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Unravelling the role of SNM1 in the DNA repair system of *Trypanosoma brucei*

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Running title: Characterising a trypanosomal DNA repair enzyme

SUMMARY

All living cells are subject to agents that promote DNA damage. A particularly lethal lesion are interstrand crosslinks (ICL), a property exploited by several anti-cancer chemotherapies. In yeast and humans an enzyme that plays a key role in repairing such damage are the PSO2/SNM1 nucleases. Here, we report that *Trypanosoma brucei*, the causative agent of African trypanosomiasis, possesses a *bona fide* member of this family (called TbSNM1) with expression of the parasite enzyme able to suppress the sensitivity yeast *pso2Δ* mutants display toward mechlorethamine, an ICL-inducing compound. By disrupting the Tb*snm1* gene, we demonstrate that TbSNM1 activity is non-essential to the medically relevant *T. brucei* life cycle stage. However, trypanosomes lacking this enzyme are more susceptible to bi- and tri-functional DNA alkylating agents with this phenotype readily complemented by ectopic expression of Tb*snm1*. Genetically modified variants of the null mutant line were subsequently used to establish the anti-parasitic mechanism of action of nitrobenzylphosphoramide mustard and aziridinyl nitrobenzamide prodrugs, compounds previously shown to possess potent trypanocidal properties while exhibiting limited toxicity to mammalian cells. This established that these agents, following activation by a parasite specific type I nitroreductase, produce metabolites that promote formation of ICLs leading to inhibition of trypanosomal growth.

INTRODUCTION

The socioeconomic development of sub-Saharan Africa has been hindered by a group of medical and veterinary infections collectively known as African trypanosomiasis. The causative agents of many of these diseases are protozoan parasites belonging to the species *Trypanosoma brucei*, organisms that live and multiply extracellularly in the bloodstream and tissue fluids of their mammalian hosts. Transmission occurs *via* the blood-feeding habits of the insect vector, the tsetse fly. Over the last 15 years implementation of improved health surveillance programmes combined with new treatment regimens has led to a dramatic fall in the estimated number of new cases of the human form of the disease, known as human African trypanosomiasis (HAT) from around 450,000 in 1997 to about 20,000 in 2012 (Barrett, 2006, WHO 2014). This situation has resulted in WHO aiming to eliminate HAT as a public health problem by 2020. In contrast, animal African trypanosomiasis, particularly in domesticated livestock, remains a major problem with these infections killing around 3 million head of cattle each year and causing an annual loss of income estimated to be about US$4.75 billion (UNFAO, 2004).

With no immediate prospect of a vaccine or chemoprophylaxis and with vector control being problematic, drug treatment represents the only option available to combat HAT. However, the current chemotherapies used are few in number and their use is controversial, as they can be costly, often require medical supervision for administration, some have limited efficacy and may cause adverse side effects, with drug resistance becoming more widespread (Wilkinson and Kelly 2009, Alsford *et al*., 2013). One way to facilitate the development of new drugs targeting HAT is to better understand the mechanism of action of existing treatments with the properties that underlie parasite selectivity incorporated into the development of new trypanocidal agents. For example, melamine rings have been incorporated into several compounds to exploit the substrate specificity displayed by the P2

adenosine transporter, a permease implicated in the uptake of pentamidine and melarsoprol (Stewart *et al*., 2004, Baliani *et al*., 2005, Chollet *et al*., 2009, Klee *et al*., 2010, Capes *et al*., 2012, Giordani *et al*., 2014). Similarly a parasite nitroreductase (NTR), an enzyme responsible for the activation of nifurtimox (Wilkinson *et al*., 2008, Hall *et al*., 2011), has been used to screen nitroaromatic libraries for anti-*T. brucei* properties (Bot *et al*., 2010, Hall *et al*., 2010, Hu *et al*., 2011, Papadopoulou *et al*., 2011, Hall *et al*., 2012, Papadopoulou *et al*., 2012, Bot *et al*., 2013, Papadopoulou *et al*., 2013). In the latter case, several NTR-activated chemicals containing nitrogen mustard or aziridine functional groups that promote DNA damage *via* formation of cross linkages, have been identified as having significant anti-parasitic activities and low mammalian cell toxicity (Bot *et al*., 2010, Hall *et al*., 2010, Hu *et al*., 2011).

Genomes are constantly challenged by endogenous and exogenous agents that promote DNA damage, with interstrand crosslinks (ICL) representing a particularly dangerous lesion (O'Connor and Kohn, 1990). Formed when the two complementary strands within the DNA double helix become covalently linked, ICL's block essential cellular process that require DNA strand separation including DNA replication and transcription, leading to chromosomal breakage, rearrangements, or cell death (Dronkert and Kanaar, 2001, McHugh *et al*., 2001, Deans and West, 2011, Sengerova *et al*., 2011). Estimates indicate that a single ICL can kill a unicellular microbe with as few as 20 being fatal to a mammalian cell (Magana-Schwencke, Henriques *et al*. 1982, Lawley and Phillips 1996). In order to preserve the integrity and functionality of DNA eukaryotic cells have evolved a series of complementary and overlapping pathways to repair ICLs, although the precise mechanisms involved in these systems are not fully understood (Deans and West, 2011). In *Saccharomyces cerevisiae* many of the major DNA repair pathways (nucleotide excision repair (NER), mismatch repair, post-replication repair/translesion synthesis and homologous recombination) have been implicated

in fixing ICL damage although only a few proteins specifically involved in ICL lesion repair have been identified (Barber *et al*., 2005, Lehoczky *et al*., 2007, Daee *et al*., 2012, Ward *et al*., 2012). Of these, Pso2p (also known as Snm1) is of great interest as cells lacking this activity are specifically and highly susceptible to ICL-forming agents including psoralen, cisplatin and mechlorethamine but not to any other forms of DNA damage (Henriques and Moustacchi, 1980, Ruhland *et al*., 1981a, Ruhland *et al*., 1981b). The precise role played by Pso2p in this repair system remains unknown although biochemical studies have shown that it displays a 5' exonuclease activity (Li *et al*., 2005). This coupled with the observation that *pso2Δ* cells exposed to ICL-inducing compounds tend to accumulate DNA double stranded breaks indicates that Pso2p does not function in the initial incision event, which in yeast is primarily controlled by NER, but may be involved in the processing of DNA ends created during the generation of ICL-associated DNA double stranded breaks (Li and Moses, 2003, Barber *et al*., 2005, Dudas *et al*., 2007). Intriguingly, Pso2p also displays a structure-specific DNA hairpin opening endonuclease activity providing evidence that it may have other functions outside ICL repair (Tiefenbach and Junop, 2012).

