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Inhibition of DNA helicases with DNA-competitive inhibitors

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Abstract—Helicases form an attractive protein family for drug discovery because they are involved in various human diseases. In this report, we show that it is possible to inhibit both the ATPase and the helicase activities of a DNA helicase with dibenzothiepins that bind at its nucleic acid binding site. These results suggest a drug discovery strategy to inhibit DNA helicases.

Helicases are ubiquitous enzymes, which utilize the energy liberated during nucleotide triphosphate (NTP) hydrolysis to separate double-stranded nucleic acids (NA) into single strands.[1] The human genome contains many helicases[2] and several of them play a role in diseases such as cancer.[3–5] Some helicases are also essential for the viability of various bacteria and viruses, and are therefore attractive targets for the development of new anti-infectious compounds.[6–8] Since their NTP-binding site is not well suited for drug discovery,[9] strategies to target these enzymes via other regions of their structure should be investigated. One of these strategies is to prevent the interaction between helicases and their NA substrate with compounds that bind at their NA-binding site.

To evaluate whether it is possible to inhibit DNA helicases with compounds interacting at their NA-binding site, we decided to use helicase IV (helIV) from *Escherichia coli* as a tool enzyme. This enzyme is well characterized[10,11] and it can be purified in a highly active form allowing an easy screen for inhibitors of both of its ATPase and helicase activities. Furthermore, helIV shows a good homology with PcrA (21 of the 26 residues located at the ATP and DNA-binding sites are identical in both proteins), which has been crystallized in the presence of its DNA substrate.[12] The similarity between these two proteins allowed us to establish a molecular model of helIV[13] that can be used as a guide to study the binding mode of helIV inhibitors.

Compounds from the Novartis compound library were screened (10 μM) in an ATPase assay.[14] After analysis of the hit list, compounds from each chemical class were selected and fresh solutions made. The purity of these solutions was verified by LC–MS and their potency (IC_{50}) determined. Compound **1** (Table 1) was identified as one of the most potent inhibitors of helIV ATPase activity. To study in more details the binding mode of this dibenzothiepin, several of its analogues were evaluated. The inhibitory properties of the most representative compounds are given in Table 1. Our SAR shows that the esterification of the carboxylic acid function (compound **2**) or the oxidation of the sulfur atom (compound **4**) reduces potency. In contrast, various substitutions at positions R^2, R^4, and R^5 are tolerated (Table 1). Since our purpose was to determine whether helIV can be inhibited with compounds that bind at its NA-binding site, the potency of these dibenzothiepins was sufficient to study their mechanism of action.

We first investigated whether the observed inhibition was the consequence of experimental artifacts. We demonstrated that compound **1** does not interfere with the reagent (malachite green) used for the ATPase assay (Supplementary Fig. 2), does not bind to DNA, and does not aggregate under the assay conditions as measured by nuclear magnetic resonance in T1ρ relaxation and waterLOGSY experiments (Supplementary Fig. 3). To determine the mode of inhibition of the

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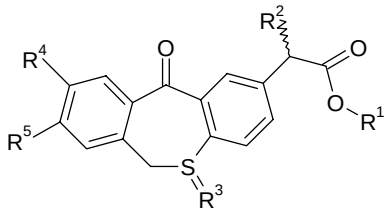
Table 1. Dibenzothiepins and their potency



Compound	R^1	R^2	R^3	R^4	R^5	ATPase[a] (%)	IC_{50}ATPase (μM)	Helicase[b] (%)
1	H	H		H	H	93 ± 10	5 ± 0.3	79 ± 4
2	CH_2CH_3	H		H	H	0 ± 0	nd	5 ± 12
3	H	CH_3		H	H	98 ± 1	3 ± 0.5	93 ± 1
4	H	H	O	H	H	2 ± 4	nd	0 ± 10
5	H	H		$NHCOCH_3$	H	99 ± 2	1.4 ± 0.2	89 ± 5
6	H	H		H	CH_3	78 ± 8	7 ± 0.8	53 ± 8

nd, not determined.
[a] Inhibition obtained in the presence of 10 μM compound, 5x $K_{m(ATP)}$ ATP, and saturating oligonucleotide concentration.
[b] 10 μM compound, 5x $K_{m(ATP)}$ ATP.

