furthermore, etoposide metabolites have the potential to form adducts with DNA and protein (Haim *et al.*, 1987; Lovett *et al.*, 2001) which may also impact on genotoxicity.

Oxidative activation of TOP2 poisons is not limited to etoposide as MPO has been implicated in the activation of mitoxantrone either directly or via the production of reactive aldehydes (Panousis *et al.*, 1995, 1997; Hazen *et al.*, 1998; Parker *et al.*, 1999; Evison *et al.*, 2015).

Using pharmacological inhibition and genetic manipulation we show that MPO activity increases etoposide and mitoxantrone-induced formation of TOP2A and TOP2B covalent enzyme-DNA complexes in cells of myeloid origin and similarly increases DSB formation. GSH depletion amplified this effect. This is consistent with the hypothesis that MPO inhibition, in a clinical setting, especially where cellular thiol levels are suppressed, could protect against genotoxic damage in the myeloid compartment by TOP2 poisons.

Materials and Methods

Reagents and antibodies. Etoposide, mitoxantrone, succinylacetone (SA), dimethyl sulfoxide (DMSO), Tween 20, Triton X-100, paraformaldehyde, DL-buthionine sulfoximine and PF-1355 (2-(6-(2,5-dimethoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2H)-yl)acetamide) were purchased

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from Sigma-Aldrich (Dorset, UK). MPOi-II (4-(5-Fluoro-1H-indol-3-yl)butanamide) was purchased from Merck-Millipore (Watford, UK). Anti-MPO ab9535 (immunofluorescence) and ab134132 (Western blotting) were from Abcam (Cambridge UK), anti-mouse γH2AX 05-636 was obtained from Merck-Millipore (Watford UK).

Cell Culture. All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Thermo Fisher Scientific,UK). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. Experiments were conducted on cells growing in log phase (2-5 x10^5cells/mL). For succinylacetone treatment of cells to down-regulate MPO, cells were treated with 200μM SA for 48 hours before addition of TOP2 poison and downstream analysis. SA was retained during TOP2 poison treatment.

Stable transfection of K562 cells with MPO cDNA. K562 cells were transfected with plasmid RC2016029 containing the MPO coding sequence with a C-terminal MYC-DDK tag in the vector pCMV 6-Entry (Origene, Rockville. USA). Transfection was performed using Dharmafect Duo (Dharmacon, UK) transfection reagent. Transfection mixtures contained 1μg/mL of MPO plasmid (Origene) or G418 control plasmid with 20μg/mL of Dharmafect Duo (Dharmacon) and 80% v/v serum-free RPMI 1640 medium. After selection with G418 (750 μg/ml) clonal lines were isolated by limited dilution. MPO-expressing lines were continuously grown in 500μg/mL G418, the selection antibiotic. MPO expression was assessed using immunofluorescence and MPO activity assays.

MPO and GSH colorimetric activity assays. MPO activity assays were performed using an Abcam MPO Activity Assay kit (ab105136, Abcam Cambridge UK). Cells were harvested by centrifugation at 1000 x g for 5 mins and homogenised in 4 pellet volumes of lysis buffer provided. Bradford assays were conducted to ensure an equal concentration of protein was used for each assay. The MPO activity assay was conducted according to manufacturer's instructions with absorbance readings measured at 415nm. GSH assays were performed using a GSH assay kit (KA0797, Abnova, Tiawan), according to the manufacturer's instructions.

Immunoblotting for MPO. Whole cell lysates of cells were prepared (Mirski *et al.*, 1993) and samples were resolved on precast 4-20% SDS-polyacrylamide gels (NuSep, Australia). Western blotting was performed by standard methods using ECL detection.

Trapped in Agarose DNA Immunostaining (TARDIS) assay. TARDIS assays to quantify TOP2 covalent protein-DNA complexes were carried out essentially as described previously (Willmore *et al.*, 1998; Cowell, Tilby, *et al.*, 2011). Briefly cells were treated for 1 hour with the desired dose of etoposide or mitoxantrone. Cells were then pelleted and washed in ice cold PBS. After re-centrifuging, cells were

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mixed with molten 2% LMP agarose (Lonza, USA) in PBS at 37°C and spread evenly onto agarose coated slides. Agarose-embedded cells were lysed in (1% w/v SDS, 20mM sodium phosphate, 10mM EDTA, pH 6.5) and non-covalently DNA bound proteins were removed using 1M NaCl. TOP2 covalent complexes were detected by immunofluorescence using rabbit anti-TOP2A (4566) or anti-TOP2B (4555) antibodies, raised to the C-terminal domain of human TOP2A and TOP2B respectively and Alexa-488 coupled anti-rabbit secondary antibodies (Thermo Fisher Scientific, UK). Slides were counterstained with Hoechst 33258 to visualise DNA. Quantitative immunofluorescence was performed by capturing images for Hoechst and Alexa-488 using a epifluorescence microscope (Olympus IX-81) fitted with an Orca-AG camera (Hamamatsu) and suitable narrow band filter sets. Images were analysed using Volocity 6.3 (Perkin Elmer) and data representation and statistics were performed using GraphPad Prism 4.0 and R.

Immunofluorescence analysis of γH2AX and MPO. After drug treatment cells were washed and pelleted in ice-cold PBS and spotted onto poly-L-lysine slides. Cells were fixed in 4% formaldehyde in PBS and permeabilised using KCM+T buffer (120mM KCl, 20mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA, 0.1% Triton X-100). After blocking in (KCM+T, 2% Bovine Serum Albumin, 10% dry milk powder) cells were probed with primary anti-MPO antibody or anti-γH2AX in blocking buffer and Alexa-488 anti-rabbit or Alexa-594 anti-mouse secondary antibodies (Thermo Fisher Scientific). Slides were counterstained with DAPI (Vector Labs, CA) and viewed using an epifluorescence microscope (Olympus IX-81). For γH2AX quantification images were captured for DAPI and Alexa-594 and quantitative analysis was performed using Volocity 6.3 (Perkin Elmer) with data representation performed in GraphPad Prism 4.0 and R.

GSH depletion. NB4 cells were treated with 150μM buthionine sulfoximine (BSO) for 4.5 hours (Griffith, 1982; Gantchev and Hunting, 1997) before addition of TOP2 poison. BSO was retained in the medium during TOP2 poison treatment.

Statistics. Data is presented as the mean values ± SEM values for n ≥3 replicate experiments; the number of replicates involved for each treatment is indicated within the figures. Statistical analysis was performed by one-way ANOVA with post-hoc Tukey's multiple comparison test. For signifying *p* values: * refers to $p < 0.05$, ** refers to $p < 0.01$, and *** refers to $p < 0.001$

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Results

MPO inhibition suppresses etoposide-induced TOP2A and TOP2B covalent DNA complex formation in NB4 cells.