Here, we report that *T. brucei* expresses a Pso2/Snm1 homologue that can readily complement for the susceptibility phenotype exhibited by *pso2Δ* yeast cells towards an ICL forming agent. Deletion of the gene, designated Tb*snm1*, from the parasite genome revealed that although the encoded enzyme is not essential for viability and growth of bloodstream form (BSF) trypanosomes, cells lacking this activity were more susceptible to bifunctional nitrogen mustard- and aziridine-based ICL-inducing agents. Using recombinant *T. brucei* expressing altered levels of Tb*snm1* we establish that the trypanocidal mechanism of several potent nitroaromatic-based agents that contain ICL-promoting grouping are dependent on an initial activation catalysed by a parasite specific type I NTR that generates metabolites which then promote DNA damage.

RESULTS

Identifying trypanocidal chemical tools for studying DNA repair.

Previous screening studies have identified nitroaromatic-based azirindyl/nitrogen mustard compounds to be effective trypanocidal agents (Bot *et al*., 2010, Hall *et al*., 2010, Hu *et al*., 2011). The antimicrobial activity of these involves a parasite specific activation step catalysed by a type I NTR that leads to metabolites postulated to promote DNA damage. To determine if the above compounds do function *via* this pathway a range of anti-cancer compounds known to mediate their cytotoxicity by promoting DNA cross linkages were screened for trypanocidal activity against BSF *T. brucei*. The structures tested included non-nitroaromatic-based aziridines and nitrogen mustards, nitrosoureas, platinum complexes, an alkyl sulfonate and non-classical DNA crosslinking agents.

Out of the non-nitroaromatic anti-cancer compounds assessed, 17 had no effect on parasite growth at concentrations of up to 30 µM, including busulfan, the only alkyl sulfonate analysed here, and all 5 non-classical DNA crosslinking agents (Table 1). These were not analysed further. For the remaining compounds, the concentration that inhibits parasite growth by 50 % (IC_{50}'s) was determined (Table 1). For all the remaining classes of DNA crosslinking agents, two or more compounds displayed trypanocidal activities with IC_{50} values ranging from 13 nM for mitomycin C, the most potent agent identified here, to approximately 35 µM for mechlorethamine and ThioTEPA.

Identification of the DNA repair enzyme TbSNM1.

In other eukaryotes, the SNM1/PSO2 family of nucleases play an important role in repairing damage caused by DNA crosslinking agents (Cattell *et al*., 2010). Analysis of the *T. b. brucei* genome database (Aslett *et al*., 2010) identified a single hypothetical gene (designated as Tb*snm1*) of 2163 bp located on chromosome 4 with potential to encode for a

79.5 kDa enzyme (TbSNM1; Gene ID: Tb927.4.1480) related to this family of enzymes. Full length TbSNM1 is 42 % identical to the *T. cruzi* homologue (GenBank accession no. XP_816034) and has 27-32% identity to the leishmanial enzymes LmSNM1 (XP_001686430) and LdSNM1 (XP_003864463). When compared to yeast, plant and mammalian counterparts sequence identity ranged from 15 to 24 %. Based on sequence, TbSNM1 can be divided into two regions (Fig. 1). The amino terminal section (residues 36-182) constitutes a non-canonical metallo-ß-lactamase (MBL; pfam12706) domain containing 4 motifs (motifs 1-4), including a characteristic HxHxDH signature (motif 2), that in other SNM1/PSO2 proteins cooperate to mediate zinc co-factor binding. The second section represents a β-CASP (named after its representative member **C**PSF, **A**rtemis, **S**NM1 and **P**SO2; pfam10996) region (residues 213-519) that contains within it a stretch of 31 amino acid comprising a DRMBL (DNA repair metallo-ß-lactamase; pfam07522) domain (residues 488-519). The β-CASP region contains a fifth zinc binding motif (motif 5) but as with other SNM1/PSO2 sequences the precise location of this has yet to be defined: *in silico* analysis of TbSNM1 indicates that D220 or H497 (motifs 5' and 5'', respectively) may fulfil this role with H497 being the most likely of the two candidate residues (Callebaut *et al*., 2002). The β-CASP domain of TbSNM1 also contains a diagnostic valine residue (position 519) that indicates that the parasite enzyme is involved in DNA processing: DNA processing MBLs contain a valine residue at the equivalent site while RNA processing MBLs contain a histidine (Callebaut *et al*., 2002).

To investigate whether the *T. brucei* enzyme is a SNM1/PSO2 homologue, Tb*snm1* minus its ATG initiation codon was amplified and cloned into a version of the yeast expression vector pYCYlac111 that contains a DNA sequence encoding for the FLAG-tag epitope. The resultant plasmid was transformed into the *S. cerevisiae* wild type and *pso2*Δ strains and expression of recombinant TbSNM1 confirmed by western blot analysis (Fig. 2A). The

susceptibility of the fungal lines to mechlorethamine, a DNA crosslinking agent, was then determined and from the resultant dose-response curves the IC_{50} value for each strain calculated (Fig. 2B and C). Yeast lacking *pso2* were clearly more susceptible to the nitrogen mustard than wild type with the null mutant displaying an IC_{50} value approximately 40% that of the control strain. When Tb*snm1* was expressed in wild type yeast a slight (1.4-fold) resistance was noted. This phenotype was also observed in the *pso2*Δ strain expressing Tb*snm1* correlating with an increase in the IC_{50} value from 1.3 μM in cells lacking Pso2p to 5.7 μM in *pso2*Δ yeast expressing FLAG-TbSNM1. These data clearly shows that TbSNM1 can complement for the *pso2*Δ mutation and that the trypanosomal enzyme is a *bona fide* SNM1/PSO2 homologue.

TbSNM1 is targeted to the T. brucei nucleus.

When analysed using the PSORTII and WoLFPSORT algorithms, TbSNM1 was predicted to be targeted to the nucleus *via* a 'four pattern' RRRH (residues 428-431) nuclear localisation signal. To confirm this, the full length Tb*snm1* gene minus its ATG initiation codon was amplified and ligated in-frame and downstream of the gene encoding for the enhanced green fluorescence protein (GFP) in a trypanosomal vector that facilitated tetracycline inducible gene expression (Alsford *et al*. 2005). The resultant construct was used to transform BSF *T. brucei* and parasite clones were selected.

To induce expression of the tagged protein, cells were incubated in the presence of tetracycline for 48 hours. Recombinant parasites were examined by Western blotting using a monoclonal antibody against GFP (Fig. 3A), with extracts derived from these cells containing a band of the expected size (~105 kDa), or were fixed and examined by confocal microscopy (Fig. 3B). For parasites expressing GFP-TbSNM1, GFP fluorescence was restricted to a large single spot, a pattern reported for trypanosomal proteins localized to nucleus (Fig. 3B). To

confirm this, cells were co-stained with the DNA dye, DAPI. When the images were compared, the pattern of localization indicated that GFP-TbSNM1 was located in the larger of two compartments (the nucleus) where DAPI is found with the smaller, faint spot corresponding to the kinetoplast, the genome found in the parasites' single mitochondrion.

Functional Analysis of TbSNM1 in T. brucei.

