Biochemical Pharmacology, Vol. 51, pp. 1373–1378, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00061-5

Novenamines as Inhibitors of Two Independent Enzymes during DNA Replication in a Toluenized *Escherichia coli* Cell System

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ABSTRACT. The amphiphilic novenamines described in this report have been shown previously to be specific inhibitors of human immunodeficiency virus type 1 reverse transcriptase-associated ribonuclease, which they inhibit when they are in the micellar state but not when they are monomeric. These compounds also inhibit the bacterial enzyme DNA gyrase, which is essential for DNA replication. Hence, the present studies were initiated to determine whether the molecular species inhibiting the gyrase reaction was the monomeric or the micellar form. For this purpose, the rate of DNA replication was measured in a toluenized *Escherichia coli* cell system in the presence of increasing concentrations of novenamines. The resulting concentration–response curves proved anomalous, suggesting the involvement of micelles or some other, noncovalently aggregated forms of the inhibitors. The results were analyzed in terms of a variety of kinetic schemes and were found to be most consistent with the model where novenamines inhibit replicative DNA synthesis predominantly as cooperative dimers and, to a lesser extent, as monomers, but not as highly aggregated micelles. Based on this analysis and the knowledge that novobiocin and all novenamine-containing analogs are powerful gyrase inhibitors, we conclude that the target of the cooperative, dimeric inhibition is the gyrase, whereas the monomers of the novenamines inhibit another enzyme species involved in the bacterial DNA replication process. BIOCHEM PHARMACOL 51;10:1373–1378, 1996.

KEY WORDS. bacterial DNA replication; gyrase; amphiphilic inhibitors; novenamines; topoisomerases

In a previous report, we showed that several novenamines inhibit the HIV-1† RNase H [1], whereas they do not inhibit the polymerase function of RT. In contrast, the parent compound, novobiocin, has no inhibitory effect on either the RT-nuclease or polymerase. The novenamines studied are strong amphiphiles and contain a single ionizable group. As a consequence, they can assume several physical forms, namely protonated monomers, ionized monomers, and ionized micelles, but not protonated micelles. In alkaline solutions and above their cmc, the compounds form ionized micelles that inhibit the nuclease, whereas the ionized monomers do not. Novenamine is a substructure of the antibiotic novobiocin and consists of the sugar noviose and a substituted coumarin residue (Fig. 1). Novobiocin and all analogs containing a novenamine residue also inhibit the bacterial enzyme DNA gyrase, which is an essential enzyme required during the replication of the bacterial chromosome [2–5]. Since the novenamines inhibit the viral RNase H exclusively in their micellar form, studies were initiated to determine whether the inhibition of gyrase also required the micellar form. The routine assessment of activity of the purified gyrase enzyme is based on a gel assay whereby the reaction products are separated by agarose gel electrophoresis [2]. By its intrinsic nature, this assay is only semiquantitative, and it does not yield results precise enough to establish good concentration–response curves.

In toluenized *Escherichia coli* cells, novobiocin and its analogs inhibit replicative DNA synthesis, but not repair synthesis, since gyrase is essential only for replication and not for repair [2]. Thus, toluenized cells constitute a convenient system for quantitating the gyrase inhibitory activity of novenamines. Hence, this system was used to study the inhibition patterns of the compounds described here. The concentration–response curves generated were anomalous and suggested the involvement of micelles or some other physically aggregated forms of the compounds in the inhibitory process. The data were analyzed mathematically, and they were found to be consistent with a kinetic model where replicative DNA synthesis is inhibited cooperatively by two novenamine molecules and, to a much lesser extent, by the monomers, but not by the micelles. Inhibition oc-