MPO activity can be efficiently reduced in cell culture systems by the heme biosynthesis inhibitor succinylacetone (SA) (Pinnix *et al.*, 1994; Kagan *et al.*, 2001; Vlasova *et al.*, 2011). Using NB4 cells, an APL line that express MPO at a high level (Hu *et al.*, 1993), we found that incubation with 200μM SA for 48 hours reduced MPO enzymatic activity to below the detection level of the assay employed, and as reported previously (Pinnix *et al.*, 1994), it significantly depleted mature MPO protein (Fig. 1A & 1B). Quantification of replicate blots indicated that mature MPO protein level was reduced to less than 25% of that in untreated cells. Notably, SA treatment for up to 72 hours did not affect TOP2A or TOP2B protein levels in NB4 cells, accelerate acidification of the medium nor reduce cell viability or cell growth (Supplemental Fig. 2 B-E). Similarly, SA did not affect TOP2A or TOP2B enzymatic activity *in vitro* (Supplemental Fig. 2 F-H). We used the TARDIS assay (Willmore *et al.*, 1998; Cowell, Tilby, *et al.*, 2011) to quantify stabilised TOP2-DNA covalent complexes in etoposide-treated cells. This assay employs sensitive quantitative immunofluorescence to analyse TOP2 content in agarose embedded "nuclear ghosts" that remain after non-covalently attached proteins and other cellular constituents have been removed from nuclear DNA by SDS-salt extraction (Supplemental Fig. 3A&B). Pre-treatment with SA significantly reduced the levels of etoposide-induced TOP2A and TOP2B-DNA complexes. This was true for both 10μM and 100μM etoposide (Fig. 1 C & D, Supplemental Fig. 3A&B). At both doses of etoposide, the magnitude of the affect was greater for TOP2B than for TOP2A. For TOP2B with 100μM etoposide SA pre-treatment resulted in a 38% reduction in TOP2-complex formation, whereas the reduction was 12% for TOP2A. For 10 μM etoposide the respective figures were 55% and 18% (Fig. 1 C & D, Supplemental Table 1). As expected, SA pre-treatment did not affect stabilised TOP2A or TOP2B-DNA complexes in K562 cells, a CML-derived cell line that does not express MPO (Supplemental Fig. 3 C&D). Etoposide quinone displays enhanced TOP2-mediated DNA cleavage activity *in vitro* compared to the parent compound (Jacob *et al.*, 2011), and this is more pronounced for TOP2B (Smith *et al.*, 2014). The observation that SA pre-treatment, which reduces MPO-mediated etoposide phenoxy radical production, partially suppressed etoposide-induced TOP2-CC formation in MPO-expressing NB4 cells supports the conclusion that oxidative metabolism of etoposide plays a role in TOP2-mediated DNA damage *in vivo*.

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MPO expression in K562 cells stimulates etoposide-induced TOP2-DNA covalent complex formation.

K562 is a CML derived cell line that does not express detectable MPO (Hu *et al.*, 1993)(Supplemental Fig. 1). We transfected K562 cells with a human MPO expression construct and isolated clonal K562 lines expressing MPO. Two $K562^{MPO}$ cell lines (MPO line 4 and MPO line 5) exhibited MPO activity at 37% ±7.5 and 46% ±6.0 of the level of NB4 cells respectively. Both cell lines expressed MPO in all cells by immunofluorescence (Fig. 2A). Parental K562 cells and $K562^{MPO}$ cell lines 4 and 5 were treated with 10μM or 100μM etoposide and stabilised TOP2-DNA complexes were quantified. The $K562^{MPO}$ cell lines displayed significantly higher levels of drug-stabilised complexes for both TOP2 isoforms compared to K562 cells lacking MPO expression at 100 μM etoposide. At 10 μM etoposide raised levels of TOP2A and TOP2B stabilised complexes were observed with MPO line 5, and for line 4 significantly raised complex levels were observed only for TOP2A (Fig. 2B). After subtracting background complex levels, $K562^{MPO}$ line 5 displayed a 1.6 and 2.0-fold increase in TOP2A- and TOP2B-DNA complexes respectively compared to K562 following 100μM etoposide treatment. For line 4, which expressed MPO at a lower level, a smaller fold increase in complexes was observed (Supplemental Table 1). By comparison, K562 cells transfected with an empty expression vector did not display increased TOP2 covalent complex formation (Supplemental Fig. 3 G&H). This further supports the notion that MPO-mediated activation contributes to etoposide-mediated DNA damage in MPO expressing cells.

MPO contributes to etoposide induced cellular DNA damage.

TOP2 poison-induced covalent DNA complexes are processed to DSBs that result in histone H2AX phosphorylation (Sunter *et al.*, 2010; Cowell, Papageorgiou, *et al.*, 2011; Cowell *et al.*, 2012) as part of a well-established response involving activation of the DNA damage kinase ATM (Burma *et al.*, 2001). Thus, γH2AX can be used as a measure of DSBs generated by etoposide treatment. The inhibition of MPO using SA resulted in a 40% reduction in γH2AX induced by 100μM etoposide (Fig. 3A, Supplemental Table 1). Notably, this is equivalent to the difference in γH2AX signal observed between 10μM and 100μM etoposide in the absence of SA. SA did not significantly affect the level of γH2AX observed following exposure to 10μM etoposide. SA had no effect on etoposide-induced γH2AX in K562 cells which do not express MPO (Supplemental Fig. 3E).

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Etoposide-induced γH2AX formation was also examined in K562^{MPO} cell lines. For 10μM etoposide, MPO expression resulted in a significant rise in γH2AX intensity (Fig. 3B), reaching a 1.8-fold increase over K562 cells for line 5 (Supplemental Table 1). At 100μM etoposide neither cell line displayed a significant increase in γH2AX over K562. However, the lack of significant effect of MPO expression when cells were treated with 100μM compared to the lower etoposide dose can be explained by the observation that γH2AX formation reaches saturation at 100μM etoposide in K562 cells (Supplemental Fig. 3F).

K562 cells transfected with empty vector were also tested for induction of γH2AX formation in comparison with non-transfected K562 cells. The empty vector control did not differ significantly from the non-transfected cells in γH2AX signal formation upon etoposide treatment (Supplemental Fig. 3I). Thus, MPO activity results in elevated H2AX phosphorylation in etoposide-treated cells.

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MPO stimulates mitoxantrone-induced accumulation of TOP2-DNA complexes and H2AX phosphorylation.

The inhibitor SA, which effectively abolishes MPO activity in NB4 cells (Fig. 1), resulted in a substantial reduction in stabilised TOP2-DNA complexes induced by mitoxantrone (Fig. 4A). After subtraction of signal detected in control cells (0μM Mitoxantrone), TOP2A and TOP2B complexes induced by 0.5μM mitoxantrone were reduced by 59 and 88% respectively, while at 1μM mitoxantrone the figures were 43% and 63% (Supplemental Table 1). Similarly, SA pre-treatment reduced the mitoxantrone induced γH2AX signal by 39% for1μM mitoxantrone (Fig. 4B).

K562^{MPO} cell line 2 expresses MPO activity at 20% of the level observed in NB4 cells (Fig. 4C). Mitoxantrone (1μM) induced 2.2 and 2.9 fold more stabilised TOP2A and TOP2B complexes in K562^{MPO} cell line 2 than in parental K562 cells (Fig. 4D, Supplemental Table 1).

