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Research Articles

The amphiphilic properties of novenamines determine their activity as inhibitors of HIV-1 RNase H

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Abstract. Few inhibitors of the RNase H function associated with the HIV-1 reverse transcriptase have been discovered to date. We observed that three novenamines, U-34445, U-35122, and U-35401, are specific inhibitors of the HIV-1 RT RNase H function. All three compounds are strong amphiphiles and contain one ionizable group. Hence, a priori, in aqueous solutions the inhibitors might exist in at least four different physical states, namely protonated monomers, ionized monomers, protonated micelles, and ionized micelles. The three inhibitors all yielded anomalous dose-response curves, indicating that the four molecular species have different inhibitory potentials. In order to identify the inhibitory species, the amphiphilic properties of these compounds were studied. It was established that in alkaline solutions, around pH 8, all compounds are ionized and form micelles at concentrations above their CMC. Both the protonated and the ionized forms of these molecules form stable insoluble monomolecular layers at the air/water interface. The anomalies of the dose-response curves can be resolved by taking into account the fact that, in solution, the relative proportion of these molecules in each physical state depends on the pH and on their analytical concentration. Thus interpreted, the results indicate that RNase H is inhibited only by the ionized micellar form of these compounds and not by their monomeric form. Around their pK_a ($\sim$pH 5), the three compounds reproducibly form uniformly sized, self-emulsified colloidal particles that may be used as an efficient drug delivery system.
Key words. HIV RNase H; inhibitors; novenamines; micelles.

Abbreviations: HIV-1, HIV-2, human immunodeficiency virus type 1 or 2, respectively; HIV RT, reverse transcriptase; RNase H, HIV-1 RT associated nuclease; CMC, critical micellar concentration; MLV, murine leukemia virus.

Specific inhibitors of the HIV-1 (cf. abbreviations above) RNase H function are rare. We discovered several novenamines that act as specific inhibitors of this enzyme. Novenamine is a substructure of the antibiotic novobiocin and consists of the sugar noviose and a substituted coumarin residue (fig. 1). Novobiocin (U-6391) and all analogs containing a novenamine moiety also inhibit the function of the B subunit of the bacterial enzyme DNA gyrase[1–3]. The novenamine analogs described here act as specific inhibitors of the HIV-1 RNase H enzyme without affecting the polymerase function. A more detailed study of the inhibition of the RNase reaction revealed that the dose-response curves are anomalous and indicated an unusual inhibitory mechanism. The molecules are strong amphiphiles and this suggested that their inhibitory action might be governed by their amphiphilicity. Hence, the amphiphilic properties of the compounds were studied.

The compounds are not inhibitory at low concentrations but gain activity after a critical concentration threshold is reached. This abrupt onset of inhibition at intermediate inhibitor concentrations suggested the involvement of micelles or other ordered structures since amphiphilic molecules have the potential to form micelles or other ordered structures in aqueous solution[4–6]. Moreover, the molecules will ionize and may thus be present either in the protonated or the deprotonated form. As a consequence, the inhibitors may, a priori, assume at least four different physical states, namely a) protonated monomers, b) deprotonated monomers, c) protonated micelles, and d) ionized micelles. We show in this paper that the novenamines may exist in aqueous solutions as protonated and ionized monomers and, in alkaline solutions, as ionized micelles. The amphiphilic nature of these molecules is further illustrated by showing that they are able to form insoluble monolayers at an air/water interface both in their protonated and ionized forms. They also form self-emulsified colloidal particles near their pK_a. Finally, a specific physical form of each compound is shown to be responsible for the inhibition of RNase H.

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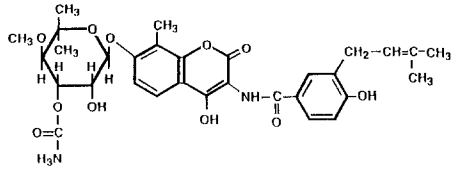
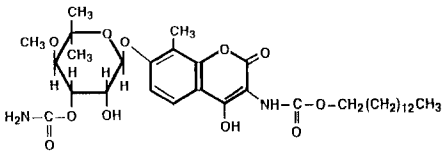
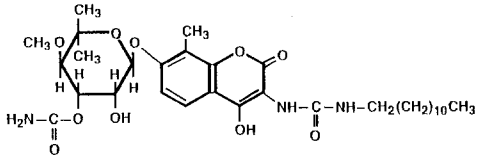