dibenzothiepins, the ATPase assay was carried out under steady-state conditions in the presence of different amounts of the compound (Supplementary data). Eadie–Hofstee plots of these experiments (Fig. 1A) show that compound **1** is an ATP non-competitive inhibitor and therefore that it does not bind at the ATP-binding site. The ability of dibenzothiepins to inhibit helIV unwinding activity was investigated in a time-resolved fluorescence quench helicase assay[13] (Supplementary data). All the compounds, which inhibited the ATPase activity of helIV (compounds **1**, **3**, **4**, and **6**), also inhibited its helicase activity, while compounds **2** and **4**, which were inactive in the ATPase assay, were also inactive in the helicase assay (Table 1). To determine whether dibenzothiepins affect the helIV–DNA interaction, the ability of compounds **1** and **2** to compete with DNA was studied in gel shift assays (Fig. 1B and Supplementary data). Compound **1** inhibits the interaction between helIV and a single-stranded oligonucleotide (dT_{25}) in a dose-dependent fashion. In contrast, the inactive analogue compound **2** does not prevent this interaction. The effect of compound **1** on DNA binding was further studied in the ATPase assay where its inhibitory effect on helIV ATPase activity was studied in the presence of different concentrations of dT_{25}. The data presented in Figure 1C indicate that compound **1** is a competitive inhibitor of dT_{25}. Altogether, these results reveal that, because dibenzothiepins prevent the interaction of helIV with DNA, they inhibit both its ATPase and helicase activities.

The DNA-binding site of helicases similar to that of helIV is constituted by different hydrophobic pockets where the bases of the DNA bind.[12] Docking studies let us to hypothesize that dibenzothiepins bind at the NA-binding site in a pocket located next to Phe411 (see below). To confirm this hypothesis, Phe411 was mutated to alanine (Phe411Ala), and the ATPase activity of the mutant enzyme was measured in the presence of saturating ATP concentration and different dT_{25} concentrations (Fig. 2A). From these experiments, K_{DNA}—which corresponds to the concentration of dT_{25} oligonucleotide required for half-maximal stimulation of ATP hydrolysis[15]—was determined. The larger K_{DNA} value for Phe411Ala indicates that the mutation reduces the affinity of the enzyme for dT_{25}. In contrast, similar $K_{m(ATP)}$ were determined for both the wild-type (wt) and the Phe411Ala proteins, indicating that the mutation does not affect ATP binding (Fig. 2A). Altogether, this shows that Phe411 is involved in DNA binding. The ability of compound **1** to inhibit wt and Phe411Ala ATPase activity was measured in the presence of 5x $K_{m(ATP)}$ concentration of ATP and 5x K_{DNA} concentration of dT_{25}. Under these conditions—the same occupancy of the NA-binding site of both enzymes by dT_{25}—compound **1** inhibits wt but not Phe411Ala (Fig. 2B). This shows that the mutation of Phe411 to alanine reduces the affinity of compound **1** for helIV-suggesting that Phe411 is involved in compound **1** binding to helIV.

To verify whether or not this effect is observed with other residues present at the NA-binding site, Tyr473—which is located in a different pocket at the NA-binding site—was mutated to alanine. This mutation also increases K_{DNA} without affecting $K_{m(ATP)}$ (Fig. 2A), confirming that Tyr473 is involved in DNA binding. The effect of compound **1** on Tyr473Ala is in sharp contrast to the effect observed with Phe411Ala (Fig. 2B). Under the same experimental conditions, compound **1** inhibits Tyr473Ala ATPase activity with a potency similar to that with which it inhibits wt ATPase activity (Fig. 2A). Therefore, not every mutation at the NA-binding site affects compound **1** binding, which suggests that Phe411 is important for the interaction between helIV and compound **1**.

We next used a molecular model of helIV[13] to determine whether the Phe411 pocket can accommodate