Like etoposide, mitoxantrone is prone to oxidation by peroxidases such as MPO, and mitoxantrone can form DNA adducts via activation by formaldehyde (Panousis *et al.*, 1995; Parker *et al.*, 1999), but less is known about the impact of this on TOP2-DNA complex formation in cells, or the downstream accumulation of DNA DSBs. The observed MPO activity requirement for maximal TOP2 complex formation as well as H2AX phosphorylation supports the idea that MPO-mediated oxidation directly or indirectly enhances the activity of mitoxantrone as a TOP2 poison.

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Glutathione depletion stimulated etoposide and mitoxantrone induced DNA damage in an MPO-dependent manner

Etoposide and mitoxantrone metabolites react with the low molecular weight thiol GSH (Yalowich *et al.*, 1996; Panousis *et al.*, 1997; Kagan *et al.*, 2001; Fan *et al.*, 2006). It follows that the effect of high MPO expression on TOP2 poison-mediated DNA damage in cells may therefore be limited by cellular GSH. In order to test this we used buthionine sulfoximine (BSO) which inhibits γ-glutamylcysteine synthetase, and thus leads to GSH depletion in cells (Griffith, 1982). The addition of BSO did not affect TOP2 activity in *in vitro* activity assays (Supplemental Fig. 2 F-H). Glutathione levels were reduced by more than 70% following pretreatment with BSO (150μM, 4.5hr, Fig. 5A). BSO-treated NB4 cells exhibited elevated levels of TOP2A and TOP2B-DNA complexes induced by 10μM etoposide (1.9 and 3.4 fold respectively), and for 100μM etoposide BSO increased TOP2B-DNA complexes (1.6 fold) but did not increase TOP2A-DNA complex levels (Fig. 5B&C, Supplemental Table 2). In addition, BSO pre-treatment significantly increased etoposide-induced H2AX phosphorylation at both doses of etoposide (2.2 and 2.0 fold increase respectively, Fig. 5D & Supplemental Table 2). Significantly, BSO had no effect on TOP2 complexes or H2AX phosphorylation induced by etoposide when cells were pre-treated with the MPO inhibitor SA. This is consistent with the previously described MPO-dependent (and therefore SA-suppressed) generation of etoposide quinone, via the phenoxy-radical (Haim *et al.*, 1986; Kagan *et al.*, 2001), leading to elevated TOP2-DNA complex accumulation and histone H2AX phosphorylation due to elevated TOP2 poison activity (Gantchev and Hunting, 1998; Jacob *et al.*, 2011). This activity is then enhanced under conditions of depleted glutathione, which would otherwise reduce the phenoxy radical and/or combine with etoposide quinone. BSO pre-treatment also resulted in elevated mitoxantrone-induced stabilised TOP2-DNA complexes and H2AX phosphorylation (Fig. 6 A-C), although the magnitude of the effect was smaller than for etoposide (Fig. 5, Supplemental Table 3).

Direct small molecule inhibitors of MPO suppress etoposide and mitoxantrone-induced TOP2A and TOP2B covalent DNA complex formation and H2AX phosphorylation in NB4 cells.

The work described above employed the heme synthesis inhibitor SA which indirectly reduces cellular MPO activity. Recently a number of direct specific MPO inhibitors have been developed. These include PF-1355 (2-(6-(2,5-dimethoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2H)-yl)acetamide) (Zheng *et al.*, 2015) and MPOi-II (4-(5-Fluoro-1H-indol-3-yl)butanamide) (Soubhye *et al.*, 2013). As expected, both of these inhibitors dramatically reduced MPO activity in NB4 cells (Supplemental Fig. 4). After 4 hours MPO activity was undetectable in PF-1355 treated cells and for MPOi-II activity was reduced by more than 90%. Pre-treatment with either inhibitor also reduced the

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levels of TOP2A and TOP2B DNA complexes and H2AX phosphorylation induced by etoposide or mitoxantrone in NB4 cells, having a greater effect on TOP2B (Fig. 7). The magnitudes of the effects were comparable to those observed with SA pre-treatment (Supplemental Table 2 & Figs 1C, 1D, 3A, 4A, 4B). As was observed for SA, PF-1355 and MPOi-II did not affect induction of TOP2A or TOP2B complexes or H2AX phosphorylation induced by etoposide or mitoxantrone in MPO non-expressing K562 cells (Supplemental Fig. 4).

Discussion

Therapy-related acute leukemia is a rare, but life threatening complication of prior treatment for a primary cancer. Therefore, interventions which specifically protect myeloid cells from the unwanted effects of cytotoxic chemotherapies would be very welcome. MPO is expressed exclusively in cells of myeloid origin and is capable of enzymatic conversion of TOP2 poisons including etoposide, mitoxantrone and anthracyclines, to species with greater DNA damaging activity. So, in principle, MPO inhibition should partially protect myeloid precursors from TOP2 poison-mediated DNA damage, while preserving the desired cytotoxic effects in the target tumour cells. This idea is supported by the data reported here showing that three chemically distinct MPO inhibitors each reduce etoposide and mitoxantrone-induced TOP2-DNA complex and γH2AX formation in NB4 cells; while conversely, exogenous MPO expression in K562 cells leads to increased etoposide and mitoxantrone-induced damage.

Notably, the suppression of MPO activity had an approximately three-fold greater effect on TOP2B complex formation than on TOP2A. The greater effect for TOP2B is of interest as this TOP2 isoform appears to be required for the majority of etoposide-induced *MLL* and *RUNX1* chromosomal breaks (Cowell *et al.*, 2012; Smith *et al.*, 2013) in a human lymphoblastoid cell line model and for etoposide-mediated carcinogenesis in a mouse model (Azarova *et al.*, 2007). The MPO-dependent enhancement of the TOP2 poisoning activity of etoposide is likely to be due to production of etoposide phenoxy radicals and etoposide ortho-quinone in MPO expressing cells (see Supplemental Fig. 2A). Etoposide phenoxy radicals have been observed *in vitro* and in cell culture systems (Haim *et al.*, 1986; Kalyanaraman *et al.*, 1989; Yalowich *et al.*, 1996; Kagan *et al.*, 2001; Vlasova *et al.*, 2011) and can be further oxidised to etoposide quinone (Haim *et al.*, 1986; Fan *et al.*, 2006) which exhibits enhanced TOP2 dependent DNA cleavage activity compared to etoposide, especially for TOP2B (Jacob *et al.*, 2011; Smith *et al.*, 2014). Etoposide ortho-quinone reacts spontaneously with GSH in solution (Fan *et al.*, 2006), and so the effect of MPO expression on TOP2-DNA complex accumulation may be limited by GSH titration of etoposide quinone within cells. Indeed, we found that when GSH

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was depleted by BSO, treatment of NB4 cells with 10μM etoposide resulted in significantly elevated TOP2A and TOP2B-DNA complex accumulation (1.9 and 3.4 fold respectively), and this effect was abolished by SA pre-treatment (Fig. 5). Notably though, although glutathione depletion amplified the effect of SA on etoposide induced TOP2-DNA complexes, we were still able to measure suppression of etoposide-induced TOP2A and TOP2B complexes in the absence of BSO.