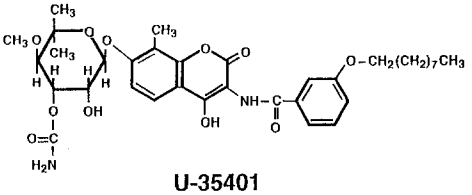
U-6391



U-34445



U-35122



U-35401

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

The compounds studied are the carboxy-novenamine ester U-34445, the carbamoyl-novenamine U-35122 and the N-benzoyl-novenamine U-35401 (fig. 1). They were chosen by virtue of their inhibitory activity toward HIV-1 RNase H. 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.

Materials and methods

The carboxy-novenamine ester U-34445, the carbamoyl-novenamine U-35122 and the N-benzoyl-novenamine U-35401 were prepared as described[7]. The pure, recombinant HIV-1 RT was provided by Dr. S. K. Sharma and his associates. The RNase H assay system measures the loss of trichloroacetic acid-precipitable radiolabelled RNA:DNA hybrid as a function of time[8]. The specific assay mixtures contained 2.5 µg and 2 µCi/ml of [^{3}H]poly(rG):poly(dC) (1:1), 20 mM Tris.HCl, pH 8.5, 2 mM $MgCl_2$, 0.02% Nonidet P-40 (Sigma), 3% glycerol and 1 µg of p51/p66 enzyme per 50 µl reaction mixture. Samples were incubated for 30 min at 25 °C, and the reaction was terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid. The precipitate was collected on a glass filter, washed, dried and its radioactivity measured.

Under our experimental conditions the RNase H-catalyzed hydrolysis of the hybrid substrate is a first-order reaction. In order to increase the accuracy of our measurements, we did not measure initial rates of hydrolysis, but, rather, measured the remaining substrate concentration after a significant portion of the substrate had been hydrolyzed. The first-order reaction is described by the equation:

$$S_u = S_0\, e^{-kt} \tag{1}$$

where S_0 is the initial substrate concentration, S_u is the substrate concentration remaining at time = t in the absence of inhibitor, and k is the experimental first-order rate constant. In the presence of a competitive inhibitor at concentration I, in great excess with respect to the enzyme concentration, the substrate concentration, S, remaining at time = t is:

$$S = S_0\, e^{-\frac{kt}{1+\frac{I}{K_i}}} \tag{2}$$

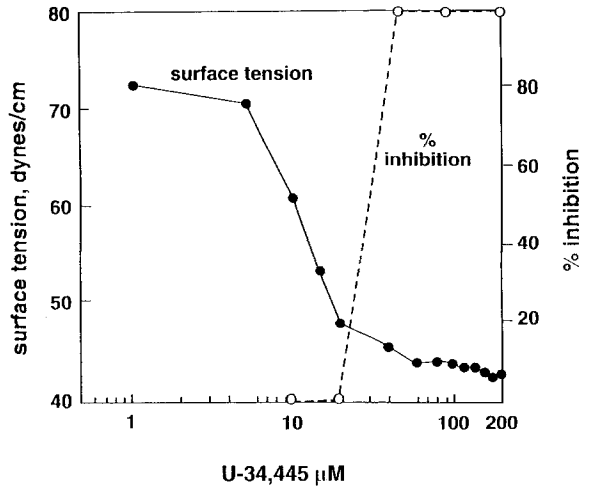
where K_i is the dissociation constant of the enzyme-inhibitor complex. If both the uninhibited and the inhibited reaction mixtures are incubated for the same length of time, then the 'percent inhibition', may be described in terms of the decrease of the experimental first-order rate constant:

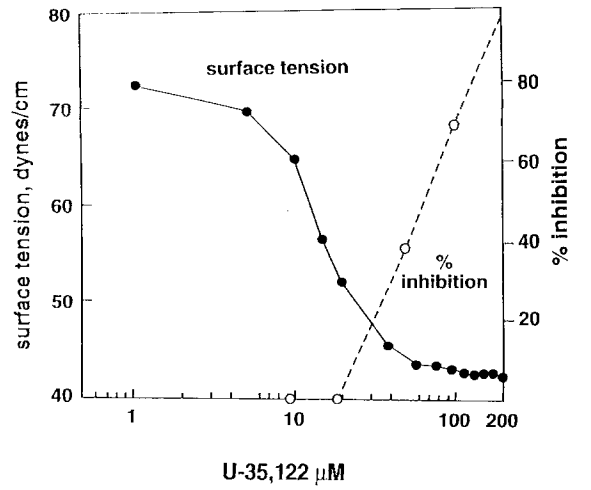
$$\%\ \text{inhibition} = 100\,\frac{\ln S - \ln S_0}{\ln S_u - \ln S_0} \tag{3}$$

