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Selective inhibition of T2AA for human PCNA over PCNA homolog in *Trypanosoma brucei*

Yasser N. Haggag[1], Mohammad A. Nossair[1], Haitham Elaadli[1,2,*] and Zachary Mackey[2]

[1]Department of Animal Hygiene and Zoonoses, Faculty of Veterinary Medicine, Alexandria University.
[2]Department of Biochemistry, Fralin Life Science Vector Borne Disease Institute, Virginia Tech, Blacksburg, VA 24061, USA.

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***Correspondence to:**
helaadli@vt.edu

ABSTRACT

Proliferating cell nuclear antigen (PCNA) coordinates many functions including cell cycle progression and DNA replication in eukaryotic cells. Inhibition of the PCNA protein function in Trypanosoma brucei could be a new strategy in controlling Human African Trypanosomiasis. In the present study, we explored the function of TbPCNA by deregulation the protein expression through the overexpression, and by applying the small molecule inhibition. Overexpression of human PCNA or T. brucei PCNA wild-type proteins inhibits the parasite proliferation. On the other hand, T. brucei that overexpress the HsPCNA M40A or TbPCNA M40A mutants grew normally, indicating that inhibition of PIP-box protein interaction is important for the lethal phenotype. In testing this hypothesis, we applied the small molecule inhibitor T2 amino alcohol (T2AA). Proliferation in parasites that overexpress HsPCNA resumed after T2AA treatment, but not parasites that overexpressed TbPCNA. We concluded that T2AA selectively inhibits HsPCNA not TbPCNA. This data presented here will be a prerequisite for investigating TbPCNA interacting proteins and discovering inhibitors that specifically target TbPCNA to kill the parasite.

1. INTRODUCTION

Trypanosoma brucei is the protozoan parasite that causes Human African trypanosomiasis also known as sleeping sickness, a meningoencephalitic disease that is most often fatal if left untreated (Jamonneau et al., 2012). DNA replication in *T. brucei*, as in all other organisms, is spatially and temporally coordinated to produce a single exact copy of the genome per cell division. In human cells, DNA replication is initiated at origins that become licensed by the components of the origin recognition complex: (Orc1-6), Ctd1, Cdc6, and the mini-chromosome maintenance proteins (Mcm 2–7) that form the pre-replication complex in G1 (Bell and Dutta, 2002). The pre-replication complex in trypanosomatids consists of the Orc1/Cdc6 protein and Cd45-Mcm2-7-GINS complex (Dang and Li, 2011). Activation of replication origins occurs at S phase and involves formation of replication forks that become primed by the DNA polymerase α/primase complex. This allows the replication machinery to bind to activated forks and begin synthesis of new DNA (Moldovan et al., 2007).

Proliferating cell nuclear antigen (PCNA) is a central component of the eukaryotic DNA replication machinery. Decades of research in model systems demonstrate that the structure of PCNA is conserved throughout eukaryotic cells and its function is essential for survival (Bauer and Burgers, 1990; Bravo and Macdonald-Bravo, 1987; Henderson et al., 1994; Jaskulski et al., 1988; Kurki et al., 1986; Prelich et al., 1987; Waseem et al., 1992; Zuber et al., 1989). Human PCNA (HsPCNA) interacts with more than 200 proteins that contain the PCNA interacting peptide motif (PIP-box) to regulate many cellular processes including chromatin reorganization (Hasan et al., 2001; Milutinovic et al., 2002), DNA repair (Dianova et al., 2001; Gary et al., 1997; Li et al., 1995; Muller-Weeks and Caradonna, 1996; Umar et al., 1996), and the cell cycle (Szepesi et al., 1994; Warbrick et al., 1998; Xiong et al., 1992). The PIP-box is represented by the consensus sequence (Qxψxxϕϕ) where ψ= (hydrophobic residues) and ϕ= (aromatic residues) that determines how well the motif can pack into the hydrophobic pocket formed by the