While these data were mostly obtained with NB4 cells which express MPO at a high level (Supplemental Fig. 1) (Hu *et al.*, 1993), enhanced etoposide-induced TOP2-DNA complex formation and H2AX phosphorylation were also observed in K562 cells exogenously expressing MPO activity at less than 50% of the level of NB4 cells. For the analogous mitoxantrone experiments, exogenously expressed MPO resulted in a 1.6 and 2.9-fold increase in TOP2A and TOP2B-DNA complexes respectively, even though MPO was only expressed at 20% of the NB4 level. Thus, it appears that MPO can stimulate TOP2-mediated DNA damage even when expressed at moderate levels more similar to those that exist in bone marrow myeloid precursor cells.

While our results with etoposide in NB4 cells can be explained by etoposide redox activity in MPO expressing cells and the finding that etoposide quinone is a more effective TOP2 poison than its parent compound *in vitro* (Jacob *et al.*, 2011; Smith *et al.*, 2014), the situation is less clear for mitoxantrone. Mitoxantrone can be oxidised by MPO, and products of this oxidation react covalently with DNA, both in cell-free systems and in cells (Panousis *et al.*, 1995, 1997). Furthermore, mitoxantrone is activated by formaldehyde, also resulting in covalent interaction with DNA (Parker *et al.*, 1999). However, it is currently unclear whether the metabolic products of mitoxantrone function as TOP2 poisons, and if so what their relative activity is compared to the parent compound. We show here that MPO downregulation significantly impairs mitoxantrone-induced TOP2-DNA complex accumulation in NB4 cells. Again the effect is larger for TOP2B than TOP2A. For 0.5μM mitoxantrone, SA pre-incubation decreased TOP2B-DNA complexes to a level that was no longer significantly above the background level obtained in the absence of mitoxantrone. Exogenous expression of MPO in K562 cells resulted in a more than doubling in TOP2A and TOP2B-DNA complexes induced by mitoxantrone. Together, these data strongly suggest that oxidative metabolism of mitoxantrone leads to increased TOP2A and TOP2B poisoning. However, our data do not distinguish between direct enhanced poisoning of TOP2 by mitoxantrone metabolites, and generation of DNA adducts or repair intermediates that act as TOP2 poisons (Kingma *et al.*, 1995; Sabourin and Osheroff, 2000).

As was observed for etoposide, GSH depletion increased the level of TOP2A and TOP2B-DNA complexes induced by mitoxantrone consistent with the involvement of a metabolite that reacts

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readily with free thiols. Furthermore, high intensity chemotherapy leads to depletion of thiols including GSH (Jonas *et al.*, 2000; Kasapović *et al.*, 2010; Kadam and Abhang, 2013), and so the high level of TOP2 poison-induced DNA damage observed in BSO-treated cells is likely to reflect the generation of genetic lesions in bone marrow cells during cytotoxic chemotherapy regimens.

MPO has become a target of interest for novel anti-inflammatory drug development (Malle *et al.*, 2007) and a number of potent specific MPO inhibitors have recently been reported (Tiden *et al.*, 2011; Forbes *et al.*, 2013; Soubhye *et al.*, 2013, 2016; Li *et al.*, 2015; Zheng *et al.*, 2015). We have used two such inhibitors, PF1355 and MPOi-II and show that like SA, they reduce etoposide and mitoxantrone-induced damage in MPO-expressing cells. Thus, novel compounds developed for a different clinical need could, in principle, be repurposed to reduce unwanted and carcinogenic TOP2 poison-induced genotoxic damage in critical bone marrow cells and could have a significant impact in the frequency of therapy-induced secondary leukemias.

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Author contributions

Participated in research design: Atwal, Austin, Cowell

Conducted experiments: Atwal, Lishman, Austin

Performed data analysis: Atwal, Cowell

Wrote or contributed to writing manuscript: Atwal, Austin, Cowell

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Footnotes

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Figure Legends

Figure 1. MPO inhibition reduces the level of TOP2-DNA covalent complexes formed by etoposide in NB4 cells. (A & B) Succinylacetone (SA) abolished MPO activity (A) and reduced mature protein levels in NB4 cells (B); NB4 cells were treated with 200μM SA for 48hr and assayed for MPO activity (A) and MPO protein level by western blotting of whole cell extracts (B). Blots were quantified by densitometry. **(C & D)**. TOP2-DNA covalent complexes were quantified by TARDIS analysis using antibodies specific to TOP2A (C), or TOP2B (D). Integrated fluorescence values were determined per nucleus (at least 500 nuclei per treatment per replicate experiment). From these, median values were obtained for each treatment and means of the medians were calculated from replicate experiments (n=3). Data are expressed as a percentage of the mean value obtained with 100μM etoposide in the absence of SA, ±S.E.M. Integrated fluorescence data corresponding to an individual experiment are also shown in Supplemental Fig. 3 A&B.

Figure 2. Expression of active MPO in K562 cells increases the level of etoposide-stabilised TOP2-DNA covalent complexes. (A) Comparison of MPO activity (left) and MPO immunofluorescence (right) in K562 cells, two MPO-expressing K562-derived cell lines ($K562^{MPO\ line4}$ $K562^{MPO\ line5}$) and in NB4 cells. **(B)** K562 and $K562^{MPO}$ cell lines were incubated with 10μM or 100μM Etoposide or a vehicle control for 1 hour. Etoposide-stabilised TOP2-DNA complexes were quantified by TARDIS analysis using antibodies specific for TOP2A or TOP2B as described for Fig. 1. Numbers of replicates are indicated.

Figure 3. MPO activity results in raised levels of etoposide-induced H2AX phosphorylation. (A) NB4 cells were pre-treated for 48hrs with SA (200μM) then incubated with etoposide (10μM or 100μM). **(B)** K562 and $K562^{MPO}$ cell lines were incubated with 10μM and 100μM etoposide or DMSO vehicle control. For both (A) and (B) γH2AX was quantified by immunofluorescence. Analysis was carried out as described for TARDIS analysis in Figure 1. Data are expressed relative to the mean values obtained with 100μM etoposide in the absence of SA (A) or in WT parental K562 cells (B). Numbers of replicates are indicated.

Figure 4. MPO activity enhances mitoxantrone-induced TOP2-DNA covalent complex formation and H2AX phosphorylation. (A & B) NB4 cells were pre-treated with 200μM SA for 48hrs followed by a 1-hour incubation with 0.5μM or 1μM mitoxantrone, or a vehicle control. TOP2-DNA covalent complexes and γH2AX were quantified as in Figs 1 & 3. Data are expressed relative to the mean values obtained with 1μM mitoxantrone in the absence of SA. **(C)** Quantification of MPO activity in $K562^{MPO}$ line 2 compared to NB4 and parental K562 cells. **(D)** MPO expression in K562 cells results in enhanced mitoxantrone-induced TOP2-DNA protein complex formation. Data are expressed relative to the mean value obtained for parental K562 cells treated with 1μM mitoxantrone. Numbers of replicates are indicated.