The value for K_i can be calculated from the relationship:

$$\%\ \text{inhibition} = 100\,\frac{I}{K_i + I} \tag{4}$$

The surface tension was measured by the du Noüy ring method, using a Cahn 27 electrobalance, and a 4 mm deep circular teflon trough of 30 mm diameter. The surface pressure-area curves were measured using a Lauda-Langmuir film balance (Brinkman Instruments, Des Plaines, Illinois, USA). Monolayers were spread from ethanol-hexane (3:7, v:v) stock solutions.





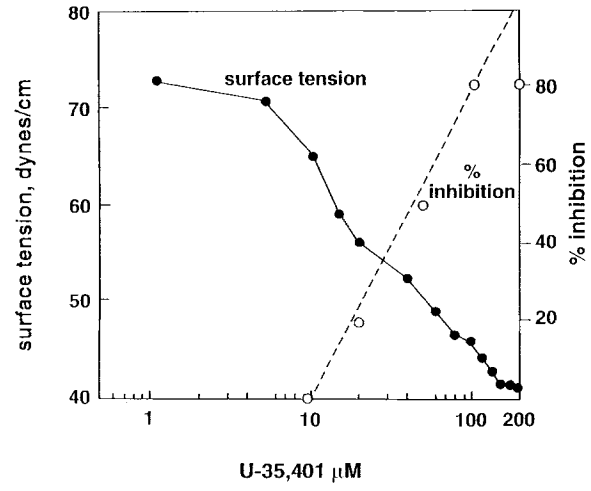


Figure 2. Surface tension measurements and RNase H inhibition of U-34445, U-35122 and U-35401.

The mean particle size of the colloidal particles was measured by Quasi Elastic Light Scattering using a Nicomp 370 Submirror Particle Sizer (Pacific Scientific, Santa Barbara California, USA). The novenamines

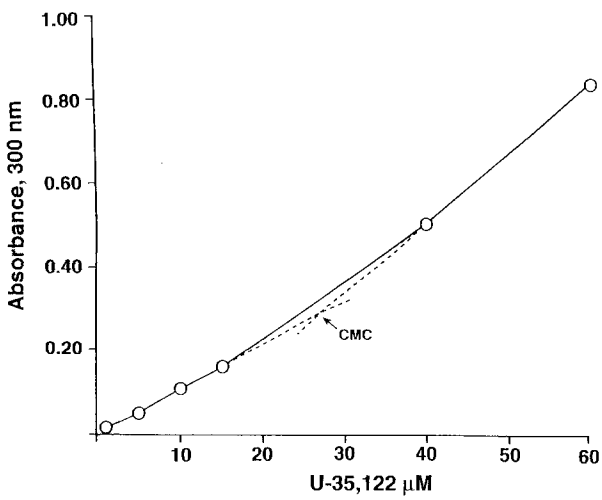


Figure 3. Optical density-concentration curve for U-35122.

were prepared as 20 mM stock solutions in methanol. These stock solutions were then diluted 100 to 200 fold in 10 mM acetate or phosphate buffers covering a pH range from pH 4 to 8. The minimal particle size the instrument can detect is slightly less than 20 nm.

For the electron microscopic studies, 2 µl aliquots of a 200 µM solution of inhibitor were applied to formvar support films on 200 mesh copper grids. Excess liquid was drained and the samples were allowed to dry. The samples were then negatively stained with 0.2% aqueous uranyl acetate, viewed and photographed in a JEOL 1200EX electron microscope.

Results

Soluble monolayers. Amphiphilic molecules preferentially accumulate at the water/air interface where partial hydration stabilizes the molecules with respect to the fully hydrated state in the bulk solution. This generates a 'soluble monolayer' where even the lipophilic domains of the amphiphilic molecules are still partially hydrated[4]. The monodisperse solute in the aqueous phase is in a partition equilibrium with those in the surface layer. The accumulation of the solute on the surface results in a decrease of the surface tension. Upon reaching the CMC, the critical micellar concentration, the monomer concentration becomes constant and independent of the analytical concentration of the solute. As a consequence, the surface tension also becomes constant and independent of the analytical solute concentration.