interdomain-connecting loop and central loop of PCNA (Bruning and Shamoo, 2004; Gulbis et al., 1996; Moldovan et al., 2007). Basic knowledge about PCNA/PIP-box interactions has translated to the pre-clinical proof-of-mechanism for the inhibitor T2AA to therapeutically target HsPCNA in cancer cells (Actis et al., 2013; Inoue et al., 2014; Punchihewa et al., 2012; Tan et al., 2012). There is a homolog of PCNA in T. brucei, TbPCNA, which is regulated through the cell cycle, and which is located at the nuclear periphery (Kaufmann et al., 2012). Currently, no information is available about mechanism and interactions of TbPCNA, with no associated proteins have been identified to date that interact with the PCNA of *T. brucei* (TbPCNA). Human PCNA can be specifically inhibited by a small molecule termed T2AA, a derivative of the thyroid hormone (Punchihewa et al., 2012). Here, we expltore how T2AA interacts with TbPCNA, the possibility of inhibition, and the molecular determinants that are involved.

2. MATERIAL AND METHODS

2.1. Cell culture:

Bloodstream form *T. brucei* (clone 90-13) were incubated in 5% CO_2 at 37^{o}C in HMI-9 medium modified to contain 20% fetal bovine serum (Hirumi and Hirumi, 1989). The parasites were cultured in media containing 100 U/ml penicillin and 100 μg/ml streptomycin. Selection medium contained 5 μg/ml hygromycin B, 2.5 μg/ml G418, and 2.5 μg/ml phleomycin. Parasites were induced by adding tetracycline to the medium at a final concentration of 1.0 μg/ml.

2.2. Overexpression of recombinant PCNA in *T. brucei:*

The TbPCNA-pLew111, HsPCNA-pLew111, $TbPCNA_{M40A}$-pLew111 or $HsPCNA_{M40A}$-pLew111expression vectors were transfected into *T. brucei* after being linearized with Not I restriction enzyme, which allowed for tetracycline inducible overexpression of TbPCNA in the parasite (Motyka et al., 2006; Wirtz et al., 1998). For electroporation, 10^7 parasites were transfected as described for (Burkard et al., 2007), transferred to 10 ml of HMI-9 media, and incubated overnight at 37^{o}C with CO_2. The next day after cell recovery, stable clones were selected and grown in media containing 5.0 μg/ml hygromycin B, 2.5 μg/ml G418, and 2.5 μg/ml of phleomycin for 7 days. Stable transfectants were induced by supplementing selection media with tetracycline to a final concentration of 1 μg/ml. Several phleomycin-resistant transfectants of each construct were screened by immunoblot analysis to identify ones that expressed HA-tagged versions of each protein upon tetracycline induction. We designated tetracycline- inducible transfectants able to overexpress willd-type $PCNA_{HA}$ as either $TbPCNA_{OE}$ or $HsPCNA_{OE}$ clones. The stable clones that expressed the M40A mutation were designated as $TbPCNA_{M40A}$or $HsPCNA_{M40A}$. The coding region for wild-type TbPCNA was amplified with the forward primer 5' AAGCTTATGCTTGAGGCTCAGGTTCTG-3' and the reverse primer 5'-CTTAAGCTCGG-CGTCGTCCACCTTTGG-3'. The $TbPCNA_{M40A}$ mutant was made using the forward primer 5' TCCATTCAAGCTAGGGATACGAGCCACGTT G-3' and the reverse primer 5'-CAACGTGGCTCGTATCCCTAGCTTGAATGGA -3'.