To assess whether TbSNM1 was essential to BSF *T. brucei* an RNAi–based approach was initially employed. A DNA fragment corresponding to an internal region of Tb*snm1* was cloned into p2T7^{Ti} (Wilkinson *et al*., 2003) and the construct transformed into BSF *T. brucei*. In the absence of tetracycline, recombinant clones were found to grow at approximately the same rate as the parental cells. Addition of tetracycline to parasites harbouring the RNAi construct did not affect the growth rate suggesting that TbSNM1 is not essential to BSF *T. brucei*. To confirm this, DNA fragments corresponding to the 5' flank of Tb*snm1* and the 3' region of the Tb*snm1* gene were cloned either side of a cassette containing blasticidin or puromycin resistance markers. The integration constructs were transformed into BSF *T. brucei* with heterozygote (Tb*snm1*$^{+/-}$) and then null mutant (Tb*snm1*$^{-/-}$) lines selected. Southern hybridisation was used to confirm each integration event demonstrating that both copies of the *Tbsnm1* gene could readily be deleted from the parasite genome (Fig. 4A and B) while qPCR data analysed using the comparative C_T method showed that a full length Tb*snm1* mRNA was not expressed (data not shown) (Schmittgen and Livak, 2008). Reduction or lack of TbSNM1 had no effect on trypanosome growth (data not shown). Therefore, TbSNM1 is non-essential to BSF *T. brucei* under normal culture conditions confirming the RNAi observations.

To evaluate whether deletion of both copies of Tb*snm1* from the *T. brucei* genome altered sensitivity to chemicals that promote DNA cross linkage, null mutant cells were grown in the

presence of these agents and the IC_{50} values for each compound determined (Table 1). Cells lacking TbSNM1 were more susceptible to a range of nitrogen mustard and aziridinyl compounds, including several of the trypanocidal nitroaromatic structures previously identified (Bot *et al*., 2010, Hall *et al*., 2010, Hu *et al*., 2011). Intriguingly, Tb*snm1*$^{-/-}$ cells exhibited a larger difference in their sensitivities to the nitrogen mustards screened than that observed when using the aziridinyl compounds. When these growth assays were extended to look at other DNA damaging agents including mitomycin C, semustine, cisplatin, MMS, H_2O_2, hydroxyurea and UV light, and to the clinically used trypanocidal drugs nifurtimox, benznidazole or difluoromethylornithine (DFMO), no difference in IC_{50} was observed.

In order to demonstrate conclusively that the altered susceptibility phenotypes were specifically due to lack of TbSNM1, a complementation strategy was used. In these experiments Tb*snm1*$^{-/-}$ cells were transformed with a vector that facilitates constitutive expression of an ectopic copy of Tb*snm1* integrated into one of the parasite's tubulin arrays: wild type cells also expressing this vector were also generated. The IC_{50} of these parasites towards selected nitrogen mustard and aziridinyl compounds was determined and compared with values obtained using wild type and Tb*snm1* null mutant lines (Fig. 5A). When the susceptibility of the complemented line to the nitrobenzyl-containing nitrogen mustard (LH34) and aziridinyl (NH1) compounds was tested, the resultant dose response curves (and associated IC_{50} values) were distinct from the Tb*snm1*$^{-/-}$ cells, which displayed increased sensitivity to both agents, and equivalent to the plots observed using Tb*snm1* expressing parasites (wild type and wild type cells engineered to express elevated levels of TbSNM1) (Fig. 5A). When the screens were extended to investigate the complemented line's susceptibility to non-nitroaromatic nitrogen mustard (mechlorethamine) and aziridinyl (triethylenemelamine) compounds, a resistance phenotype was noted, with the dose response curves (and associated IC_{50} values) in the complemented line mirroring that obtained for wild

type parasites expressing elevated levels of TbSNM1 (Fig. 5A): trypanosomes (wild type and Tb*snm1* null mutants) expressing an ectopic copy of Tb*snm1* were up to 2.1-fold more resistant to mechlorethamine and triethylenemelamine than wild type.

The above complementation studies indicate that parasites (wild type or Tb*snm1*$^{-/-}$) ectopically expressing Tb*snm1* are resistant to non-nitroaromatic DNA crosslinking agents but not to the nitroaromatic-containing compounds. One reason for this could reflect that the latter structures function as prodrugs and must undergo an NTR catalysed activation step before mediating their trypanocidal DNA damaging activities.

Linking prodrug activation with DNA damage.

To identify any link between the DNA damaging and the NTR-activating pathways, both copies of the Tb*snm1* gene were deleted from *T. brucei* cells expressing an ectopic copy of Tb*ntr* and the susceptibilities of these recombinant cells towards selected nitrogen mustard and aziridinyl compounds determined (Fig. 5B). When treated with mechlorethamine or triethylenemelamine, both Tb*snm1* expressing cell lines (wild type and trypanosomes expressing elevated levels of Tb*ntr*) displayed similar dose response curves and therefore had similar IC_{50}'s to either agent (Fig. 5B). When these studies were expanded to investigate the susceptiblity of parasites lacking TbSNM1, the Tb*snm1*$^{-/-}$ null mutant line and Tb*snm1*$^{-/-}$ cells expressing the ectopic copy of Tb*ntr* displayed equivalent IC_{50} values, with both being more sensitive to mechlorethamine and triethylenemelamine than wild type (Fig. 5B). Importantly, no difference in IC_{50} was observed using either of the lines lacking Tb*snm1* indicating that Tb*ntr* plays no role in metabolising either mechlorethamine or triethylenemelamine.

When the nitrobenzyl-containing DNA crosslinking agents LH34 and NH1 were tested against the parasite lines expressing altered levels of Tb*snm1* and/or Tb*ntr* a different outcome was observed (Fig. 5B). For Tb*snm1*$^{-/-}$ parasites or wild type cells expressing an

ectopic copy of Tb*ntr*, treatment with either damaging agents resulted in increased susceptibility when compared against controls, with Tb*ntr* over expressing trypanosomes being more sensitive to LH34 and NH1 than the null mutant lines: Tb*snm1*$^{-/-}$ cells and *T. brucei* over expressing Tb*ntr* were 18.0- and 24.0-fold more susceptible to LH34, respectively, with a 2.7- and 31.1-fold increase in sensitivity noted towards NH1. For Tb*snm1*$^{-/-}$ null parasites expressing an ectopic copy of Tb*ntr* this increase in potency was magnified further with these cells showing a 80.0- and 38.7-fold increase in susceptibility towards LH34 and NH1, respectively, when as compared against wild type.

DISCUSSION

Currently, very little is known about the mechanisms *T. brucei* employs to repair ICL damage even though this parasite is exposed to such deleterious insults throughout its cell and life cycles. In other unicellular eukaryotes such as budding and fission yeast, the processing of ICLs occurs through the concerted activities of several major DNA repair pathways with one enzyme, Pso2p, playing a central and specific role in fixing such lesions. Although non-essential for yeast viability, the importance of Pso2p is only apparent in its absence on exposure to ICL-inducing, bifunctional alkylating agents but not to monofunctional alkylating agents, ionizing radiation or ultraviolet light (Henriques and Moustacchi, 1980, Ruhland *et al*., 1981a, Ruhland *et al*., 1981b). Here, we report the characterisation of TbSNM1, a trypanosomal Pso2p homologue, and demonstrate that this enzyme plays a key role in processing ICL lesions when generated by bifunctional nitrogen mustard and aziridinyl compounds including several nitroaromatic-based agents previously shown to have potent anti-trypanosomal properties with low toxicity to mammalian cells (Bot *et al*. 2010, Hall *et al*., 2010, Hu *et al*., 2011).

