A novel DNA gyrase inhibitor rescues *Escherichia coli* dnaAcos mutant cells from lethal hyperinitiation

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**Objectives:** In order to search for novel antibacterial compounds we used a previously developed screening strain designed specifically to discover inhibitors of the bacterial initiator protein, DnaA. This strain (SF53) is not viable at 30°C due to overinitiation. Therefore, compounds that are able to restore growth to SF53 cells are likely to cause either partial or complete inhibition of DnaA function. In this study we used SF53 cells to screen the Library of Pharmacologically Active Compounds (LOPAC).

**Methods:** An SF53 screen of LOPAC in 384-well plates was performed. The effects of compounds identified as positive were studied further by growth assays specific for replication proteins as well as an *in vitro* assay of the activity of purified DNA gyrase.

**Results:** One of the compounds that tested positive in this screening was the benzazepine derivate (+)-6-chloro-PB hydrobromide (S143). We found that the substance did not target DnaA directly, but that it most probably reduces overinitiation by inhibiting DNA gyrase. Benzazepines have not previously been reported as gyrase inhibitors.

**Conclusions:** These findings indicate that a screening with SF53 will be able to identify compounds that also target other replication proteins (in addition to DnaA). Screening of LOPAC with SF53 cells led to the discovery of a novel DNA gyrase inhibitor.

**Keywords:** DnaA, initiation, DNA replication

**Introduction**

DnaA is the key protein for the initiation of DNA replication in *Escherichia coli* and other bacteria. It recognizes specific DNA sequences within oriC, where it binds and promotes DNA strand opening.\(^1\)–\(^3\) The opening of the double helix allows loading of DnaB and recruitment of the rest of the replication machinery.\(^1\)–\(^3\) The DnaA protein is considered a good target for novel antibiotics because its sequence is conserved among most bacteria and is only weakly related to eukaryotic initiators.\(^6\) A dnaAcos strain, SF53, was developed with the purpose of screening for DnaA inhibitors.\(^5\) The conditional mutant, dnaA219, has a cold-sensitive phenotype due to overinitiation.\(^6\) Introduction of the rnhA deletion into this strain allows initiation at stable R-loop structures (oriRs) if the DnaA protein is inactive.\(^5,7,8\) Thus, growth of SF53 at the non-permissive temperature, in the presence of compounds in a screening assay, should indicate either partial or complete inhibition of DnaA function.

The type II topoisomerases, DNA gyrase and topoisomerase IV, alter the topological state of DNA. They have a high degree of sequence similarity and use the energy of ATP hydrolysis.\(^9\) DNA gyrase is the only enzyme able to introduce (–) supercoils into DNA. Negative supercoiling of the chromosome is essential for initiation of DNA replication and transcription.\(^10\) Both DNA gyrase and topoisomerase IV are the targets of several drugs such as the synthetic compounds fluoroquinolones and the naturally occurring coumarins produced by certain species of *Streptomycyes*.\(^11\) While quinolones are responsible for stabilizing the double-stranded breaks formed by topoisomerase, the coumarins inhibit the ATPase activity of DNA gyrase and topoisomerase IV by competing with ATP binding to the GyrB and the ParE subunits of the enzymes, respectively.\(^12\)–\(^15\) In this report we show that a novel DNA gyrase inhibitor, discovered in the screening for DnaA inhibitors, is able to counteract hyperinitiation in the SF53 strain.
Materials and methods

Bacterial strains and growth conditions

The strains of *E. coli* K12 used in this study are listed in Table 1. Cells were grown routinely in Luria–Bertani (LB) medium or in AB minimal medium supplemented with 1 mg/L thiamine, 0.2% glucose and 0.5% casamino acids (CAAs) (glucose/CAA medium). Cell growth was followed by measurement of the OD 450 (the optical density at 450 nm) in glucose/CAA medium and the OD600 in LB medium. The screening was performed in glucose/CAA medium and cell growth monitored by measurement of the OD600. SF53 was grown overnight at 42°C and the screening was performed at 30°C.

