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Inhibition of *Mycobacterium tuberculosis* topoisomerase I by m-AMSA, a eukaryotic type II topoisomerase poison

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ABSTRACT

m-AMSA, an established inhibitor of eukaryotic type II topoisomerases, exerts its cidal effect by binding to the enzyme–DNA complex thus inhibiting the DNA religation step. The molecule and its analogues have been successfully used as chemotherapeutic agents against different forms of cancer. After virtual screening using a homology model of the *Mycobacterium tuberculosis* topoisomerase I, we identified m-AMSA as a high scoring hit. We demonstrate that m-AMSA can inhibit the DNA relaxation activity of topoisomerase I from *M. tuberculosis* and *Mycobacterium smegmatis*. In a whole cell assay, m-AMSA inhibited the growth of both the mycobacteria.

1. Introduction

Topoisomerases are ubiquitous enzymes which maintain topological homeostasis within the cells during a variety of DNA transaction processes such as replication, transcription, chromosome segregation, etc. [1,2]. The reaction catalyzed by the topoisomerases involves the formation of a phosphotyrosine covalent adduct and a single-stranded (type I) or double-stranded (type II) break in the DNA during the two sequential trans-esterification reactions [2–4]. Owing to their mechanism of action, topoisomerases have been exploited extensively for the development of anti-bacterial as well as anti-cancer agents [5–9]. They have been targeted by various classes of molecules with different modes of action such as the trapping of the topoisomerase–DNA complex, catalytic inhibition, or accumulation of the cleavage complex [8,10]. In eukaryotes, both type I and type II topoisomerases are targeted by a variety of small molecules [11]. For example camptothecin, a plant alkaloid, and its semi-synthetic derivatives, selectively target the type I topoisomerase (TopoI) by inhibiting the resealing of the cleaved DNA [12]. Topoisomerase II (TopoII) is the target of another class of molecules, the anthracyclines, which intercalate into the DNA and trap the protein-mediated cleavage complex [10]. In addition, several other intercalating agents are known to possess anti-topoisomerase activity. Amsacrine, an acridine derivative, originally designed as an intercalating agent has been shown to act as a eukaryotic topoisomerase II poison [13]. It has been elucidated that there is a correlation between the intercalating property and the ability of the molecule to accumulate the protein–DNA covalent complexes by reducing the rate of re-ligation. m-AMSA, one of the isomers of amsacrine, has been shown to exhibit anti-neoplastic activity [11,14]. The FDA-approved drug is used in the treatment of Hodgkin's and non-Hodgkin's lymphomas as well as against refractory acute lymphocytic and nonlymphocytic leukemias [15,16]. It can inhibit both the isoforms of human topoisomerase II – α and β [17,18] by accumulating the protein–DNA covalent adducts [19,20].

A different set of compounds target bacterial type IIA topoisomerases, which constitute an essential group of enzymes. The type IIA enzymes are inhibited by the quinolones [21], coumarins [22], proteinaceous poisons [23,24] and a number of other small molecules [11], disturbing different steps of the catalytic cycle. In contrast to this large repertoire of compounds that target the type II enzymes, there is a dearth of such inhibitory molecules against the type I topoisomerases of bacteria.

In our efforts to develop inhibitors against type I topoisomerases from mycobacteria, *in silico* screening was carried out on a *Mycobacterium tuberculosis* topoisomerase I (MttopoI) structural model. Amsacrine was found to have a favorable docking score in the screening, and commercially available m-AMSA was assayed against different type I topoisomerases. Here, we describe the

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inhibition of *M. tuberculosis*, *Mycobacterium smegmatis* and *Escherichia coli* topoI by this compound. The growth of *M. smegmatis* and *M. tuberculosis* cells was affected upon treatment with the compound.

2. Materials and methods

2.1. Enzymes and chemicals

MttopoI [25], *M. smegmatis* topoI (MstopoI) [26] and *E. coli* topoI (EctopoI) [27] were purified as described previously. m-AMSA was purchased from Sigma–Aldrich and a 10 mM stock was prepared in DMSO. Negatively supercoiled pUC18 plasmid DNA was purified using Qiagen midiprep kits.