In terms of its sequence, TbSNM1 displays the main characteristics found in other PSO2/SNM1 proteins, possessing adjacent MBL and β-CASP domains that together form the enzyme's zinc-binding central catalytic core (Cattell *et al*., 2010). To confirm the *in silico* identification a complementation approach was undertaken. This involved ectopically expressing the trypanosomal enzyme in a yeast *pso2Δ* line and then evaluating the susceptibility of the resultant cells to mechlorethamine, a bifunctional alkylating agent routinely used as an ICL-inducing agent. In this genetic background the parasite protein was able to revert the susceptibility phenotype displayed by the *pso2Δ* line resulting in an additional slight (approximately 2-fold) resistance towards this nitrogen mustard. This confirmed that the trypanosomal enzyme is a genuine Pso2p homologue and that it plays a role in the processing of ICL lesions. Further, as TbSNM1 can complement for the *pso2* mutation then the parasite enzyme may interact with the same partner proteins as its yeast counterpart. For example, Pso2p contains an ubiquitin binding zinc finger (UBZ) C2HC motif upstream of its catalytic core (Yang *et al*., 2010). By analogy with hSNM1A, the only human PSO2/SNM1 homologue able to complement the yeast *pso2* mutation (Hazrati *et al*., 2008), this signature sequence is able to facilitate binding to monoubiquinated PCNA leading to recruitment of this repair enzyme to ICL-stalled replication forks (Yang *et al*., 2010). Interestingly, *in silico* searches failed to identify any known UBZ C2HC domain or any other type of ubiquitin interaction motifs (UIM) in the parasite protein sequence. Therefore, if formation of PSO2/SNM1-containing DNA repair complexes at the site of ICL damage does involve PCNA ubiquitylation then the molecular mechanisms underlying TbSNM1 recruitment to such lesions occurs through an as yet uncharacterised UIM or *via* interactions involving a conserved adapter protein. Recently, it has been shown that the β-CASP domain of Pso2p can be phosphorylated leading to the suggestion that this event may play a role in modulating the enzyme's exo- or endo-nucleolytic activity (Munari *et al*., 2014). Whether

313 TbSNM1 undergoes a similar posttranslational modification and how this effects its nuclease
314 activity has yet to be established.

315 The endogenous function of TbSNM1 is non-essential to replicating *T. brucei*: both copies
316 of *Tbsnm1* could be deleted from the genome of BSF trypomastigote parasites. However, the
317 importance of this enzyme to the trypanosome only became evident following exposure to
318 ICL-inducing compounds: null mutant cells were more susceptible to bi- and tri-functional
319 alkylating agents as compared to controls while these recombinant cells display an equivalent
320 sensitivity to wild type when exposed to MMS, UV irradiation and H_2O_2, treatments
321 normally repaired by homologous recombination, nucleotide excision repair or base excise
322 repair pathways. This trait was solely due to loss of TbSNM1 activity as expression of an
323 ectopic copy of Tb*snm1* in the null mutant genetic background restored the recombinant
324 parasites IC_{50} near to wild type levels. Intriguingly, the range of compounds that elicits the
325 change in susceptibility in the Tb*snm1*$^{-/-}$ trypanosomes although similar to that noted for the
326 yeast *pso2Δ* line does have some notable differences (Henriques and Moustacchi, 1980,
327 Ruhland *et al*., 1981a, Ruhland *et al*., 1981b). For example, yeast *pso2* mutants are reported
328 to be more susceptible to cisplatin and mitomycin C while *T. brucei* lacking TbSNM1 display
329 sensitivities similar to that exhibited by wild type parasites. This may be because that
330 although both compounds can function as ICL-inducing agents they can also mediate their
331 cytotoxic activities *via* other mechanisms including promoting formation of intrastrand
332 crosslinks, activating signal transduction pathways, stimulating redox cycling, acting as
333 enzyme inhibitors or alkylating other biological molecules (Sharma and Tomasz, 1994,
334 Pagano *et al*., 2003, Siddik, 2003, Rabik and Dolan, 2007, Paz *et al*., 2012). One (or a
335 combination) of these alternative modes of action (or possibly another unidentified
336 mechanism) may account for cisplatin's and mitomycin C's trypanocidal properties therefore
337 negating the requirement for a TbSNM1-dependent ICL repair pathway.

Previous trypanocidal screening programmes have identified nitrobenzylphosphoramide mustards (NBPMs) and aziridinyl nitrobenzamides (ANBs) as having potent anti-parasitic activity (Bot *et al*. 2010, Hall *et al*., 2010, Hu *et al*., 2011). These agents function as prodrugs and must be activated before they can mediate their cytotoxic effects, a reaction catalysed a NADH dependent type I NTR. This reduction causes the conversion of a conserved electron withdrawing nitro-group present on the compound's benzyl ring to an electron donating hydroxylamine derivative (Bot *et al*. 2010, Hall *et al*., 2010, Hu *et al*., 2011). This action effectively acts as an electronic switch that is believed to turn on the alkylating ability of the nitrogen mustard or aziridinyl moiety causing ICL-mediated DNA damage. Using Tbs*nm1* null mutant parasites engineered to express elevated levels of TbNTR we have now demonstrated a link between prodrug activation and ICL formation. Here we observed that wild type parasites exhibited the highest IC_{50} values towards LH34 (a nitrobenzylphosphoramide mustard) and NH1 (an aziridinyl nitrobenzamide) while trypanosomes lacking TbSNM1 and cells over expressing TbNTR had intermediate sensitivities. Interestingly, Tb*snm1* null mutants that also express elevated levels of TbNTR were the most prone to both compounds. This implies that following uptake, LH34 and NH1 are transported into mitochondrion where they undergo TbNTR mediated reduction to form the bioactive products. These observations suggest that in parasites where the NTR activity is over expressed this conversion occurs at a faster rate than in wild type cells resulting in increased sensitivity to the compound. A portion of the resulting metabolites are then able to access the nucleus where they induce ICL formation. In the absence of TbSNM1, mutant cells are less able to repair this type of DNA damage, resulting in an increased susceptibility to the ICL-inducing agent. In parasites where both TbSNM1 and TbNTR levels have been altered, this susceptibility phenotype is exacerbated. Intriguingly, the difference in sensitivities between Tb*snm1*$^{-/-}$ cells expressing elevated TbNTR levels from those over

363 expressing TbNTR alone was greater for LH34 than for NH1. This may be attributable to
364 properties of the substrate/TbNTR-generated metabolites, possibly reflecting differences in
365 cell and/or organelle uptake (TbNTR is a mitochondrial protein (Wilkinson *et al* 2008) or
366 how the substrates interact with, or how the metabolites are released from, TbNTR (*in vitro*
367 nitrobenzyl phosphoramide nitrogen mustard-based compounds interact with TbNTR more
368 readily than the aziridinyl nitrobenzamide (Hall *et al*., 2013). Additionally, as this study only
369 considers ICL formation and repair in the nuclear genome, it is plausible that LH34 and NH1
370 reduction products may also affect the mitochondrial genome with the NH1 metabolites
371 preferentially affecting this DNA containing region and not the nucleus.