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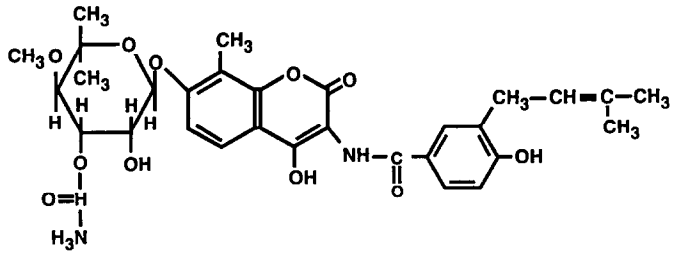
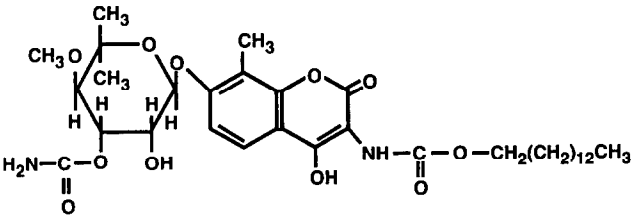
† *Abbreviations:* HIV-1, human immunodeficiency virus type 1; RT, HIV-1 reverse transcriptase; RNase H, RT-associated ribonuclease; and cmc, critical micelle concentration.

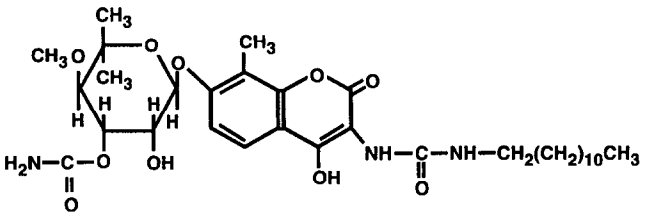
Received 5 October 1995; accepted 8 December 1995.



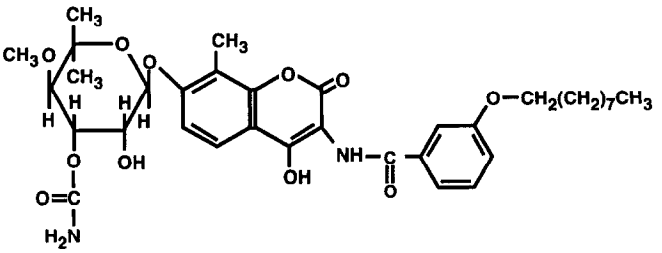
U-6391



U-34445



U-35122



U-35401

FIG. 1. Chemical structures for novobiocin (U-6391) and the novenamines U-34445, U-35122, and U-35401.

curred either on two independent enzymes or on two independent sites on the same enzyme; one of these sites was exclusively inhibited by the dimers—either formed in solution or at the active site—and the other site by the monomers.

MATERIALS AND METHODS

Chemistry of the Novenamines Studied

The compounds studied were the carboxy-novenamine ester U-34445, the carbamoyl-novenamine U-35122, and the *N*-benzoyl-novenamine U-35401 (Fig. 1). All compounds act as specific inhibitors of the HIV-1 RNase H function and do not inhibit the polymerase function of this enzyme. The three novenamines differ not only by the length of their aliphatic chains but also by the chemistry of the spacer groups connecting the chains to the novenamine moiety. Specifically, the C_{14} chain of U-34445 is connected with a carboxyl group to the novenamine. In the case of U-35122, the spacer is a carbamoyl group, and the chain is twelve carbon atoms long. In U-35401, a C_9 chain is linked to the novenamine through a benzoyl group.

Strain

The mutant *polA*$^-$ strain *E. coli* M21, obtained from Dr. R. E. Moses, was used throughout these studies.

Growth and Toluene Treatment of Cells

Cells were grown in a medium containing per liter: tryptone (Difco), 10 g; NaCl, 5 g; thymine, 10 mg; thiamine, 200 μg; at pH 7.2. They were grown to an optical density of 0.6 $O.D._{570}$ unit, harvested by centrifugation, and washed once with a 0.25 culture volume of 0.05 M potassium phosphate buffer, pH 7.5. The cells were then suspended in 0.1 vol. of the original culture medium in the same phosphate buffer containing 1% toluene and stirred at room temperature for 10 min [6, 7]. Following toluene treatment, the cells were removed by centrifugation, the pellet and tube walls were rinsed thoroughly with 0.05 M phosphate buffer, pH 7.5, and the pellet was suspended with the same buffer in 0.1 vol. of the original culture medium. This cell suspension was divided into small aliquots and stored at −70° until used.