2.2. Homology modeling and in silico docking of molecules

Three bacterial topoI structures available in the PDB were used for modeling. These included 1ECL (closed state, no DNA or Mg^{2+} bound), 1MW8 (closed state with non-covalent DNA bound, no Mg^{2+} bound) and 1MW9 (closed state, no DNA or Mg^{2+} bound). A homology model of the MttopoI with a closed site, no DNA or Mg^{2+} bound (A2VM29 based on 1ECL/1MW9) was also available in ModBase [28]. The bacterial topoII structure (2RGR) and the topoIII structure (1I7D) available in PDB were also used. Therefore, a homology model for the EctopoI was first created with the gate open and with Mg^{2+} bound by aligning topoI subdomains with topoIII subdomains. The Mg^{2+} site was generated from the topoIII residue coordinates. The MttopoI homology model with the gate open and Mg^{2+} bound was built by using the same sequence alignment used for 1ECL in the ModBase and the EctopoI homology model as a scaffold. This was achieved by downloading the sequence P0A620 in FASTA format and using "align sequence to template" in Discovery Studio (sequence identity 38.3 and sequence similarity 54.8). This was then used to create a homology model with Mg^{2+} and a covalently bound DNA fragment. The DNA in this final model was based on the DNA position in the EctopoII crystal structure 2RGR and was achieved using pyMOL [29].

The final MttopoI homology model with Mg^{2+}, a DNA fragment bound and with an open site was used for docking using LibDock (Discovery Studio, Accelrys, San Diego, CA) [30]. The binding site was centered on Mg^{2+} with a diameter of 8 Å. The protocol included 10 hotspots and a docking tolerance of (0.25). The FAST conformation method was used along with the steepest descent minimization with CHARMm. A set of FDA-approved drugs was collected and exported from the Collaborative Drug Discovery Database (www.collaborativedrug.com, Burlingame, CA).

2.3. DNA relaxation assays

The relaxation of supercoiled pUC18 DNA was carried out as described previously [31]. Enzyme inhibition assays were carried out with different orders of addition. The compound was allowed to bind to MttopoI or to the negatively supercoiled pUC18 at 37° C for 15 min, following which, either the DNA or the MttopoI was added to the reaction respectively and incubation was further continued at 37° C for 30 min. To assay the extent of inhibition by m-AMSA on a pre-formed protein–DNA non-covalent complex, MttopoI was pre-incubated with the pUC18 for 15 min on ice, following which, increasing concentrations of the compound were added to the reaction and incubated for 30 min at 37° C. The samples were electrophoresed in a 1.2% agarose gel for 12 h at 2.5 V/cm, stained with ethidium bromide (0.5 μg/mL) and the DNA bands were visualized using a gel documentation system (Bio-Rad, Hercules, CA, USA).

2.4. Oligonucleotide cleavage assay

Cleavage assays were carried out with a 5′-end-labeled 32-mer harboring the STS (Strong Topoisomerase Site) annealed to a complimentary sequence and also with a single-stranded 5′-end-labeled 32-mer harboring the STS. Since m-AMSA is a DNA intercalating agent, the double-stranded substrate was preincubated with various concentrations of the compound to assess the effect of the compound intercalation on the cleavage reaction. The pre-incubation of the substrate and m-AMSA was carried out in a buffer containing 40 mM Tris–HCl (pH 8.0), 20 mM NaCl, 1 mM EDTA and 5 mM $MgCl_2$ at 37° C for 15 min. Following this, MttopoI was added and the reactions were incubated at 37° C for 30 min. The reactions were stopped with 45% formamide and heated at 95° C for 2 min. The products were resolved in a 12% denaturing PAGE and analyzed by a phosphorimager (model BAS 1800; Fujifilm).