Figure 5. Glutathione depletion increases etoposide-mediated TOP2-DNA covalent complex formation and H2AX phosphorylation. (A) BSO pre-incubation (150μM, 4.5 hours) resulted in 70% reduction of total and reduced glutathione in NB4 cells. **(B-D)** NB4 cells were incubated in the presence or absence of SA (200μM) for 48hours, BSO (150μM) for 4.5 hours, with both or with

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neither, followed by addition of 10μM or 100μM etoposide for 1 hour. TARDIS and γH2AX assays were performed as described in Figs 1 & 3. Numbers of replicates are indicated.

Figure 6. Glutathione depletion potentiates mitoxantrone-mediated TOP2 covalent complex formation and H2AX phosphorylation. NB4 cells were pre-incubated with SA, BSO or both as described for Fig 4, followed by addition of 1μM mitoxantrone for 1 hour. TARDIS and γH2AX assays were performed as described in Figs 1 & 3.

Figure 7. Direct MPO inhibition reduces the level of TOP2-DNA covalent complexes formation and H2AX phosphorylation induced by etoposide or mitoxantrone in NB4 cells. (A & B) NB4 cells were pre-treated with MPO inhibitors PF-1355 (10 μM, 4hr) or MPOi-II (5 μM, 4 hr) before adding 10 μM or 100 μM etoposide, or a vehicle control for one hour. **(C & D)** NB4 cells were pre-treated with MPO inhibitors as in A & B before adding 0.5 μM or 1 μM mitoxantrone or a vehicle control for one hour. TOP2-DNA covalent complexes (A & C) and γH2AX (B & B) were quantified as in Figs 1 & 3. Numbers of replicates are indicated.

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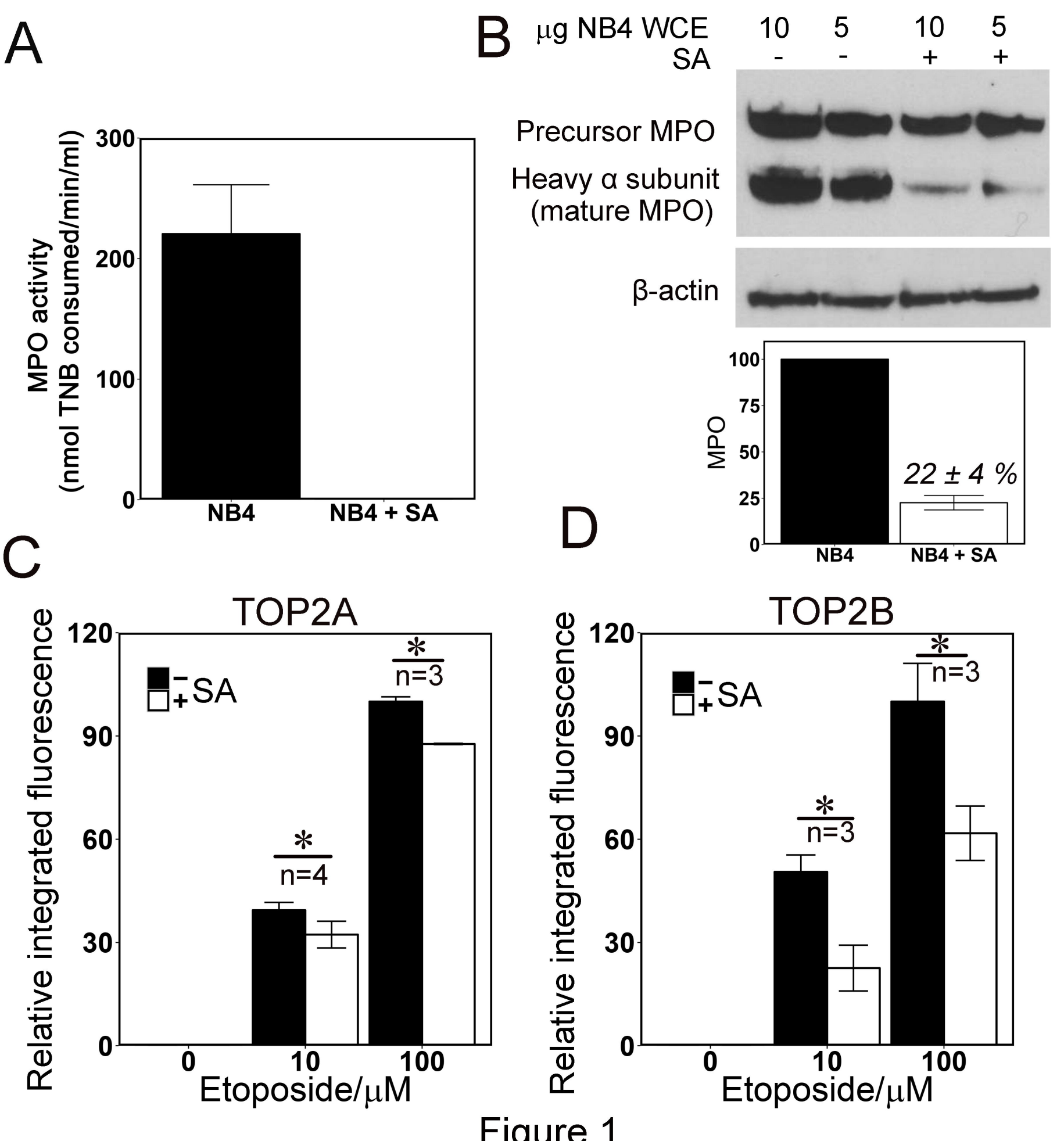
A
MPO activity
(nmol TNB consumed/min/ml)
300
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100
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NB4 + SA
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μg NB4 WCE
10
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10
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SA
-
-
+
+
Precursor MPO
Heavy α subunit
(mature MPO)
β-actin
MPO
100
75
50
25
0
22 ± 4 %
NB4
NB4 + SA
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TOP2A
Relative integrated fluorescence
120
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Etoposide/μM
D
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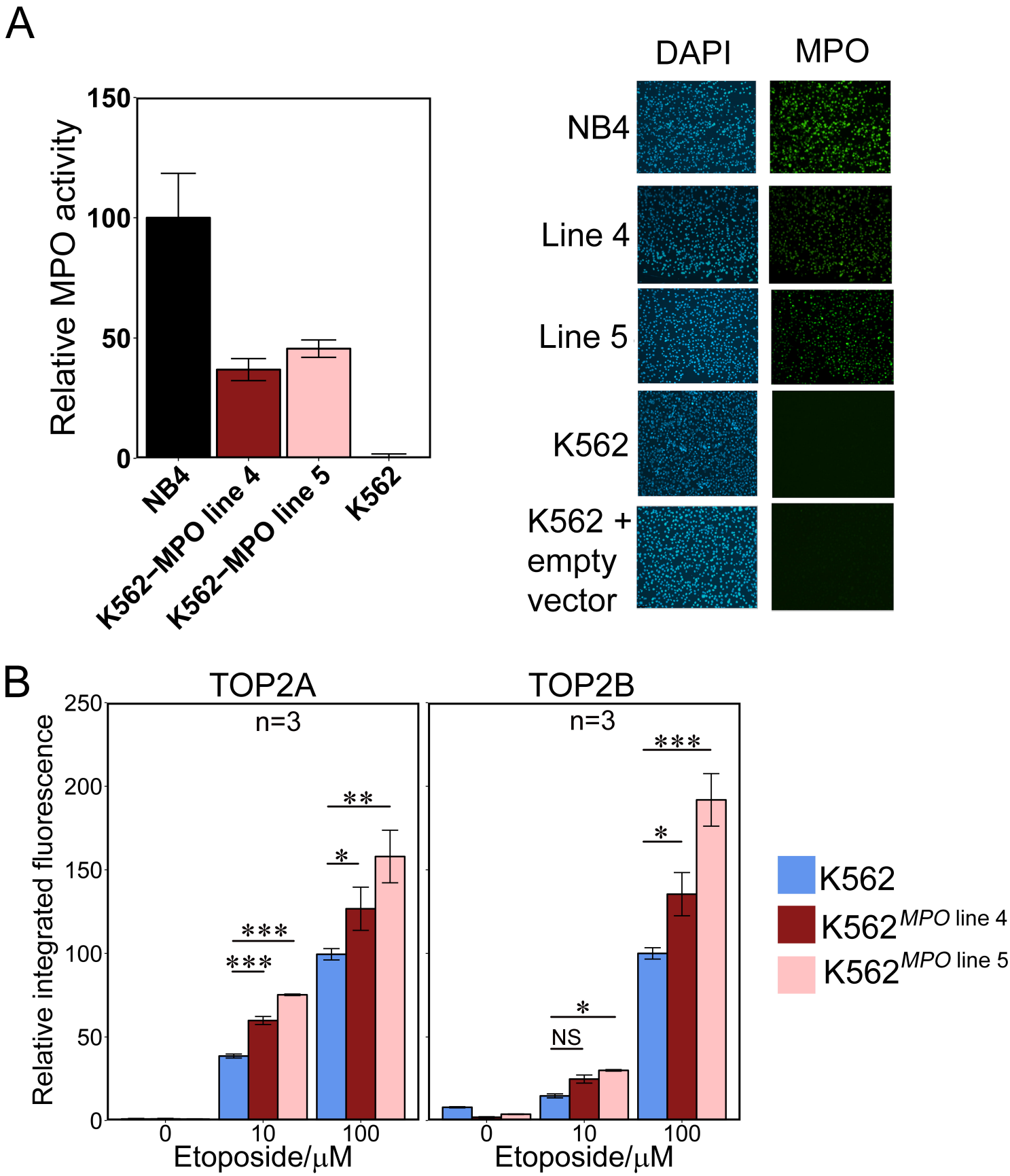
100
Etoposide/μM