DNA Synthesis

For measuring the ATP-dependent DNA replication, the reaction mixture contained, in a total volume of 150 μL: Tris · HCl buffer, pH 8.0, 50 mM; KCl, 0.1 M; magnesium-acetate, 10 mM; ATP, 2 mM; dCTP, dGTP, and dATP, 0.5 mM each; [^{35}S]TTP, 0.02 mM containing 1.25 μCi/sample

and approximately 1×10^8 cells/sample. Incubations were carried out at 37° for 30 min. The reaction was terminated by the addition of 150 μL of cold 10% trichloroacetic acid. The precipitates were collected on glass filters, extensively washed with 5% trichloroacetic acid, and counted for radioactivity.

RESULTS

Inhibition Kinetics

The concentration–response curves obtained with the novenamines studied in the DNA replication system were anomalous in that they were not hyperbolic as one would expect for a simple, low-molecular-weight, competitive inhibitor. The data were thus analyzed using several alternative mathematical models. Somewhat surprisingly, the experimental results were most consistent with a model where two different enzymes—or, alternatively, two distinct functional domains of the same enzyme molecule—are blocked competitively by the inhibitors. Moreover, the analysis predicts that one active site is inhibited exclusively by the monomeric form and the other exclusively by the dimeric form of the inhibitor. This dimer may be formed by cooperative binding at the active site or pre-formed at low concentrations in the solution. According to this model, we have the relations:

$$E_1 + I \overset{K_1}{} C_1 \tag{1}$$

for the monomer-inhibited enzyme and

$$E_2 + 2I \overset{K_2}{} C_2 \tag{2}$$

for the dimer-inhibited enzyme, where E_1 and E_2 are the concentrations of the free forms of the two enzymes, I is the concentration of the inhibitor, K_1 and K_2 are the inhibitory dissociation constants for the two enzymes, and C_1 and C_2 are the concentrations of the two enzyme-inhibitor complexes. Moreover, if the initial substrate concentration is $S_o \ll K_m$ for both enzymes, then:

$$E_{1,o} = E_1 + C_1 \tag{3}$$

and

$$E_{2,o} = E_2 + C_2 \tag{4}$$

where $E_{1,o}$ and $E_{2,o}$ are the total or analytical concentrations of the two enzymes. Further,

$$K_1 = \frac{E_1 I}{C_1} \tag{5}$$

$$K_2 = \frac{E_2 I^2}{C_2} \tag{6}$$

and

$$v = \alpha E_1 + \beta E_2 \tag{7}$$

where v = velocity of the reaction, and α and β represent the proportional constants of the two enzymes contributing to the total velocity v. As equations 5 and 6 are solved for C_1 and C_2, respectively, we have:

$$C_1 = E_1 \frac{I}{K_1} \tag{8}$$

$$C_2 = E_2 \frac{I^2}{K_1} \tag{9}$$

Also, we can solve for E_1 in terms of constants and I:

$$E_{1,o} = E_1 + E_1 \frac{I}{K_1} \tag{10}$$

$$E_1 = \frac{E_{1,o}}{1 + \frac{I}{K_1}} \tag{11}$$

and for E_2 in terms of constants and I:

$$E_{2,o} = E_2 + E_2 \frac{I^2}{K_2} \tag{12}$$

$$E_2 = \frac{E_{2,o}}{1 + \frac{I^2}{K_2}} \tag{13}$$

Then,

$$v = \frac{\alpha E_{1,o}}{1 + \frac{I}{K_1}} + \frac{\beta E_{2,o}}{1 + \frac{I^2}{K_2}} \tag{14}$$

and

$$v = \frac{v_{u,1}}{1 + \frac{I}{K_1}} + \frac{v_{u,2}}{1 + \frac{I^2}{K_2}} \tag{15}$$