2.5. Growth inhibition

M. smegmatis mc^2155 or *M. tuberculosis* $H_{37}Ra$ were grown to OD_{595nm} = 0.6 in Middlebrook 7H9 broth supplemented with 0.2% glycerol and 0.5% Tween-80. The cultures were diluted to a final OD_{595nm} = 0.05 with fresh 10 ml 7H9 broth and aliquoted into a 100-well growth plate. Serial dilutions of m-AMSA were made and the untreated culture was taken as control. The growth was monitored at A_{595nm} over 48 h with continuous shaking at 200 rpm. The readings were analyzed by GraphPad Prism software (*ver.* 5.0). To determine the minimum inhibitory concentration (MIC) values of the compound, a resazurin reduction microplate assay (REMA) was carried out [32]. The *M. smegmatis* and *M. tuberculosis* cultures were grown for 2 days or 7 days respectively, in the presence of the compounds. Resazurin dye was added to the cultures at a final concentration of 0.02% and incubated further for 1 h or 14 h for *M. smegmatis* and *M. tuberculosis* respectively.

3. Results and discussion

3.1. In silico screening

On docking into the homology model for MttopoI, 2316 compounds from the library of FDA-approved drugs failed to dock. The remaining 317 were ranked by the Libdock Score (range ~21–150). Out of the several molecules selected for testing, one compound of particular interest was the topoII inhibitor amsacrine (m-AMSA) which had a score of 94.6 and ranked 75th (Fig. 1A). The molecule appears to have a Pi-stacking interaction between its acridine group and the Mg^{2+} bound to the Toprim domain in the amino terminal region of the enzyme (Fig. 1B).

3.2. Inhibition of DNA relaxation by m-AMSA

The favorable docking score of m-AMSA in the *in silico* screening described above led us to examine its inhibitory potential against topoisomerase I from *M. tuberculosis*, *M. smegmatis* and *E. coli*. The relaxation activities of the enzymes were inhibited by the drug in a concentration dependent manner. The reactions catalyzed by MttopoI and MstopoI were completely inhibited at 10 μM (Fig. 2A and Fig. S1) while the inhibition of EctopoI was seen between 10 and 25 μM concentration of the drug (Fig. 2B). The inhibition of the topoisomerase I from different bacteria indicates that the drug may be binding to a region which is shared between the enzymes from different species. Although both the mycobacterial topoI and EctopoI acquire an overall toroidal structure, there are differences in the primary amino acid sequences, which may