Compounds used in growth experiments and DNA supercoiling assay

The Sigma product numbers of the compounds are given in parentheses: (+)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide (S143); (+)-1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride (D047); 6-chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (S2816 SKF); and 6-chloro-2,3,4,5-tetrahydro-1-(4-hydroxyphenyl)-1H-3-benzazepine-7,8-diol monohydrobromide (F6800). See Figure 1 for structures.

DNA supercoiling assay

The effect of S143 on the activity of purified DNA gyrase was determined in reaction mixtures (30 μL) containing 35 mM Tris–HCl pH 7.5, 24 mM KCl, 4 mM MgCl₂, 2 mM dithiothreitol, 1.8 mM spermidine, 1 mM ATP, 6.5% glycerol, 100 mg/L BSA, 250 ng of relaxed plasmid pHT1 DNA (TopoGen), 1 U of *E. coli* gyrase (TopoGen) and various concentrations of drug (0.5 μL). Samples were incubated for 60 min at 37°C, before adding 6 μL of stop buffer loading dye (5% Sarkosyl/0.125% Bromophenol Blue/25% glycerol). An aliquot of 30 μL of chloroform/isoamyl alcohol (24:1 mixture) was added and the sample was vortexed and centrifuged. The aqueous blue phase containing the DNA was loaded on a 1% agarose gel. The bands containing supercoiled DNA (marked by an arrow in Figure 5) were quantified with ImageQuant 5.0.

Results

Screening of the Library of Pharmacologically Active Compounds (LOPAC)

Approximately 1400 compounds of the LOPAC were screened twice with the SF53 (dnaA219 rnhA) strain. The screening was performed in 384-well plates with 20 μL of diluted cell cultures of SF53 that had been frozen and pre-checked for the absence of suppressors. The various compounds were added to each well at a concentration of 50 μM. The plates were incubated at 30°C for 24 h and the OD₆₀₀ was measured. If the OD₆₀₀ exceeded a threshold value of 0.1 the chemical was identified as a potential DnaAcos suppressor. One of the compounds that tested positive in this screening was the benzazepine derivative 6-chloro-PB hydrobromide (S143).

The benzazepines are bicyclic compounds consisting of fused benzene and azepine rings. The 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepines are known as selective dopamine receptor agonists, primarily used as research tools. Several analogues of S143 are available for purchase and we decided to test three of them (Figure 1).
Effect of various concentrations of S143 and its analogues on SF53 growth

We tested the effect of S143 and its analogues on growth of SF53 in glucose/CAA medium at 30°C. Various concentrations of the substances ranging from 0.1 to 1.0 mM (~30–400 mg/L) were added to a diluted culture, and the OD₄₅₀ was then measured after incubation with shaking at 30°C for 24 h (Figure 2). S143 was the only substance, of the four analogues tested, that was able to restore growth to SF53. Only a narrow range of S143 concentrations permitted suppression of the SF53 growth defect, with 0.2 mM (74 mg/L) as the optimal concentration. At higher concentrations of S143 the growth of SF53 was severely reduced. The complete inhibition of growth clearly demonstrates that S143 targets an essential function.

Effect of S143 and its analogues on E. coli wild-type growth

We next wanted to investigate what effect S143 and its analogues had on E. coli wild-type cell growth. Overnight cultures of CM735 grown in LB medium at 37°C were diluted to an OD₆₀₀ of 0.001 in 100 mL of fresh LB medium and incubated with shaking until cells reached an OD₆₀₀ of 0.15. Cells (10 mL) were then transferred into flasks and 0.25 mM of the various substances were added. Incubation continued with shaking at 37°C and the OD was measured every 30 min. As can be seen from Figure 3(a), all of the four analogues inhibited the growth of CM735, albeit to different degrees. Whereas S143 was the strongest inhibitor, D047 seemed to be the weakest. The fact that the three analogues of S143 exhibited a weaker inhibitory effect than S143, combined with a tendency to precipitate at higher concentration, might explain why they cannot restore growth to SF53. We also tested various concentrations of S143 (Figure 3b). The inhibition of CM735 increased with increasing S143 concentrations, but above a concentration of 0.5 mM (185 mg/L) the effect on CM735 was only slightly enhanced; there was only a minor difference in growth between 0.5 and 1.0 mM (185 and 371 mg/L). This may indicate that S143 had reached the maximum level of inhibition.