where $v_{u,1}$ and $v_{u,2}$ are the velocities of the uninhibited reactions catalyzed by E_1 and E_2, respectively. Equation 15 correctly describes the kinetics of this system even if the condition $S_o \ll K_m$ is not satisfied, but then the calculated, apparent inhibitory constants, K_i^{app}, are related to the true inhibitory constants, K_i by the equation

$$K_i^{app} = K_i \frac{K_m}{S_o + K_m} \tag{16}$$

In either case, the percent inhibition can then be calculated as

$$\% \text{ inhibition} = 100\,\frac{v_{u,1} + v_{u,2} - v}{v_{u,1} + v_{u,2}} \qquad (17)$$

Accordingly, the percent inhibition versus I data at a given enzyme and substrate concentration can be analyzed using the following equation:

$$\% \text{ inhibition} = 100\left(1 - \frac{\dfrac{v_{u,1}}{v_{u,1} + v_{u,2}}}{1 + \dfrac{I}{K_1}} - \frac{1 - \dfrac{v_{u,1}}{v_{u,1} + v_{u,2}}}{1 + \dfrac{I^2}{K_2}}\right) \qquad (18)$$

where K_1, K_2, and the fraction $v_{u,1}/(v_{u,1} + v_{u,2})$ are the independent parameters that govern the inhibition kinetics. The fraction $v_{u,2}/(v_{u,1} + v_{u,2})$ is not an independent parameter, since it is equal to $1 - v_{u,1}/(v_{u,1} + v_{u,2})$.

The experimental data were analyzed in terms of Equation 18, using a nonlinear least-squares regression program.

Inhibition of DNA Replication

As mentioned above, the inhibition patterns obtained with U-34445, U-35122, and U-35401 in the replication system were anomalous in that they were not hyperbolic as one would expect for simple, low-molecular-weight, competitive inhibitors (Fig. 2, a–c). Instead, at low concentrations (up to 0.25 μM), no inhibition was observable, and inhibition suddenly increased from none to approximately 70% over the 0.25 to 5 μM range. Slightly above 5 μM, a discontinuity occurred in the curves; the inhibitions increased only slightly from 5 to 500 μM to approach 100%. Analysis of the concentration–response curve for U-34445 in terms of Equation 18 yielded the following equilibrium constants: K_1 = 88.7 μM for the monomeric form of U-34445 and K_2 = 1.9 ± 0.17 (SD) μM^2 for the dimeric form (Fig. 2a). Hence, the dimers are inherently more potent than the monomers. The analysis also yielded a value of 0.25 for the parameter $v_{u,1}/(v_{u,1} + v_{u,2})$, representing the fractional contribution of the monomers to inhibition.