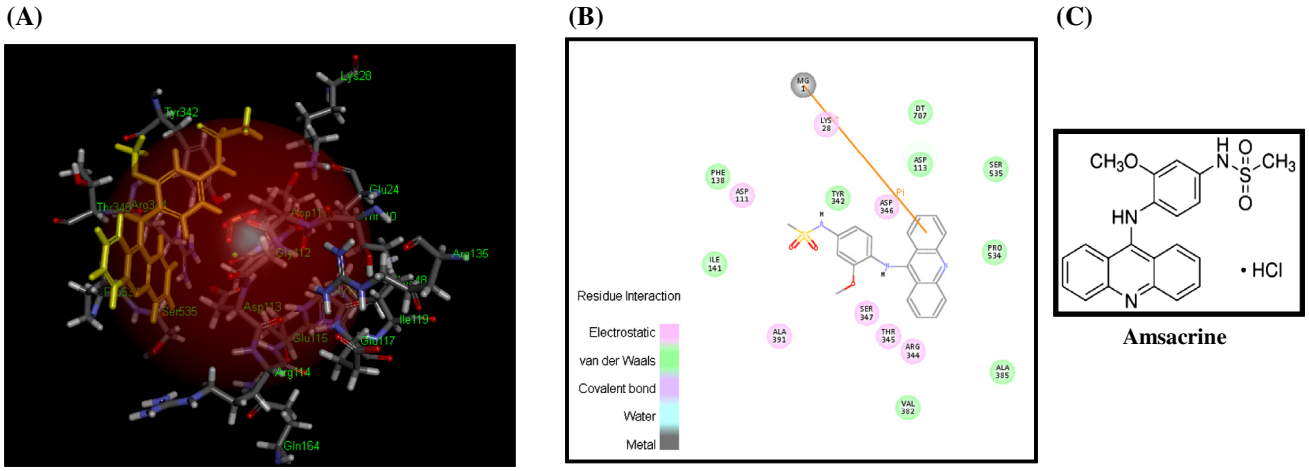

Fig. 1. m-AMSA docked in homology model of *M. tuberculosis* TopoI: (A) The homology model was generated as described in Section 2 and the compounds were docked using LibDock. Amsacrine is shown in yellow, and residues around the central Mg^{2+} are shown for clarity. (B) 2D representation of the 3D binding site illustrating the key residues for interaction and shows a Pi stacking interaction. (C) 2D structure of amsacrine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

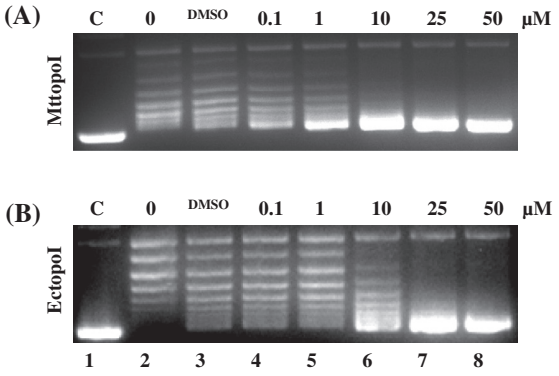

Fig. 2. m-AMSA inhibits the DNA relaxation activity of MttopoI and EctopoI:1 unit of (A) MttopoI or (B) EctopoI was incubated with different concentrations of the compound (lanes 4–8) at 37° C for 15 min following which, 500 ng of supercoiled pUC18 was added. The incubation was further continued at 37° C for 30 min and the reaction was terminated by addition of 0.6% SDS-agarose dye. The reaction products were resolved on a 1.2% agarose gel followed by staining with (Ethidium Bromide) EtBr. Lane 1, supercoiled pUC18; lane 2, relaxation control; lane 3, solvent control; lanes 4–8 reactions with various concentration of m-AMSA.

account for the differential interaction of the compound with the enzymes.

3.3. Extent of inhibition depends on order of addition

m-AMSA inhibits type II topoisomerases by interacting with the enzyme and the DNA. The head group interacts with the enzyme while the acridine moiety intercalates into the DNA [13]. The intercalating property contributes to the inhibition of the relaxation reaction catalyzed by topoII, though it is not the sole determinant of the mechanism. To understand the interaction of the compound with topoI, DNA or the topoI-DNA complex, DNA relaxation assays were carried out with different orders of addition of the DNA, enzyme and the compound (Fig. S2). When the compound was pre-incubated either with the enzyme or the DNA, a complete inhibition of the reaction was observed at 10 μM and 25 μM respectively (Figs. S2A and S2B). In contrast, when the compound was added to the pre-formed DNA–enzyme complex, the inhibition was not complete even at 50 μM (Fig. S2C). From these data, it appears that the compound needs to first interact either with the protein or the DNA, prior to the formation of the topoI–DNA complex for inhibition of the reaction. In the first two orders of addition described above, the molecule is free to interact either with the enzyme or intercalate into the DNA, resulting in better inhibition. However, once the enzyme is bound to the DNA, m-AMSA cannot access the binding site and its interaction with the otherwise free components in the reaction is compromised, thus affecting the efficiency of the inhibition. From these observations, we deduce that the formation of the topoI–DNA complex would occlude the binding site(s) of m-AMSA on the enzyme as well as the DNA.