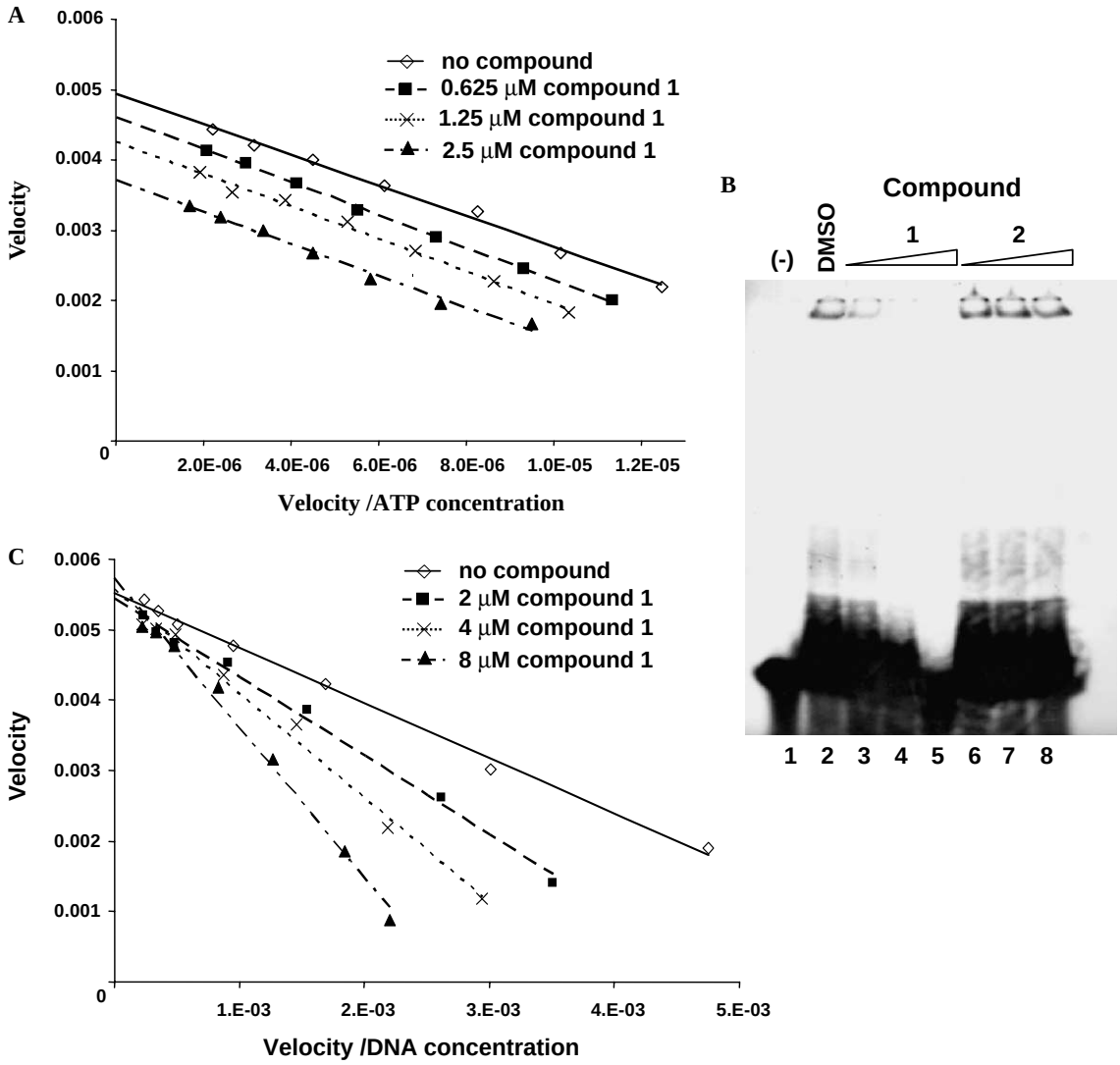


Figure 1. Inhibitory properties of dibenzothiepins. (A) The ATPase activity of helIV was measured in the absence or in the presence of the indicated compound **1** concentration. An Eadie–Hosftee plot (velocity vs velocity/ATP concentration) of one of these experiments is shown. (B) helIV and a radiolabeled dT_{25} oligonucleotide were incubated in the absence (5% DMSO) or in the presence of 8.75, 17.5, and 35 μM compound **1** (lanes 3–5) or compound **2** (lanes 6–8). The reactions were loaded onto a native polyacrylamide gel and the formation of helIV–dT_{25} complexes detected by autoradiography. (-) represents the probe in the absence of protein. (C) The ATPase activity of helIV was measured in the absence or in the presence of the indicated amounts of compound **1** at various dT_{25} concentrations. A velocity versus velocity/DNA concentration plot of one of these experiments is shown.

compound **1**. Once superimposed on the DNA base that binds to the corresponding pocket in PcrA,[12] compound **1** makes stacking interactions with Phe411, but does not contact Tyr473 (Fig. 3). If compound **1** interacts in such a way with helIV, only the mutation of Phe411 to Ala but not that of Tyr473 to Ala should disrupt its binding as observed in our experiments. Furthermore, the carboxylic function of compound **1** is in the vicinity of the guanidinium moiety of Arg446, suggesting that they form a salt bridge (Fig. 3). Dibenzothiepins that cannot make this ionic interaction should then have a decreased potency. In agreement with this, an ester form of compound **1**—compound **2**—is inactive in our assays (Table 1). Similarly, the presence of an oxygen atom at position R^3 in compound **4** should lead to sterical clashes between Phe411 and the inhibitor, explaining why this dibenzothiepin has a low potency (Table 1). The agreement between the observations made from the modeling study and the biochemical results shows that dibenzothiepins inhibit helIV because they bind at its NA-binding site in a pocket located next to Phe411.