2.3. Generation of TbPCNA mutant:

The $TbPCNA_{M40A}$ point mutation was made from TbPCNA cDNA using the wild type and mutant primers and employing the annealing overlapping PCR method (Bryksin and Matsumura, 2010). The $HsPCNA_{M40A}$ plasmid was generously provided by gift from (G. L. Moldovan at Penn State Medical School).The PCR products of these constructs were subcloned into the pLew111 vector at the HindIII/AflII sites for expression in *T. brucei* and in the BamHI/HindIII site of the pTrcHisA vector (Thermo Fisher) for expression in *E. coli.*

2.4. Expression and purification of PCNA in *E.coli:*

The TbPCNA coding region was subcloned into the BamHI/HindIII sites of pTrcHis A (Thermo Fisher) and expressed with a 6×His tag in BL21-DE3. The recombinant protein was purified by nickel agarose affinity chromatography (Thermo Fisher) as recommended by the manufacturer.

2.5. Immunoblots

Expression of hemagglutinin-tagged TbPCNA expressed in *T. brucei* was examined by immunoblot as previously described (Mackey et al., 2004). Stain-Free gels used to resolve polypeptides for immunoblots were obtained from (BioRad).

2.6. Isothermal titration Calorimetry (ITC)

ITC experiment was carried out by the stepwise injection of 10 μL of a 100-200 μM T2AA solution into a 300 μL solution of 20 μMTbPCNA. Binding of the ligand to the protein was detected by heat changes in the mixture and measured using the Microcal PEAQ-ITC (Malvern)

3. RESULTS AND DISCUSSION

3.1. Overexpression of PCNA in *T. brucei*

A representative immunoblot showed that basal levels of $TbPCNA_{HA}$ were detected in non-induced $TbPCNA_{OE}$ clones. Such a result indicated that the

promoter in this plasmid was not tightly regulated. Detection of high basal levels of $TbPCNA_{HA}$ in non-induced $TbPCNA_{OE}$ clones was consistent with the 20% read-through originally reported using the parental pLew82 plasmid (Wirtz et al., 1998) which is the backbone of pLew111. Several independent $TbPCNA_{OE}$ clones were diluted to 10^5/ml and cultured in HMI-9 media containing tetracycline at T_0 to test the effects that overproducing wild type and mutant PCNA constructs had on proliferation in *T. brucei*. Mean levels of proliferation were arrested in the $TbPCNA_{OE}$ clones as early as 24 h and lasted through day 6 post induction.

3.2. Specific inhibition of stable PCNA/PIP-box protein interactions in *T. brucei*

We recently discovered that overexpressing either TbPCNA or HsPCNA in bloodstream form *T. brucei* triggered the parasites to cease proliferation and arrest in G2/M of the cell cycle (Valenciano et al., 2015). Figures 1 (C and D panels) demonstrate the efficiency by which overexpressing PCNA homologs arrest proliferation in *T. brucei*. We did site directed mutagenesis in the TbPCNA and HsPCNA open reading frames, changing methionine-40 to alanine (M40A) in both homologs. M40 is part of the evolutionary conserved central loop that forms the hydrophobic pocket of PCNA with the IDCL and the C-terminus (Carrasco-Miranda et al., 2014; Tsurimoto and Stillman, 1991; Warbrick et al., 1995) to mediate hydrophobic interactions with PIP-box proteins (Muller et al., 2013). We discovered that parasites overexpressing either the $TbPCNA_{M40A}$ mutant (Figure 2, bottom panel) or the $HsPCNA_{M40A}$ mutant (Figure 2, top panel) proliferated normally.

From these observations, we hypothesized that PCNA/PIP-box protein interactions are critical for triggering arrested proliferation in parasites that overexpressed either PCNA homolog.