3.4. m-AMSA stimulates the DNA cleavage activity of MttopoI

m-AMSA was the first compound shown to poison the mammalian topoisomerase II [19]. The compound has been shown to induce the accumulation of protein-associated DNA strand breaks in treated cells as well as *in vitro* [19,33]. To assess the ability of the compound to promote such strand breaks in DNA in the presence of MttopoI, oligonucleotide based DNA cleavage assays were carried out with a double-stranded 32-mer. The substrate was pre-incubated with the compound to allow intercalation following which MttopoI was added to the reaction. With increasing concentrations of m-AMSA, stimulation of the cleavage was observed although the stimulation was only up to ~1.7-fold (Fig. 3A, lanes 5–7). In contrast, the DNA cleavage assay performed with the single-stranded oligonucleotide did not show stimulation of cleavage activity (Fig. S3). From these results, it appears that the intercalation of the compound to the DNA is required for its action, similar to its action against the eukaryotic topoisomerases. A low degree of cleavage stimulation suggests the binding of the enzyme to the drug with low affinity unlike m-AMSA binding to eukaryotic type II topoisomerase. From these studies, it is apparent that in addition to DNA intercalation, m-AMSA has to interact with the enzyme to impact DNA double-stranded breaks.

3.5. Inhibition of the growth of M. smegmatis and M. tuberculosis

Since m-AMSA inhibited DNA relaxation and induced the stimulation of topoI mediated DNA cleavage, the cell growth inhibitory potential of the compound was tested against *M. smegmatis* mc^2155 and *M. tuberculosis* $H_{37}Ra$ cells. The cell growth of *M.*

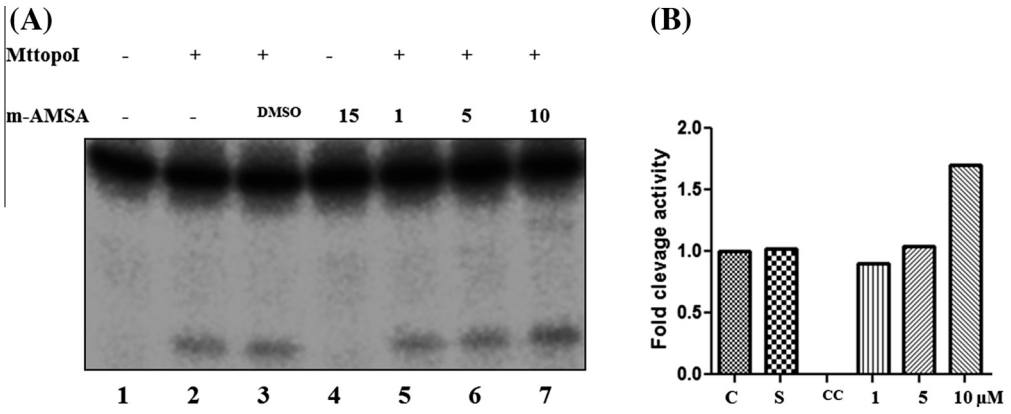

Fig. 3. m-AMSA stimulates cleavage: Different concentrations of m-AMSA (lanes 5–7) were incubated with 5′end-labeled specific 32-mer annealed to a complimentary sequence, at 37° C for 15 min following which MttopoI was added and the reaction was allowed to proceed at 37° C for 30 min. The reactions were terminated by addition of 45% formamide and heating at 95° C for 2 min. The products were resolved in a 12% denaturing PAGE and analyzed by phosphorimager. Lane 3 is the solvent (DMSO) control. C: cleavage reaction with MttopoI in absence of compound; CC: compound control, oligonucleotide incubated only with the compound; S: solvent (DMSO) control.