372 We have now demonstrated that *T. brucei* expresses a *bona fide* homologue of the
373 PSO2/SNM1 nuclease family. The trypanosomal enzyme displays characteristics of its yeast
374 counterpart and is able to repair the DNA damage caused by bi- and tri-functional alkylating
375 agents. By exploiting parasites lacking this enzyme we were able to demonstrate that
376 following TbNTR-mediated activation nitrobenzylphosphoramide mustard and aziridinyl
377 nitrobenzamide agents, compounds previously shown to have potent trypanocidal properties
378 with little/no cytotoxicity in mammalian cells, generate metabolites that promote ICL
379 formation. Although not essential to survival of the medically relevant form of *T. brucei*, in
380 the future TbSNM1 could be targeted through the use of inhibitors to improve the potency of
381 other drugs that do cause parasite death through formation of the extremely lethal ICL.

382

383 **EXPERIMENTAL PROCEDURES**

384 ***Cell culturing.*** Bloodstream form *Trypanosoma brucei brucei* (MITat 427 strain; clone 221a
385 and a derivative (2T1) engineered to express elevated levels of TbNTR-myc) were
386 maintained in HMI-9 (Invitrogen) medium supplemented with 3g l^{-1} sodium bicarbonate,
387 0.014 % (v/v) β-mercaptoethanol and 10 % (v/v) foetal calf serum (Hirumi and Hirumi, 1989,

Wilkinson *et al*., 2008) at 37 °C under a 5 % (v/v) CO_2 atmosphere. The 2T1 cells were grown in the presence of 1 μg ml^{-1} phleomycin and 2 μg ml^{-1} puromycin. Transformed *T. brucei* cells were grown in the presence of 2.5 μg ml^{-1} hygromycin, 10 μg ml^{-1} blasticidin and/or 2 μg ml^{-1} puromycin.

S. cerevisiae strains BY4742 (*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0*) and a *pso2*Δ derivative obtained from the Open Biosystems (Thermo Scientific) knock-out collection were maintained in yeast extract-peptone broth containing 2 % (w/v) glucose. Transformed cells were grown in Synthetic Complete Dropout medium lacking leucine (Sigma).

Chemicals. The DNA damaging agents were obtained from Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute except CB1954, H_2O_2, methyl methanesulphonate (MMS) and hydroxyurea (all Sigma-Aldrich), NH1 (Helsby *et al*., 2004) and LH7, LH17, LH32-34 and LH37 (Hu *et al*., 2003, Li *et al*., 2003, Hu *et al*., 2011). Nifurtimox and benznidazole were obtained from Simon Croft (London School of Hygiene and Tropical Medicine) and DFMO from Mike Barrett (University of Glasgow).

Plasmids. The vectors used to delete Tb*snm1* from the *T. brucei* genome were generated as follows. Primers were designed to amplify 897 or 909 bp fragments from the 5' Tb*snm1* untranslated region and 3' region of the Tb*snm1* gene, respectively. These were cloned sequentially either side of a puromycin- (*pac*) or blasticidin- (*bla*) containing resistance cassette. The constructs were linearized (SacI/KpnI for the *pac* vector or SacII/KpnI for the *bla* vector) then introduced into BSF *T. brucei* using the Human T-cell Nucleofector® kit and an Amaxa® Nucleofector™ (Lonza AG) set to program X-001. Integration of the DNA constructs into the *T. brucei* genome results in deletion of 60% of the Tb*snm1* open reading

frame (amino acids 1 to 425) including all of the non-canonical MBL domain. As this region is essential for Pso2p/SNM1 function (Li and Moses, 2003) removal of the MBL encoding DNA sequence from the trypanosomal genome would generate parasites lacking TbSNM1 activity, effectively producing Tb*snm1* null mutant cells.

The Tb*snm1* trypanosomal expression vector was generated as follows: a 2166 bp DNA sequence corresponding to full length Tb*snm1* was amplified from *T. brucei* genomic DNA using the primers *cctgcagg*ATGGCAGGTGGA GCTGCAGGT and *gcgcgcc*TTATTCTGAGTC ACTACTCAG (lower-case italics correspond to restriction sites incorporated into the primers to facilitate cloning), digested with SdaI/SgsI and ligated into the corresponding sites of vector pTubEX-LmSpSyn (Taylor *et al* 2008), replacing Lm*spsyn*. The NotI/XhoI digested construct was introduced into *T. brucei* wild type and *Tbsnm1*$^{-/-}$ cells using nucleofection and recombinant clones selected.

For the localisation construct a 2166 bp DNA sequence corresponding to full length Tb*snm1* was amplified from *T. brucei* genomic DNA using the primers *tctaga*GCAGGTGGAGCTGC AGGTAAG and *gagatct*TTATTCTGAGTCACT ACTCAG (lower-case italics correspond to restriction sites incorporated into the primers to facilitate cloning), the fragment digested with XbaI/BglII and ligated into the XbaI/BamHI sites of vector pRPa$^{GFP-AT2}$ (Aslford *et al*., 2005) to replace the Tb*at2* coding sequence. The cloning was carried out such that the gene coding for the green fluorescence protein (GFP) was inserted in-frame at the 5' end of the *Tbsnm1*-derived DNA fragment. The AscI digested construct was introduced into *T. brucei* 2T1 parasites.

To construct the yeast complementation vector Tb*snm1* was amplified from the trypanosomal localisation plasmid using the primers *tctaga*GCAGGTGGAGCTGCAGGTAA G and *aagctt*TTATTCTGAGTCACTACTCAG (lower-case italics correspond to restriction sites incorporated into the primers to facilitate cloning). The resultant fragment was digested

with XbaI/HindIII and ligated into the corresponding sites of a pYCYlac111 derivative containing a DNA sequence encoding for the FLAG-tag epitope (Novoselova *et al*. 2013). The plasmid was transformed into yeast strains BY4742 and *pso*2Δ. In this system recombinant TbSNM1 is tagged at its amino-terminus with a FLAG-tag epitope detectable with the anti-FLAG monoclonal antibody (Sigma).

Localisation. BSF trypanosomes expressing GFP-TbSNM1 were washed twice in phosphate buffered saline (PBS), fixed in 2 % (w/v) paraformaldehyde/PBS and washed again in PBS. Aliquots of the cell suspension (10^5 cells) were then air dried onto microscope slides. Parasite DNA was stained using Vectashield Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectorshield Laboratories) and slides were viewed using a Leica SP5 confocal microscope.