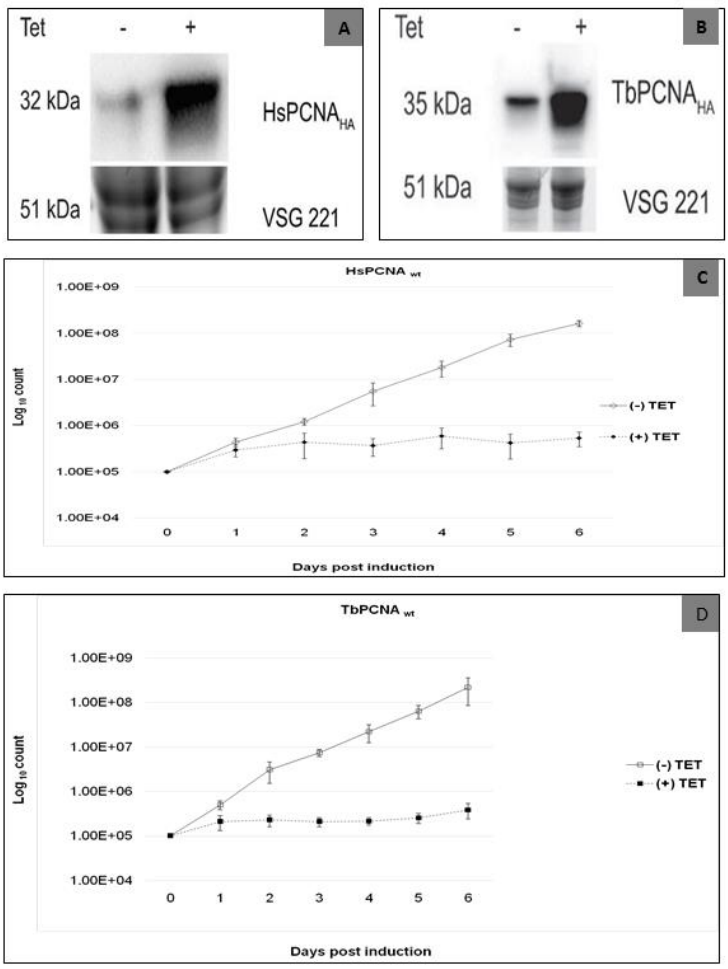


Figure 1. Overexpression of PCNA induces lethal *T. brucei*. Growth curves representing. A,B show Immunoblot analysis to detect HsPCNA HA and TbPCNA HA by α- HA antibodies after overexpression in T.brucei. VSG loading control represents Stain-FreeTM image obtained from gel prior to transfer. C,D growth curves represent the mean cumulative count over 6 d for $HsPCNA_{OE}$ and $TbPCNA_{OE}$ clones grown in media without (solid lines) or with (dashed lines) tetracycline. Graphs represent the mean counts with standard error from 3 independent clones repeated in triplicate

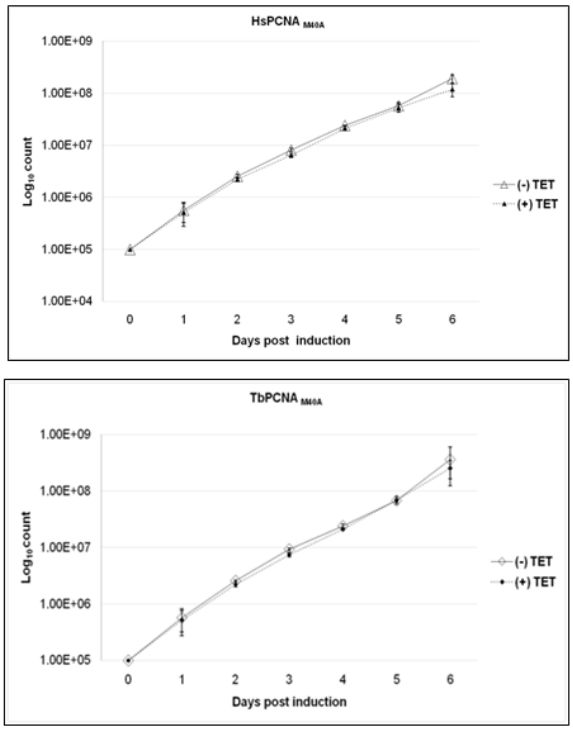


Figure 2. PIP-box protein is important for lethal phenotype. Growth curves representing the mean cumulative count over 6 d for HsPCNA $_{M40A}$ and TbPCNA $_{M40A}$ clones grown in media without (solid lines) or with (dashed lines) tetracycline. Graphs represent the mean counts with standard error from 3 independent clones repeated in triplicate.