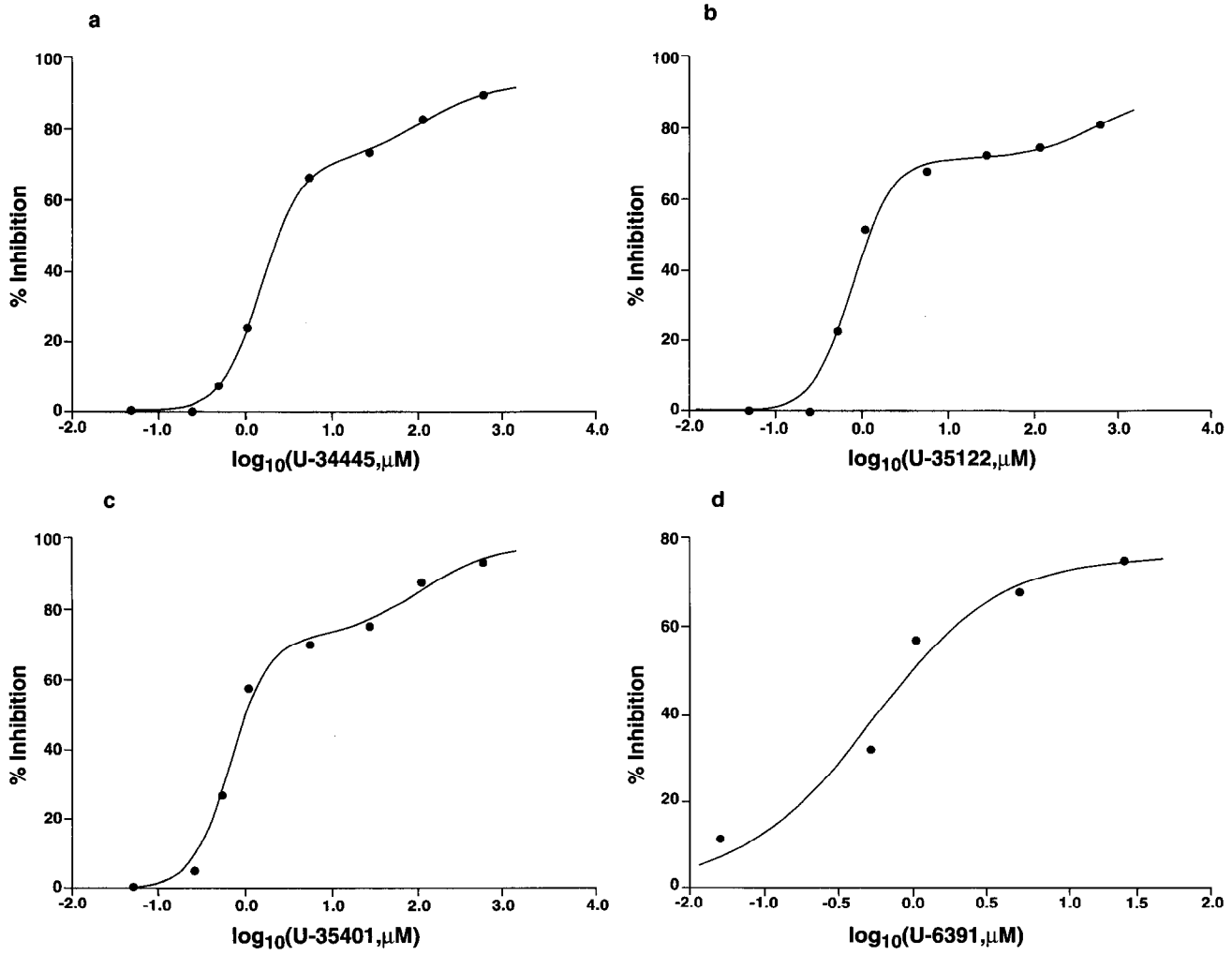


FIG. 2. Computer-generated concentration–response curves, based on Equation 18, for U-34445 (a), U-35122 (b), U-35401 (c), and novobiocin (U-6391) (d). The points represent the experimental data.

The parameter $v_{u,2}/(v_{u,1} + v_{u,2})$, which is equal to $1 - v_{u,1}/(v_{u,1} + v_{u,2})$, then yields a value of 0.75 and represented the fractional contribution of the dimers to inhibition. In other words, the monomers contribute 25% and the dimers 75% to the total inhibition. The analysis of the inhibition data for U-35122 yielded $K_1 = 498$ μM and $K_2 = 0.5 \pm 0.11$ μM^2 (Fig. 2b). Again, the K_i for the monomer was extremely large and reflected a very weak binding affinity of the monomers as compared with that of the dimers for their respective target sites. We also calculated $v_{u,1}/(v_{u,1} + v_{u,2}) = 0.22$ and by difference $v_{u,2}/(v_{u,1} + v_{u,2}) = 0.78$. Again, these results indicated that the monomeric and dimeric inhibitions are both operative, but the dimers are significantly more potent. With U-35401, the value for K_1 was 90 μM and the value for K_2 was 0.37 ± 0.08 μM^2 (Fig. 2c), with $v_{u,1}/(v_{u,1} + v_{u,2}) = 0.26$ and $v_{u,2}/(v_{u,1} + v_{u,2}) = 0.74$.