Antiproliferative assays. All assays were performed in a 96-well plate format. *T. brucei* BSF parasites were seeded at 1 x 10^4 ml^{-1} in 200 μL growth medium containing different concentrations of compound. For UV irradiation, parasites were exposed to doses up to 900 J m^{-2} using a Stratalinker® UV crosslinker (Stratagene). After incubation at 37 ^oC for 3 days, 2.5 μg resazurin (20 μL of 0.125 μg ml^{-1} stock in phosphate buffered saline) was added to each well and the plates incubated for a further 6-8 hours (Jones *et al*., 2010). Cell densities were determined by monitoring the fluorescence of each culture using a Gemini Fluorescent Plate Reader (Molecular Devices (UK) Ltd, Wokingham, UK) at an excitation wavelength of 530 nm, emission wavelength of 585 nm and a filter cut off at 550 nm. The drug/treatment concentration that inhibits cell growth by 50% (IC_{50}) was established using the non-linear regression tool on GraphPad Prism (GraphPad Software Inc.).

463 ***Yeast complementation assay.*** All assays were performed in a 96-well plate format. The cell
464 density of overnight yeast cultures were equalised according to absorbance at 405 nm in
465 medium containing different concentrations of mechlorethamine. The growth of each strain
466 was then followed by monitoring the change in absorbance at 405 nm using an Absorbance
467 Microplate Reader (BioTek Instruments Ltd). The % growth for each mechlorethamine-
468 treated culture after 18 hours relative to untreated samples was determined.
469

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474

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TABLE

Table 1. Susceptibility of *T. brucei* lines to DNA damaging agents. The cell lines analysed were *T. brucei* (wild type) and *T. brucei* Tb*snm1*$^{-/-}$ null mutants (Tb*snm1*$^{-/-}$). IC_{50} values are given in μM except for UV irradiation which is in J m^{-1}. LH7, LH17, LH32-34, LH37, CB1954 and NH1 represent structures previously identified as trypanocidal agents (Bot *et al*., 2010, Hall *et al*., 2010, Hu *et al*., 2011). nd is not determined.

compound	*T. brucei* IC_{50}	
	wild type	Tb*snm1*$^{-/-}$
nitrogen mustards		
chlorambucil, cyclophosphamide, uramustine, trofosfamide, ifosfamide, bendamustine	>30.000	nd
mechlorethamine	34.240 ± 1.270	8.210 ± 1.180
melphalan	8.660 ± 0.660	3.960 ± 0.320
estramustine	9.370 ± 1.150	nd
prednimustine	13.870 ± 1.330	nd
LH7	10.870 ± 0.240	0.580 ± 0.050
LH17	4.160 ± 0.130	0.380 ± 0.040
LH32	0.245 ± 0.079	0.021 ± 0.004
LH33	0.215 ± 0.008	0.015 ± 0.000
LH34	0.067 ± 0.006	0.006 ± 0.001
LH37	0.097 ± 0.009	0.005 ± 0.000
aziridines		
thioTEPA	37.830 ± 1.730	13.880 ± 0.970
triethylenemelamine	1.130 ± 0.150	0.300 ± 0.020
mitomycin C	0.013 ± 0.001	0.010 ± 0.001
CB1954	3.900 ± 0.420	0.690 ± 0.050
NH1	0.120 ± 0.004	0.044 ± 0.013
nitrosoureas		
carmustine, nimustine, NSC270516	>30.000	nd
lomustine	16.650 ± 0.440	17.310 ± 0.210
streptozotocin	21.800 ± 5.020	nd
semustine	4.760 ± 0.050	3.780 ± 0.070
alkyl sulfonate		
busulfan	>30.000	nd
non-classical DNA crosslinking agents		
altretamine, pipobroman, dacarbazine, temozolomide, mitobronitol	>30.000	nd
platinum-based		
oxaliplatin, nedaplatin	>30.000	nd
cisplatin	2.280 ± 0.130	3.400 ± 0.280
carboplatin	5.030 ± 0.040	nd
Other agents		
hydroxyurea	105.970 ± 10.190	88.200 ± 7.300
H_2O_2	43.710 ± 5.950	50.06 ± 7.520
UV irradiation	214.000 ± 13.000	196.000 ± 30.000
MMS	16.125 ± 1.379	14.020 ± 1.343
DMFO	24.150 ± 3.940	24.240 ± 6.710
nifurtimox	2.850 ± 0.020	2.250 ± 0.090
benznidazole	46.140 ± 1.440	37.680 ± 1.630

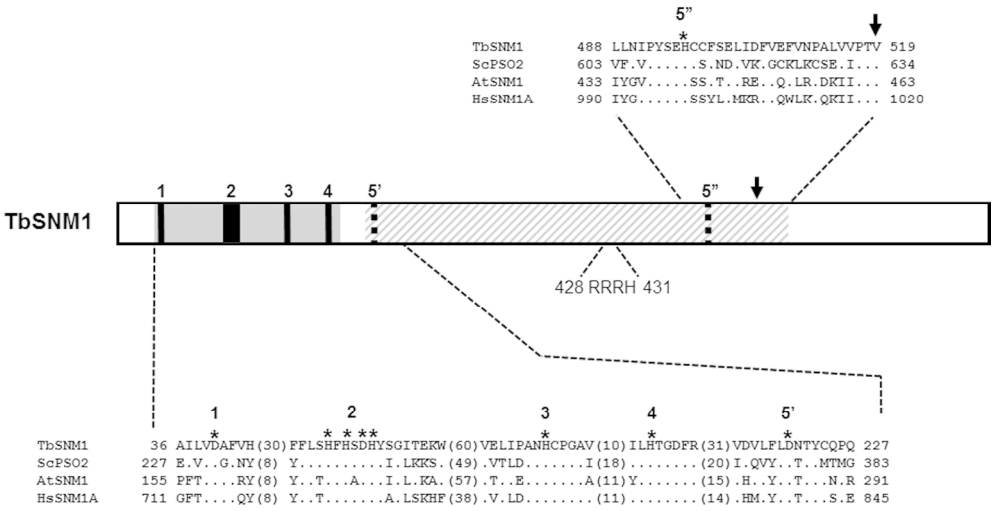
FIGURE LEGENDS

Fig. 1. Sequence analysis of TbSNM1. The sequence corresponding to the metallo-ß-lactamase (MBL; grey box) and β-CASP (hatched box) domains of TbSNM1 was aligned with other members of the SNM1A/PSO2 family of nucleases. The residues that are common with the TbSNM1 sequence are represented by dots. Sequence differences when compared with TbSNM1 are shown. In the alignments, amino acids marked with an asterisks (solid line in TbSNM1 schematic) correspond to motif 1-4, regions postulated to coordinate the metal (zinc) co-factor binding. The two possible residues that may represent motif 5 (5' or 5''; dotted line in TbSNM1 schematic) are also shown. The down arrow highlights the amino acid that distinguishes DNA from RNA processing metallo-β-lactamases while the RRRH sequence corresponds to a putative nuclear 'pattern 4' targeting signal. The sequences aligned are: *T. brucei* TbSNM1 (GenBank AAZ10739), *Saccharomyces cerevisiae* ScPSO2 (NP_013857), *Arabidopsis thaliana* AtSNM1 (NP_189302) and *Homo sapiens* HsSNM1A (NP_001258745).