To test this hypothesis, we used the small molecule inhibitor T2AA as the chemical tool to broadly inhibit PCNA/PIP-box protein interactions. When T2AA was used to treat tetracycline induced parasites that overexpressed TbPCNA, a marginal decrease in the proliferation of these parasites was observed (Figure 3D). Proliferation in *T. brucei* overexpressing HsPCNA rose significantly and reached a maximum when the dose of T2AA reached 6 μM and then decreased, providing further evidence that PCNA/PIP-box protein interactions had a critical role in triggering cell cycle arrest in parasites that overexpress HsPCNA (Figure 3B). Bell-shaped dosage curves are not unusual for drugs and might occur as a result of two binding sites (Inoue et al., 2014) or other mechanisms such as colloidal formation (Owen et al., 2014). This contrasting observation indicates that T2AA selectively inhibits HsPCNA over TbPCNA.

3.2. Comparison of T2AA binding to TbPCNA and HsPCNA by Isothermal titration calorimetry

The observation that T2AA showed preference between HsPCNA and TbPCNA in the parasite has potentially clinical application. It led us to the hypothesis that PCNA homologs from divergent species can be selectively inhibited. We tested this hypothesis by using isothermal titration calorimetry (ITC) to directly examine the binding of T2AA to HsPCNA and TbPCNA. The efficient binding and high affinity of T2AA to HsPCNA were evidenced by the significant release of heat upon injecting T2AA as observed by the sigmoidal isotherm in (Figure 5A). No significant release of heat was observed in the isotherm upon injecting T2AA into TbPCNA (Figure 5B). The lack of a thermodynamic profile for TbPCNA was consistent with T2AA not binding to TbPCNA. The thermodynamic profile of HsPCNA shows its binding constant (K_d) for T2AA was 1 μM, by fitting the isotherm for HsPCNA into a single binding site model (Figure 5C). The highly negative enthalpy (-ΔH) for HsPCNA indicates that the binding is mediated by electrostatic interactions, which is indicative of the specificity of T2AA to HsPCNA.

Here we demonstrate the small molecule T2AA inhibits HsPCNA interactions with higher selectivity than it does with TbPCNA. We have also identified key determinants that contribute to the selectivity of this small molecule between the two proteins. The consequences of deleting or deregulating PCNA homologs in other model eukaryotic organism: yeast, mammals, and Drosophila have been very well characterized demonstrating that the PCNA gene and its functional product are essential for cell survival. Much less is known about how components of the DNA replication machinery are regulated or the consequences of deregulating their levels in *T. brucei*. We recently demonstrated that downregulating TbPCNA levels in *T. brucei* reduced TbPCNA mRNA and abrogated parasite growth demonstrating that this gene product is important for *T. brucei* to proliferate normally (Valenciano et al., 2015). Future studies will examine how substitution of key binding residues in TbPCNA contributes to its reduced affinity for T2AA. It was intriguing to us why we see this differential effect of T2AA on human versus T. brucei PCNA. By taking a closer look at the IDCL sequence alignments between the two proteins, it was revealed that methionine (M*) is present in TbPCNA at the position of the key binding residue Q131 in HsPCNA, which forms hydrogen bonds with the phenolic group of T2AA (Inoue et al., 2014; Punchihewa et al., 2012). Q131 is critical for high binding affinity between HsPCNA and p21 by forming a hydrogen bond to Y151 of p21 (Kroker and Bruning, 2015).

In summary, this study with small molecules provides compelling evidence for the concept of selective inhibition between PCNA homologs from divergent species which may have clinical applications.