The three novenamines studied were each dissolved in a 0.1 M Tris.HCl buffer, pH 8.3, and the surface tension of these solutions was measured as a function of the increasing concentrations of the compounds. Each of the novenamines possesses a free hydroxyl group which has a pK_a of ~5. Hence, in this alkaline buffer, the solutes are present exclusively in the deprotonated form.

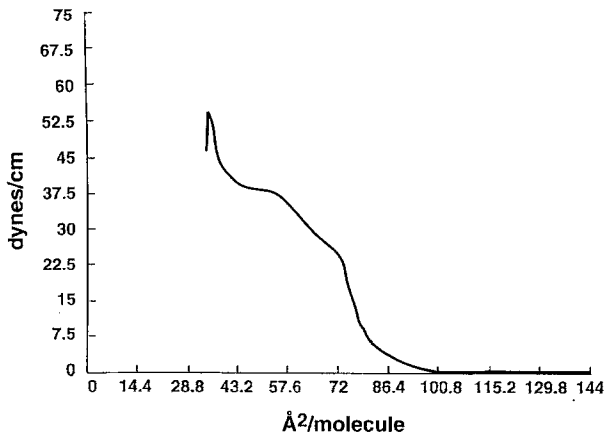


Figure 4. Force-area curve for U-35122 at pH 4. Note the two transitions areas.

As shown in figure 2, the surface tension of solutions of each of the three compounds becomes constant at high concentrations, thereby demonstrating that all three molecules form micelles at high concentrations. We found that the CMC for the carboxyl-ester U-34445 was 25 μM, the one for the carbamoyl-novenamine U-35122 was 30 μM and the N-benzoyl-novenamine U-35401 had a CMC of 47 μM. Further evidence for micelle formation was obtained by spectrophotometric measurements. Due to the different microenvironments and because of the internal filter effect in micelles, monomers possess a molar absorptivity somewhat different from that of micelles. Thus, the CMC is evident as a discontinuity in a plot of UV absorbance at a specific wave length against the analytical concentration of the compound. As an example, the plot obtained with U-35122 is shown in figure 3.

Insoluble monolayers. Amphiphilic molecules spread onto the air/water interface can form insoluble monomolecular layers. The molecules organize themselves at the water–air interface in such a way that the hydrophilic ends are hydrated while the lipophilic ends are fully dehydrated. The ability of an amphiphilic monomer to form stable insoluble monolayers requires a delicate balance between their hydrophilic and lipophilic domains. In addition, the stability of the monolayer depends on specific interactions between the dehydrated lipophilic chains. The most readily assessable property of insoluble monolayers is their surface isotherm, where the surface pressure is measured as a function of the surface area available per molecule. In response to a lateral force or pressure applied to such monolayers, several distinct states, referred to as transition states, may be formed which are limited to certain pressure ranges. These states represent different conformations of the molecules on the surface. The transition from one state to another manifests itself as a discontinuity in the surface pressure-area curves. The surface states may be defined mathematically and classified according to the parameters of the surface isotherms[4]. As an example a force-area curve for U-35122 is shown in figure 4 which possesses two transition states.

The novenamines studied have a $pK_a \sim pH$ 5. They were tested for their potential to form insoluble monolayers at the air/water interface both at pH = 4 and at pH = 8 where they are present in their respective protonated or ionized forms. At pH = 4, U-34445 exhibited no obvious gaseous phase. Instead, as is true for many lipophilic molecules that tend to self-associate, the first detectable state is a liquid expanded state which is characterized by a 'liftoff area', an area/molecule above which there is no measurable surface pressure. For U-34445 at pH = 4, the liftoff area was 65 Å^2 per molecule (table 1). Moreover, a collapse area of 35.8 Å^2 per molecule and no transition phase were measured. Both U-35122 and U-35401 had much larger liftoff areas (105, 106 Å^2 per molecule) and their collapse areas per molecule were somewhat smaller (32.6, 29.4 Å^2 per molecule) as compared to the one for U-34445. U-35122 had two transition phases, U-35401 had one such phase. The collapse pressures were quite high and of similar magnitude (49.1 to 54.8 dynes/cm) for the three compounds. It is thus apparent that, in their protonated forms, these novenamines form stable insoluble monolayers at the air/water interface. These monolayers are highly compressible, indicating that the surface molecules are readily reoriented to present a smaller cross-sectional area. At low pH's, the length of the unbranched carbon chain present in each of the compounds, did not have any significant effect on the

Table 1. Force-area curves for U-34445, U-35122, and U-35401.