Fig. 2. Complementation of the yeast *pso2*Δ mutation. A. Western blot analysis was carried out using a monoclonal antibody to the FLAG-tag epitope on cell extracts made from *S. cerevisiae* BY4742 (wild type) and *pso2*Δ strains expressing the FLAG epitope (control) or FLAG-TbSNM1. A band of ~80 kDa (indicated by **) was observed in lysates derived from cell expressing the recombinant trypanosomal protein. A cross reactive epitope (*) and Ponceau S staining of the membrane (not shown) were used as loading controls. B & C. The susceptibility of wild type and *pso2*Δ yeast strains expressing FLAG or FLAG-TbSNM1 to different concentrations of mechlorethamine. All data are mean values ± standard deviations from experiments performed in triplicate. In C, the values given in parenthesis represent the fold difference in IC_{50} values (in μM) relative to wild type controls.

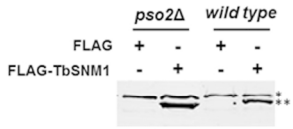
730 **Fig. 3. Localisation of TbSNM1 in bloodstream form *T.brucei*.** A. Expression of GFP-
731 TbSNM1 was examined by probing a blot containing cell lysates from *T. brucei* wild type
732 (lane 1) and GFP-TbSNM1 expressing cells (lane 2) using an anti-GFP antibody (upper
733 panel). Protein from 1.5 x 10^7 cells was loaded in each track and a cross reactive epitope
734 (lower panel) and by Coomassie staining (not shown) were used as loading controls. B.
735 Parasites expressing GFP-TbSNM1 were co-stained with DAPI (DNA) and the cells
736 examined by confocal microscopy. The TbSNM1 signal is coincidental with the nucleus (n;
737 large DAPI spot); the smaller DAPI spot corresponds to the kinetoplast (k), the trypanosome
738 mitochondrial genome.
739
740 **Fig. 4. Disruption of Tb*snm1* in *T. brucei*.** A. Diagram of the Tb*snm1* alleles and the effects
741 of gene disruption. A 5' Tb*snm1* flanking sequence and a 3' Tb*snm1* coding region were
742 amplified and cloned sequentially either side of a puromycin (*pac*) or blasticidin (*bla*)
743 cassette (plus *T. brucei* tubulin intergenic elements required for processing of mRNA (hashed
744 boxes). The dotted lines correspond to the probe used to check integration. The position of
745 the predicted *Mlu*I sites plus the band sizes (in kbp) obtained after hybridisation are shown.
746 B. Southern blot analysis of *Mlu*I digested genomic DNA from *T. brucei* (lane 1), Tb*snm1*$^{+/-}$
747 *bla* and Tb*snm1*$^{+/-}$ *pac* heterozygous clones (lanes 2 and 3 respectively) and a Tb*snm1*$^{-/-}$ null
748 mutant line (lane 4). Blots were hybridized with labelled 3' region of sequences. Sizes given
749 are in kbp.
750
751 **Fig. 5. Susceptibility of *T. brucei* lines expressing altered levels of TbSNM1 to DNA**
752 **damaging agents.** A. Growth inhibitory effects (expressed as IC_{50} values in μM or nM) of
753 the *T. brucei* wild type, Tb*snm1*$^{-/-}$ null mutant, Tb*snm1*$^{-/-}$ expressing an ectopic copy of
754 Tb*snm1* (Tb*snm1*$^{-/-}$+ Tb*smn1*$^{++}$) and *T. brucei* expressing elevated levels of Tb*snm1* (wild

type + Tb*smn1*$^{++}$) lines towards DNA damaging agents. Integration of the Tb*snm1* expression vector into a single tubulin array was confirmed by Southern hybridization and expression evaluated through qPCR (data not shown). B. Growth inhibitory effects (expressed as IC_{50} values in µM or nM) of *T. brucei* wild type, Tb*snm1*$^{-/-}$ null mutant, *T. brucei* expressing an ectopic copy of Tb*ntr* (wild type + Tb*ntr9e10*$^{++}$ and Tb*snm1*$^{-/-}$ expressing elevated levels of Tb*ntr* (Tb*snm1*$^{-/-}$ + Tb*ntr9e10*$^{++}$) towards DNA damaging agents. Expression of Tb*ntr* was evaluated through qPCR (data not shown). Data in panels A and B are mean values ± standard deviations from experiments performed in quadruplicate.

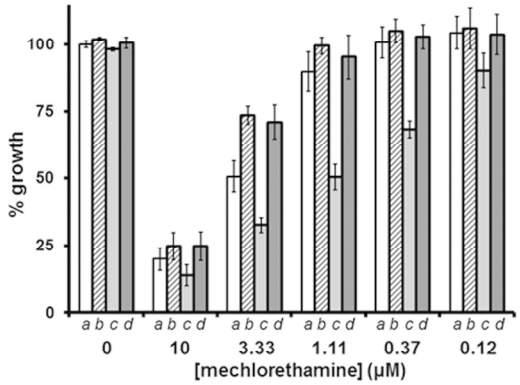


250x129mm (300 x 300 DPI)