Effect of S143 on growth of cells without DnaA

So far our results show that S143 allows SF53 to grow, and that it inhibits growth of E. coli wild-type cells. To test if S143 targets DnaA we used two strains lacking the rnhA gene. In cells without RNaseH, recombination enzymes perform replication initiation at multiple sites (oriKs) and can therefore tolerate deletion of the oriC site and inactivation of DnaA. One of the

Figure 2. Effect of various concentrations of S143 and D047 on the growth of SF53 at 30°C. The OD of a culture of SF53 cells after 24 h of incubation with shaking at 30°C is shown as a function of the concentration of the added substance. S2816 and F6800 were also tested, but are not shown since they had no effect and yielded similar results to D047. S2816 and F6800 showed a tendency to precipitate above a concentration of 0.25 mM (~100 mg/L). The experiment was repeated four times.

Figure 3. Effect of S143 and its analogues on bacterial growth. (a) Growth curves of the wild-type strain CM735 at 37°C after addition of 0.25 mM of the compounds S143 (93 mg/L), D047 (73 mg/L), S2816 (100 mg/L) or F6800 (97 mg/L). (b) OD₆₀₀ curves of the wild-type strain CM735 at 37°C after addition of the indicated concentrations of the S143 compound.
strains harbours the dnaA wild-type gene (SF50) while in the other strain dnaA was deleted (SF51). If S143 targets DnaA the cells should not be seriously affected since they have an alternative mode of initiation. However, growth of both strains was inhibited in the presence of S143, the dnaA-deleted strain more so than the dnaA wild-type strain (Figure 4). This clearly indicates that DnaA is not the target of S143. The concentration of S143 in this experiment was 0.2 mM (74 mg/L), which is the same concentration that allowed growth of SF53.

**Gyrase inhibitors**

Since the compound S143 does not target DnaA it most probably influences another essential factor acting in replication or initiation of replication. Several proteins play a crucial role in replication, among them DNA gyrase by controlling the supercoiling level of the chromosome. S143 was tested for inhibition of DNA gyrase supercoiling activity in vitro by using drug concentrations up to 1 mM (371 mg/L). As can be seen from Figure 5, S143 was found to significantly inhibit DNA supercoiling activity, with an ~50% inhibitory concentration between 0.1 and 0.25 mM (37 and 93 mg/m). This is in the same concentration range that allows growth of SF53. It is thus possible that S143 partially inhibits gyrase and thereby reduces the lethal overinitiation of SF53.

**Overexpression of DNA gyrase**

We therefore investigated the in vivo effect of S143 on DNA gyrase. If S143 targets DNA gyrase, a strain overproducing gyrase should not be inhibited by S143 to the same degree as a wild-type strain. In the *E. coli* strain PJ4240, the two genes, gyrA and gyrB, encoding the two subunits of the gyrase enzyme are combined in one locus (the gyrA locus) under the control of a single inducible lac-type promoter. (The chromosomal gyrB gene is replaced with a gyrB::lacZ fusion.) Since DNA gyrase is an essential enzyme this strain must be grown in the presence of IPTG and previous experiments have shown that an IPTG concentration of 0.4 mM results in an expression of DNA gyrase of ~3-fold relative to the wild-type level. An overnight culture of PJ4240 grown in glucose/CAA medium and IPTG at 37°C was diluted to an OD650 of 0.001 in 50 mL of fresh glucose/CAA medium with 0.4 mM IPTG and incubated with shaking until the cells reached an OD650 of 0.15. Cells (10 mL) were then transferred into flasks with and without S143. Cell growth continued with shaking at 37°C and the OD was measured every 30 min. While the cell growth of the control (MC1000) was clearly inhibited by 0.25 mM (93 mg/L) S143, the growth of PJ4240 overproducing gyrase was, on the contrary, slightly stimulated (Figure 6). These results suggest that S143 indeed targets DNA gyrase.