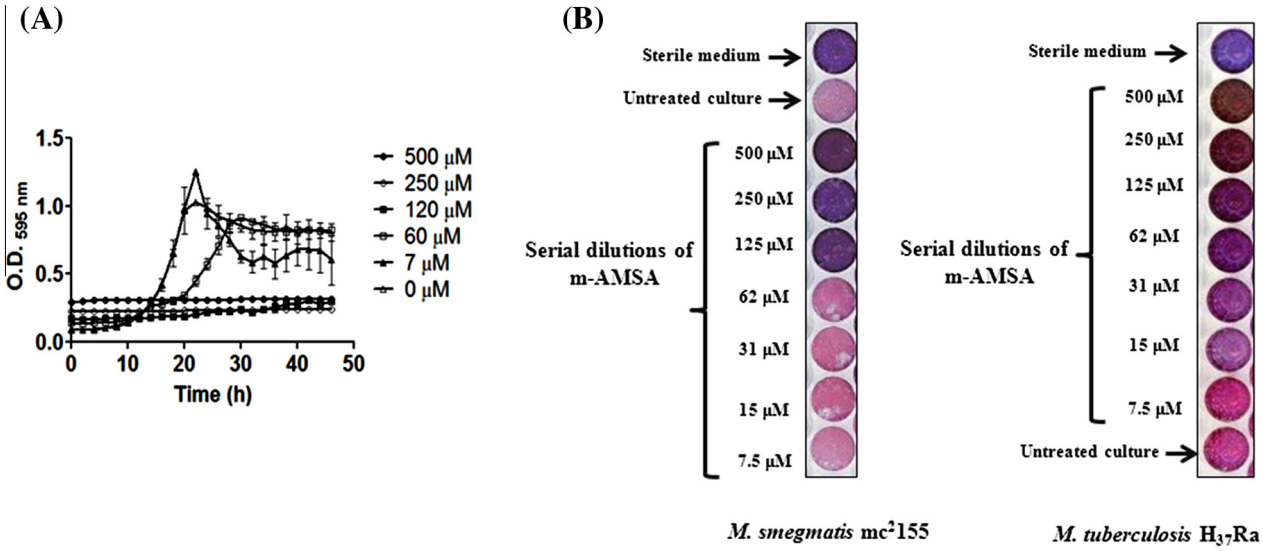

Fig. 4. Inhibition of *M. smegmatis* and *M. tuberculosis* growth by m-AMSA and determination of MIC values: (A) *M. smegmatis* mc^{2}155 and *M. tuberculosis* H_{37}Ra cells were grown in presence of increasing concentrations of m-AMSA. The growth was followed over a period of two days with OD_{595nm} being measured every 2 h. The growth curve was plotted using GraphPad Prism (*ver.* 5.0). Sterile medium and untreated culture were used as control. (B) *M. smegmatis* mc^{2}155 or *M. tuberculosis* H_{37}Ra cells were grown in presence of increasing concentrations of m-AMSA. Resazurin dye was added to a final concentration of 0.02% to each well. The plate was incubated at 37° C for 1 h (*M. smegmatis*) or 14 h (*M. tuberculosis*) to determine the MIC values. Sterile medium and untreated culture were used as control.

smegmatis mc^{2}155 cells was delayed at 60 µM of the drug while complete inhibition was achieved at higher concentrations (120 µM) (Fig. 4A). In the resazurin assay for cell viability, the MIC values of m-AMSA for *M. smegmatis* was 60 µM while for *M. tuberculosis* it was found to be higher (125 µM) (Fig. 4B). The differences in the MIC values for the *M. smegmatis* and *M. tuberculosis* cells could be an attribute of their physical properties. The former is a fast growing mycobacterium while the latter grows slowly. Further, there are a number of differences in the membrane composition and surface properties between the two bacteria [34]. Moreover, the rate of cellular metabolism and efficiency of uptake of molecules [35] could also account for the higher susceptibility of *M. smegmatis* for m-AMSA compared to *M. tuberculosis*. Overall, the combination of *in silico* and *in vitro* approaches resulted in the identification of m-AMSA as an inhibitor of eubacterial topoisomerases. Importantly, the findings in this manuscript illustrate the interaction of the drug both with the bacterial topoI and DNA to cause inhibition. During prolonged cancer chemotherapy, inhibition of an essential bacterial enzyme by an anticancer molecule would be of benefit against bacterial infections. Given its potent activity on mammalian topoII leading to cell death, it is unlikely to be clinically used against *M. tuberculosis*. However, structure–activity relationship studies with the drug may lead to better lead molecules which are specific towards the mycobacterial topoI. A DNA intercalating compound with the affinity to interact with enzyme would be an effective inhibitor, a point to be considered seriously in chemical strategies in the design of new molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.029.

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