A



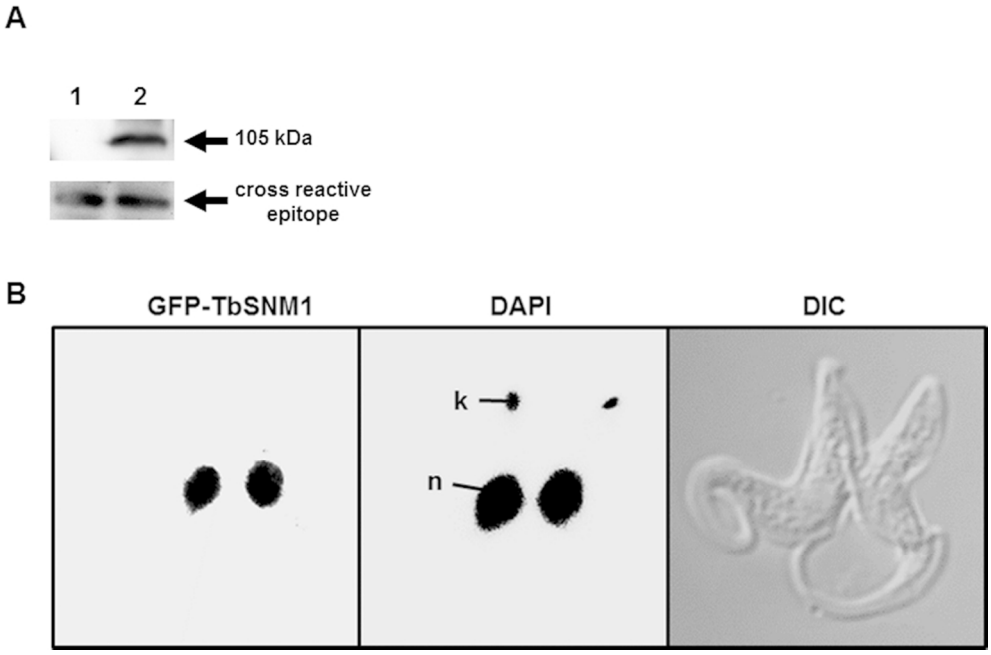
B



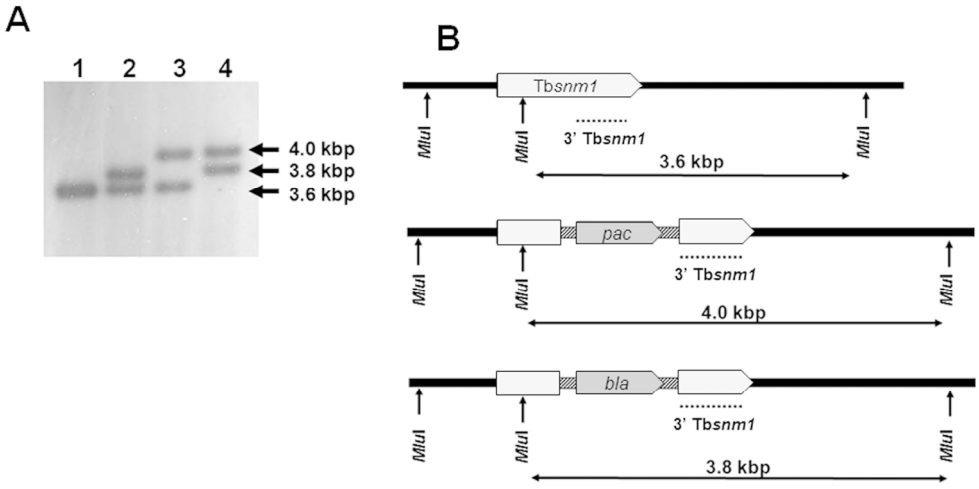
C

	Yeast IC_{50} (μM)			
	wild type + FLAG	wild type + FLAG-TbSNM1	*pso2*Δ + FLAG	*pso2*Δ + FLAG-TbSNM1
mechlorethamine	3.6 ± 0.4	5.1 ± 0.5 (1.4)	1.3 ± 0.6 (0.4)	5.7 ± 0.7 (1.6)

150x108mm (300 x 300 DPI)



99x66mm (300 x 300 DPI)



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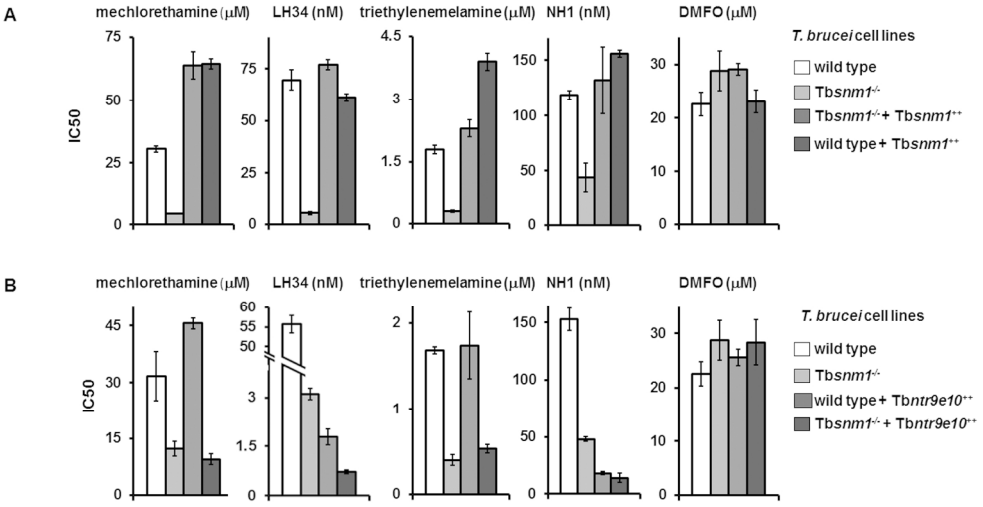
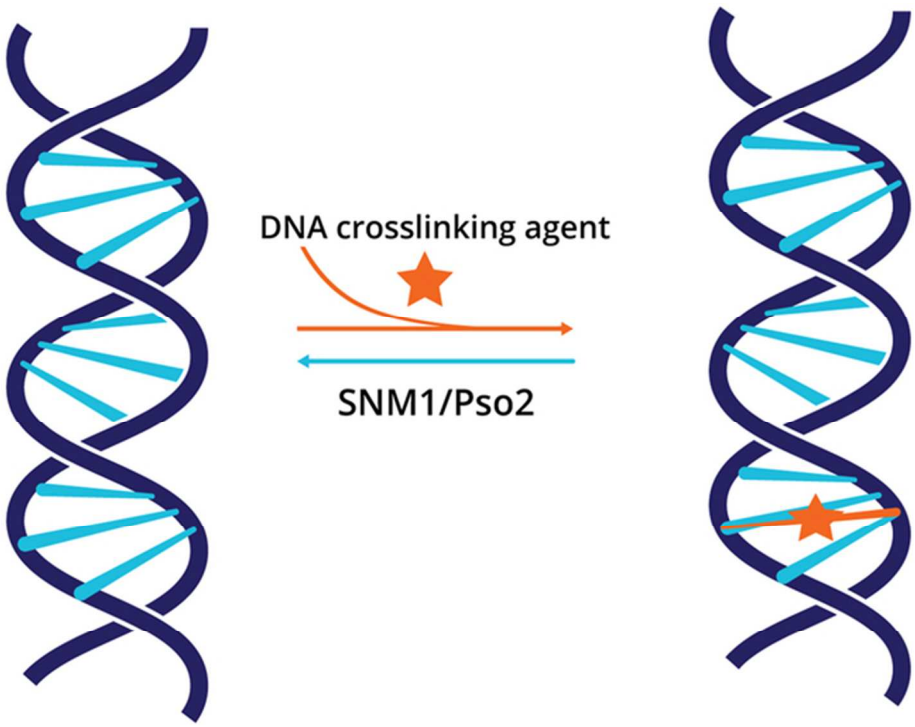


199x103mm (300 x 300 DPI)

Trypanosomiasis is of medical and veterinary importance across sub-Saharan Africa. *Trypanosoma brucei*, the causative agent of these infections, expresses a DNA repair enzyme that exhibits characteristics typical of PSO2/SNM1 family of nucleases. This activity although non-essential for the growth of bloodstream form parasites does play a key role in fixing the damage caused by DNA interstrand crosslinking agents.



59x43mm (300 x 300 DPI)