As already demonstrated by others [2], the parent compound, novobiocin, acted as a specific inhibitor of the gyrase in our system. It did not show the complex behavior observed with the three novenamines studied here. Instead, novobiocin acted as a monomeric inhibitor of one of the two enzyme sites, and the concentration–response curve was hyperbolic, indicating competitive inhibition (Fig. 2d). We calculated $K_i = 0.47 \pm 0.13$ μM and $v_{u,1}/(v_{u,1} + v_{u,2}) = 0.76$. In other words, 24% of the total rate was insensitive to novobiocin inhibition and, thus, novobiocin was unable to block completely the replicative process in our system.

As mentioned in the introduction, the three novenamines studied form ionized micelles at and above their cmc values [1]. In contrast, novobiocin does not form micelles when tested over a concentration range extending from 1 to 200 μM. The cmc for U-34445 is 25 μM, for U-35122 30 μM and for U-35401 47 μM (see Ref. 1), and these concentrations are much higher than the respective K_i values of the dimers reported here. Considering the excellent fit of the experimental results to the kinetic model embodied in Equation 18, we conclude that the micellar forms are not required for inhibition of the DNA replication in our system.

DISCUSSION

The kinetic model used here to analyze the results considers that: (a) two independent enzymatic activities are inhibited by the novenamines; (b) the two activities may reside on two different enzyme species or on the same enzyme; and (c) one activity is inhibited exclusively by the monomers while the other is inhibited by dimers formed in solution or, cooperatively, at the active site. This model is fully consistent with the experimental concentration–response curves (see Fig. 2, a–c) and with the proposal that the inhibitors act as ionized monomers and dimers, but not in the form of micelles. When assayed against the purified DNA gyrase, novobiocin and all novenamine-containing analogs are potent inhibitors and block the activity of this enzyme completely [3]. Based on this observation, we conclude that the major inhibitory activity of the three novenamines studied here is directed towards the gyrase function in the DNA replication system. Moreover, the dimers are inherently more potent than the monomers as they inhibited the replicative activity to the extent of 68–72%. Hence, the dimeric forms of the compounds must inhibit the gyrase. This conclusion is further substantiated by the observation that the parent compound novobiocin, which acts as a specific inhibitor of gyrase, was unable to block completely DNA replication in the system: at saturating concentrations, the maximal extent of inhibition attained was about 76% (Fig. 2d). Therefore, 24% of the replicative activity remained insensitive to novobiocin inhibition and represented a gyrase-independent component of the reaction. As mentioned above, the dimeric forms of the three novenamines studied inhibited DNA replication to the extent of 68–72% which is nearly the same value as the one obtained with novobiocin (76%), and this lends further credence to the conclusion that the dimers inhibit the function of the gyrase in the replication system. As the dimers were unable to suppress completely the replicative activity in the system, at very high analytical concentrations the monomers became effective as well and in concert with the dimers arrested DNA synthesis completely. The kinetic model does not reveal whether the dimers and monomers inhibit two different enzymes or two distinct functional sites on the same enzyme. However, since the effect of the dimers fully accounts for the inhibition of the gyrase, the monomers must inhibit a different enzyme involved in the gyrase-independent replication process and not a second, independent functional site of the gyrase. Considering that approximately 20 different protein species participate in the replication of the bacterial chromosome [8], it is somewhat tenuous to speculate as to what other enzyme might be impaired by the monomers. The analysis shows that at lower novenamine concentrations the inhibitory effect is restricted to the dimers, while the monomers remain inactive. It is only at very high analytical concentrations that the monomers become active although they are obviously present in the system at all concentrations. The replication complex studied here is a multi-enzyme complex, and this suggests that the dimers need to bind to the complex first. In turn, the association of the dimers with the complex must lead to a conformational change of the complex which then allows for the monomers to bind to another enzyme within the complex in a secondary step, thus completely blocking DNA synthesis.

We thank Dr. R. E. Moses for supplying us with the polA⁻ E. coli *strain and Janet K. Zelenock for editing the manuscript.*

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