Figure 1


A
DAPI
MPO
NB4
Line 4
Line 5
K562
K562 + empty vector
Relative MPO activity
150
100
50
0
NB4
K562–MPO line 4
K562–MPO line 5
K562
B
TOP2A
TOP2B
n=3
n=3
Relative integrated fluorescence
250
200
150
100
50
0
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NS
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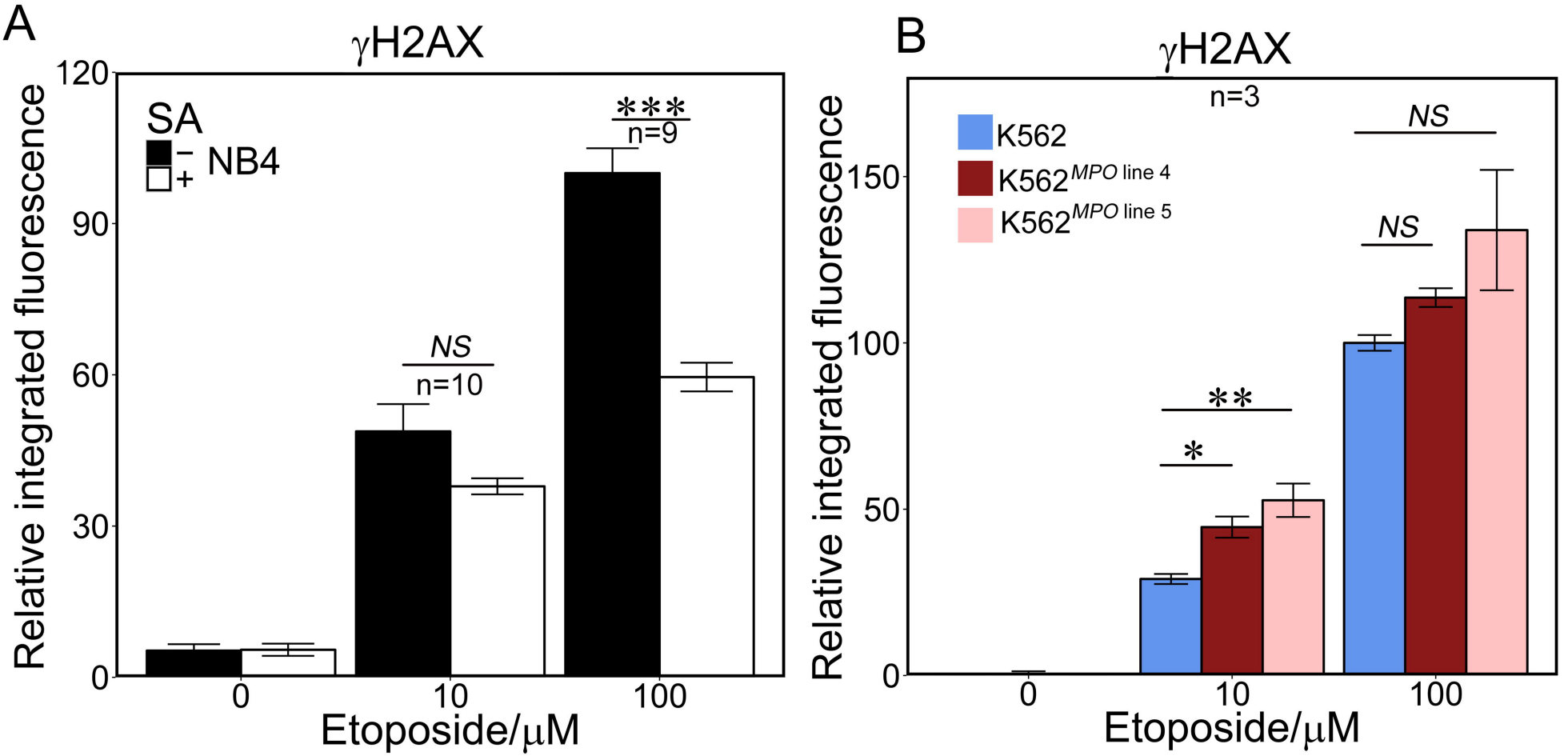
0
10
100
Etoposide/μM
K562
K562MPO line 4
K562MPO line 5

Figure 2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γH2AX
SA
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Relative integrated fluorescence
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90
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NS
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Etoposide/μM
B
γH2AX
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K562
K562MPO line 4
K562MPO line 5
Relative integrated fluorescence
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NS
NS
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Etoposide/μM