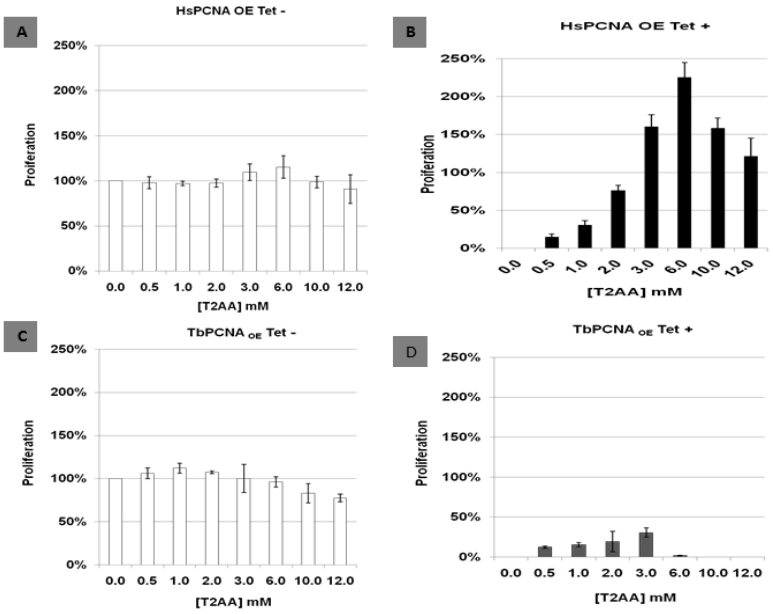


Figure 3. Proliferation restoration assay for *T. brucei*. *T. brucei* were treated with various conentrations of T2AA as indicated in the graphs. Graphs represent the mean percent with standard error from 6 independent experiments, each done on separate days.

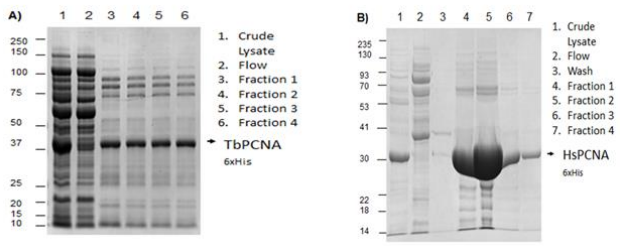
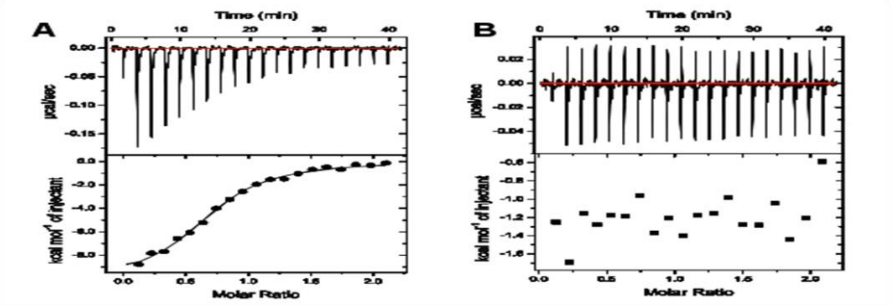


Figure 4. Expression and purification of PCNA expressed in *E.coli*. (A) Coomassie stained gel of TbPCNA purified from Ni-agarose affinity column. (B) Coomassie stained gel of HsPCNA purified from Ni-agarose affinity column.



Sample	K_d (μM)	ΔG (kcal/mol)	ΔH(kcal/mol)	-TΔS (kcal/mol)	n
hPCNA-T2AA	1.07	-8.15	-10.14	1.98	0.7

Figure 4. Binding of T2AA with HsPCNA and TbPCNA. (A) Binding isotherm of the Interaction of T2AA compound with HsPCNA. (B) Binding of T2AA with TbPCNA at 25 ° Raw heat changes upon binding are shown in the top panel and the integrated data are shown in the bottom panel.

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