**Effect of S143 on growth of *E. coli* cells with decreased susceptibility to fluoroquinolones or coumarins**

Point mutations in the topoisomerase genes are frequently the cause of resistance to quinolones and coumarins. To investigate whether *E. coli* strains with decreased susceptibility to fluoroquinolones or coumarins also have decreased susceptibility to the compound S143, we used two strains with mutations in DNA gyrase: C600gyrA<sup>S83L</sup>Δ<sup>D87Y</sup>C and LE316gyrB<sup>V164G</sup>C. While the mutations gyrA<sup>S83L</sup>Δ<sup>D87Y</sup>C confer decreased susceptibility to fluoroquinolones, the mutation gyrB<sup>V164G</sup>C confers low-level coumermycin resistance and temperature sensitivity. Overnight cultures of these strains were diluted to an OD<sub>600</sub> of 0.001 in LB. C600gyrA<sup>S83L</sup>Δ<sup>D87Y</sup>C cells were incubated with shaking at 37°C, and LE316gyrB<sup>V164G</sup>C at 30°C, until the cells reached an OD<sub>600</sub> of 0.15. Cells (10 mL) were then transferred to flasks with or without S143. Incubation was continued with shaking at the same temperatures and the OD was measured every 30 min. Growth of both the gyrA and gyrB mutant cells was inhibited by S143 to a degree similar to that of wild-type cells (Figures 3a, 3b, 6a and 7). After 2 h of growth the cell cultures with 0.25 mM (93 mg/L) S143 had an ~50% reduction in turbidity compared with the cell cultures without S143 (Figure 7). The observation that DNA gyrase mutations, conferring decreased susceptibility to fluoroquinolones and coumarins, did not influence the effect of S143 on cell growth might indicate that
S143 targets a site different from both fluoroquinolones and coumarins.

**Discussion**

As part of the search for new antimicrobial drugs, a screen for substances targeting the bacterial initiator protein DnaA was developed. Applying this screen on the LOPAC we have discovered a novel DNA gyrase inhibitor, the benzazepine (+)-6-chloro-PB hydrobromide. This inhibitor is considerably less potent than already known gyrase inhibitors—and that is probably the reason why it was picked in this screening. DNA gyrase is an essential enzyme, and a strong inhibitor of this protein would not have permitted growth of SF53. The fact that S143 inhibits cell growth at concentrations comparable to the 50% inhibitory concentration in the *in vitro* DNA gyrase assay suggests that diffusion through the membrane is not a limiting factor. The mechanism of action of this substance is yet to be investigated, and it is possible that a chemical modification of S143 will result in a more active compound. Such a compound may be worth developing since an increasing problem of resistance has been reported for the quinolone class of inhibitors of bacterial type II topoisomerases even for naturally highly susceptible species such as *E. coli*.

For a successful initiation of replication to occur several components in addition to DnaA are necessary: DnaB, DnaC, IHF, primase, gyrase and DNA polymerase III. Also RNA polymerase is essential for initiation with both wild-type DnaA protein and the hyperinitiating DnaA<sub полярных гибровых скопов</sub> mutation. It was previously shown that the template topology is important during initiation—formation of an initiation complex requires a negatively supercoiled *oriC* plasmid. Deletion of *topA*, encoding topoisomerase I, suppresses the temperature-sensitive *dnaA46* mutation. One explanation for this is that loss of topoisomerase I activity will increase the negative supercoiling, which again increases the likelihood of stable strand separation, and hence reduces the need for DnaA activity. Several other studies also indicate that DNA gyrase is important for initiation, probably by controlling the superhelicity of the template.

Here we have shown that partial inhibition of DNA gyrase may counteract lethal hyperinitiation and make the SF53 strain viable.
The effect was observed with a limited range of S143 concentrations, because an excessive amount directly inhibits replication and other essential cellular functions. The fact that the rnhA dnaA mutant cells were severely affected by the gyrase inhibitor indicates that the template topology is also important for replication from oriKs. These results indicate that screening for drugs that target DnaA will also lead to discovery of inhibitors of other proteins necessary for initiation of replication and possibly elongation of replication. Thus, screening with SF53 can be used to find interesting inhibitors that specifically target DNA replication.

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