Figure 3

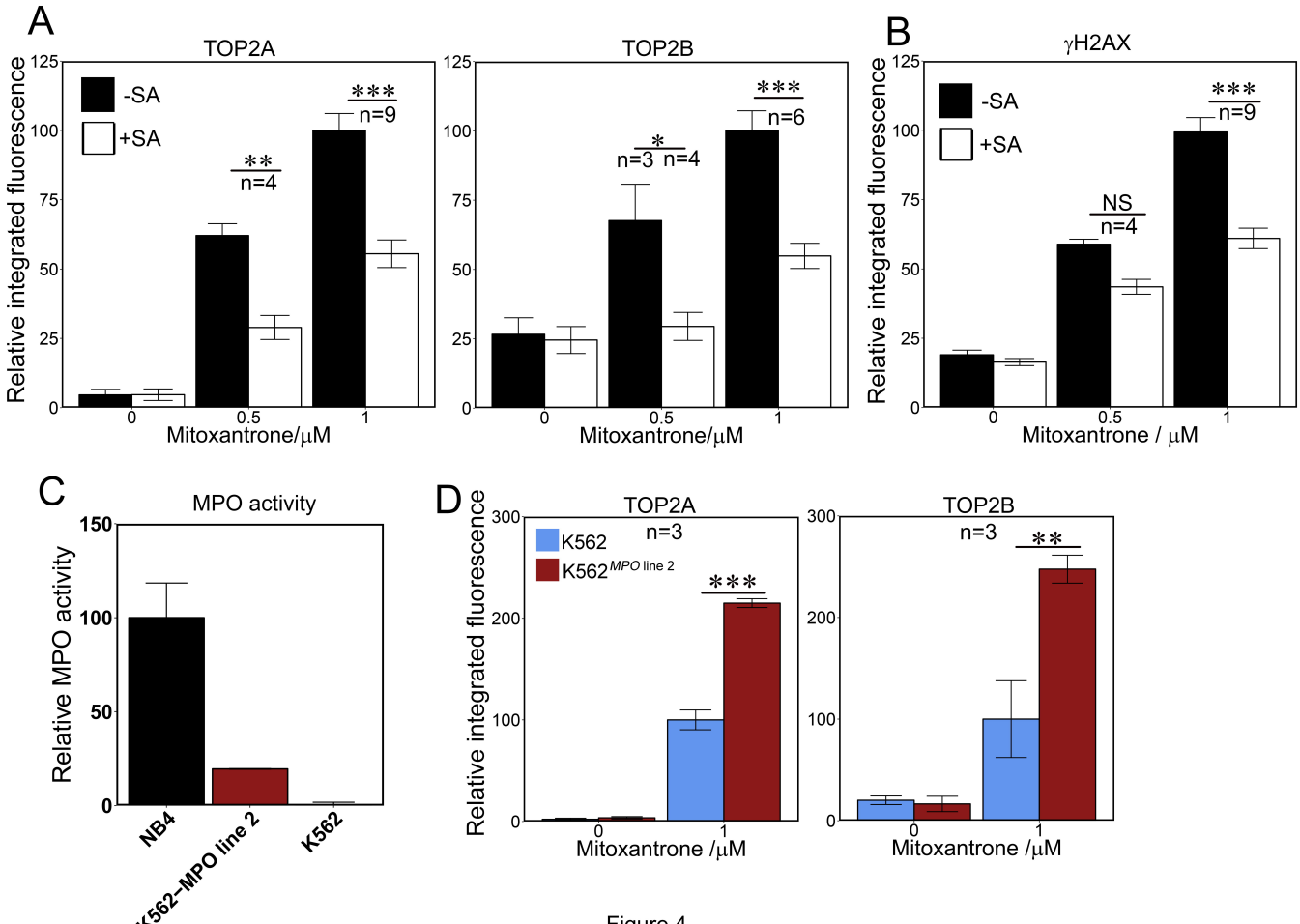
A
TOP2A
TOP2B
-SA
+SA
**
n=4
***
n=9
*
n=3 n=4
***
n=6
Relative integrated fluorescence
Mitoxantrone/μM
B
γH2AX
NS
n=4
***
n=9
Mitoxantrone / μM
C
MPO activity
Relative MPO activity
NB4
K562-MPO line 2
K562
D
TOP2A
TOP2B
n=3
K562
K562MPO line 2
***
**
Relative integrated fluorescence
Mitoxantrone /μM

Figure 4

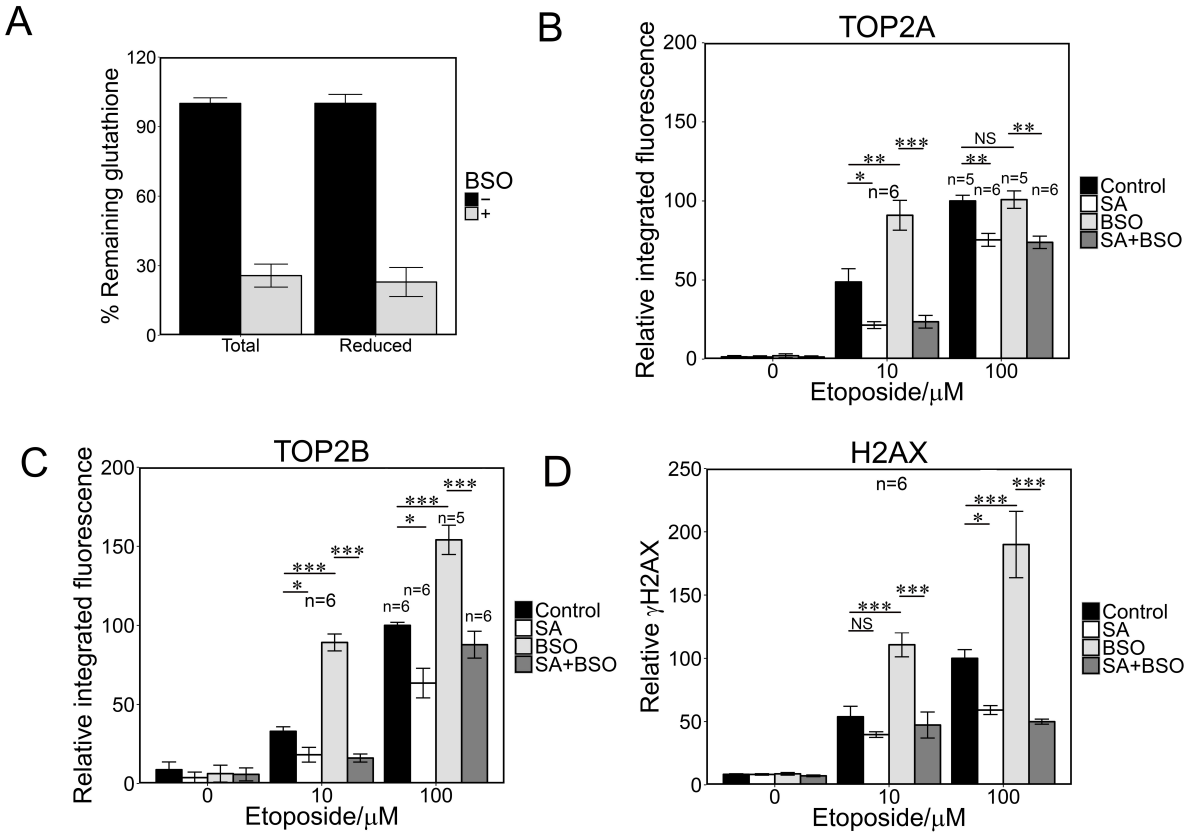
A
% Remaining glutathione
120
90
60
30
0
Total
Reduced
BSO
–
+
B
TOP2A
Relative integrated fluorescence
200
150
100
50
0
0
10
100
Etoposide/μM
Control
SA
BSO
SA+BSO
n=6
n=5
NS
C
TOP2B
Relative integrated fluorescence
D
H2AX
Relative γH2AX
250
n=6
NS