We show in this report that it is possible to inhibit helIV with compounds that interact at its NA-binding site. This indicates that DNA helicases similar to helIV could be targeted with this approach. For example, various helIV homologues are essential genes for Gram-positive bacteria[16,17], therefore, these helicases are potential drug targets to identify new antibiotics to combat *Staphylococcus aureus*, *Bacillus anthracis* or *Listeria monocytogenes*. When more structures of DNA helicases in complex with their NA substrate will be available, the drug discovery strategy we have validated here with helIV could be fully exploited to identify new drugs.

A

	$K_{m(ATP)}$(μM)	K_{DNA}(μM)	IC_{50} (μM)
Wild type	224 +/-13	0.8 +/- 0.2	5 +/- 0.3
Phe411Ala	292 +/- 10	5.8 +/- 0.5	N.D.
Tyr473Ala	271 +/-21	7.0 +/- 0.2	5.5 +/- 0.6

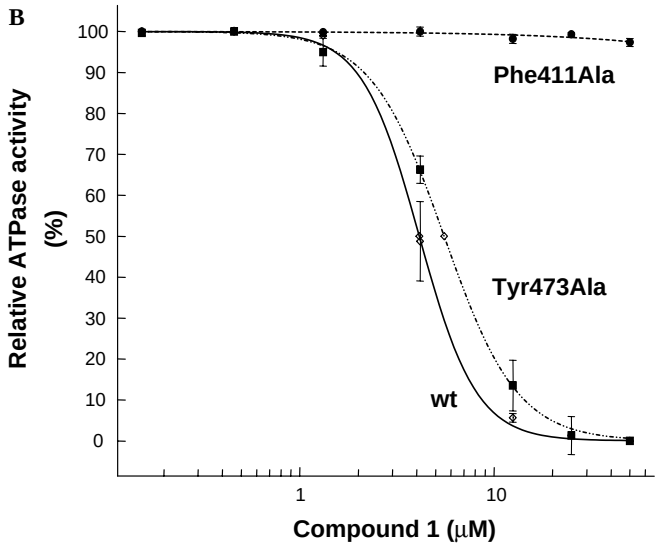


Figure 2. Mapping of the dibenzothiepin binding site. (A) Steady-state kinetic parameters for wt and mutant helIV proteins. N.D., not determined. (B) Inhibition of the ATPase activity of wt and mutant helIV by various concentrations of compound **1** (0.15, 0.46, 1.32, 4.17, 12.5, 25, and 50 μM). Hydrolytic activities in the absence of inhibitor were taken as 100%. Plots show means of triplicates.

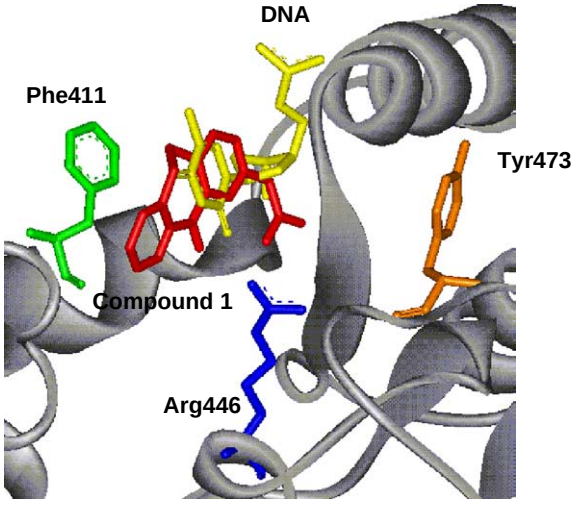


Figure 3. Proposed dibenzothiepin binding mode. Compound **1** (red) has been manually docked into the pocket located next to Phe411 (green). The DNA base found in the corresponding pocket in PcrA is represented in yellow, Tyr473 in orange, and Arg446 in blue.

Large efforts are ongoing to identify helicase inhibitors[18,19] with so far little success. This could be the consequence of the choice of the current targets, which may not be suitable for drug discovery. However, some encouraging data have been reported recently with the Rho transcription termination factor,[20] the herpes simplex virus helicase primase[21] or hepatitis C virus RNA helicase.[22]

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.10.110.

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14. Steady-state ATP hydrolysis was monitored by the measurement of inorganic phosphate release using acidic molybdate and malachite green.[23] HelIV (2.5 nM) protein was pre-incubated for 10 min at 37 °C in ATPase buffer (50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 1.5 mM dithiothreitol, 0.05% Tween 20, and 0.25 μg/ml BSA) with a dT_{25} oligonucleotide. The reaction was started by the addition of ATP and conducted for 20 min at 37 °C (the reaction is linear up to 25 min). The reaction mixture was then transferred into the molybdate/malachite green solution and the absorbance was immediately read at 630 nm (OD_{630}). A standard curve (known inorganic PO_4 concentrations vs OD_{630}) was used to determine the amount of inorganic phosphate produced during the reaction.
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