Figure 5

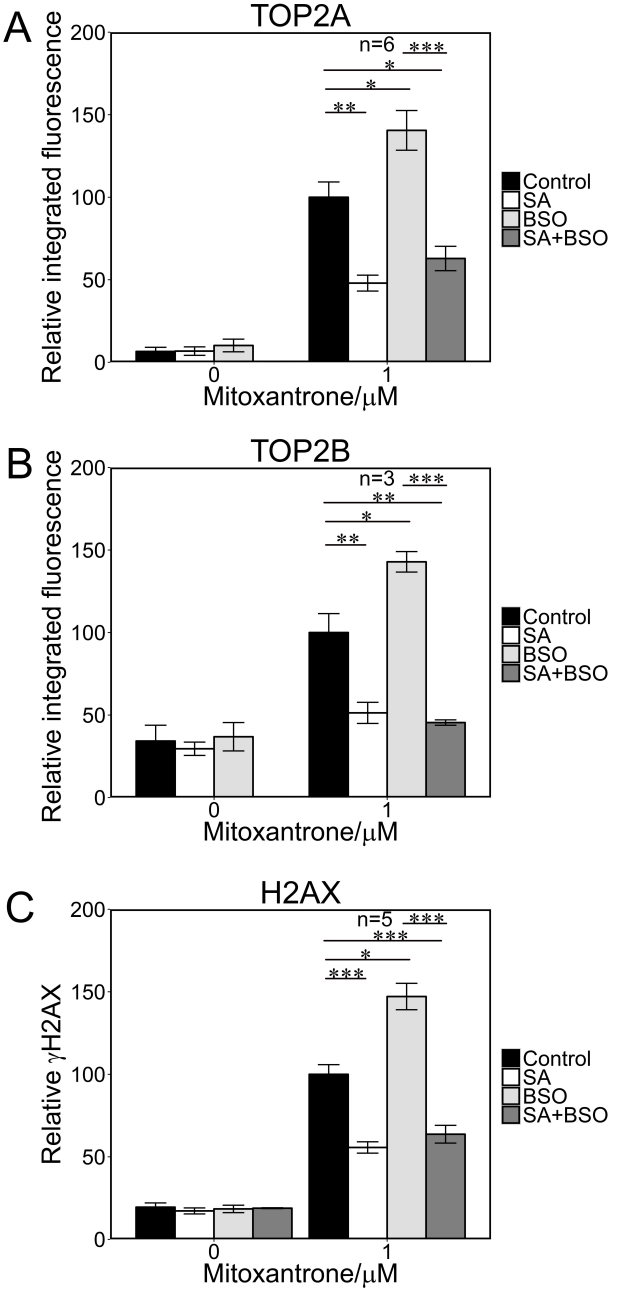
A
TOP2A
n=6
***
*
*
**
Relative integrated fluorescence
200
150
100
50
0
0
1
Mitoxantrone/μM
Control
SA
BSO
SA+BSO
B
TOP2B
n=3
***
**
*
**
Relative integrated fluorescence
200
150
100
50
0
0
1
Mitoxantrone/μM
Control
SA
BSO
SA+BSO
C
H2AX
n=5
***
***
*
***
Relative γH2AX
200
150
100
50
0
0
1
Mitoxantrone/μM
Control
SA
BSO
SA+BSO

Figure 6

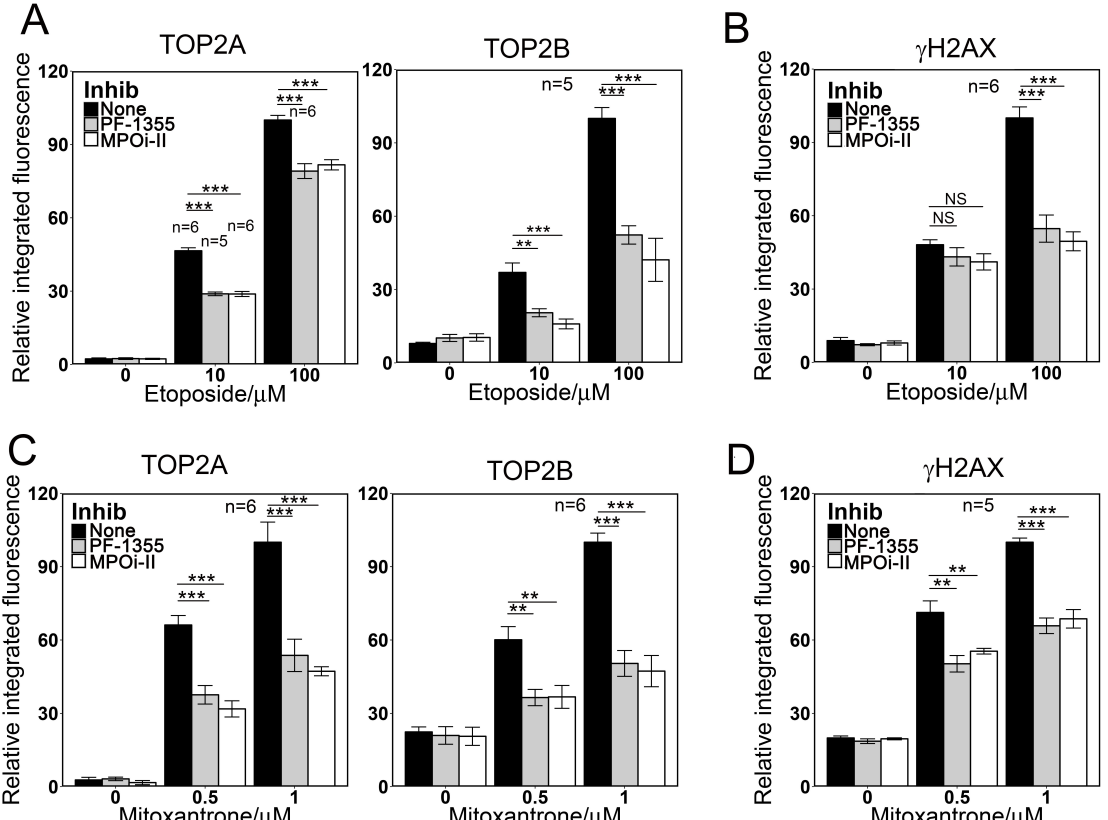
A
TOP2A
TOP2B
B
γH2AX
C
TOP2A
TOP2B
D
γH2AX
Inhib
None
PF-1355
MPOi-II
Relative integrated fluorescence
Etoposide/μM
Mitoxantrone/μM
n=5
n=6
NS
***
**

Figure 7