Compound	pH	Liftoff areas	Area of transition	Collapse area	Collapse pressure
		Å^2/molecule	Å^2/molecule	Å^2/molecule	dynes/cm
U-34445	4	65.3	–	35.8	51.6
U-35122	4	105.6	67.1/50.5*	32.6	49.1
U-35401	4	106.4	34.3	29.4	54.8
U-34445	8	143.3	85.1	33.1	≈35
U-35122	8	138.3	79.2	–	≈30
U-35401	8	140.9	66.7	–	≈20

* This compound has two transition phases.

Table 2. Relationship between particle sizes and pH.

pH	U-34445 particle	U-35122 particle	U-35401 particle
	size; nm	size; nm	size; nm
4.0	150 ± 58	ppt[a]	ppt
4.2	100 ± 36	ppt	ppt
4.5	120 ± 50	ppt	ppt
4.7	120 ± 48	ppt	ppt
5.0	100 ± 38	130 ± 50	ppt
5.3	130 ± 50	160 ± 60	ppt
5.7	120 ± 43	150 ± 60	130 ± 50
6.0	120 ± 44	120 ± 50	120 ± 50
6.3	N.d.[b]	150 ± 70	140 ± 70
6.5	140 ± ??	100 ± 50	130 ± 40

[a] Precipitate.
[b] Not determined.

collapse areas nor the collapse pressures. However, U-34445, containing a C_{14} chain, had a much smaller liftoff area as compared to the ones for U-35122 (C_{12} chain) and U-35401 (C_9 chain). The relatively large liftoff areas measured for these novenamines suggest that a large portion of their lipophilic domains lay flat on the water surface at the air/water interface.

At pH = 8 all compounds are ionized. Somewhat unexpectedly, they still formed relatively stable insoluble monolayers. Their liftoff areas ranged from 138.3 to 143.3 Å^2 per molecule (table 1). The liftoff areas were thus larger and more uniform as compared to the corresponding protonated forms at low pH. Each of the novenamines also showed one transition phase. These areas of transition decreased progressively as the carbon side-chain is reduced from C_{14} as present in U-34445 to C_9 as present in U-35401. The stability of the monolayers is considerably reduced at this high pH as reflected by their estimated collapse pressures ranging from ~20 to ~35 dynes/cm. The collapse areas could not be determined accurately for U-35122 and U-35401 and the collapse pressures for all three compounds had to be estimated. Moreover, the collapse pressures decreased with decreasing chain length. The large liftoff areas per molecule suggest that the entire lipophilic domains of these compounds lay flat on the water surface.

Particle self-emulsification. The observation that the amphiphilic compounds studied here form insoluble monolayers at the water–air interface suggest that these molecules might form colloidal particles in solution at a pH near their pK_a. Thus, each compound was studied in solution over a pH range around their pK_a (e.g. pH 4 to 6.5) to establish if particles are formed and, if so, whether the particle sizes were pH dependent. The particle size and size distribution were determined by quasi-elastic light scattering. The results are listed in table 2.

In aqueous buffered solutions at a concentration of 100 μM, the novenamine U-34445 showed a mean particle size of approximately 120 nm. This value remained relatively constant over the pH range from 4 to 6.5. The pK_a of the compound is around pH 5, hence, the particle size remained constant for both the protonated and deprotonated forms. At pH 8 no colloidal particles were detected, and the compound must be present as deprotonated micelles and monomers, since the CMC for U-34445 is 47 μM as described below. At a concentration of 100 μM the novenamine U-35122 formed precipitates or large particles of diverse sizes at pH 4 to 4.7. From pH 5 to 6.5 a single population of discrete particles was observed. Above that pK_a the particle sizes decreased further until a constant, minimal particle size was attained. The size of these minimal particles was very uniform as demonstrated by the narrow standard deviation of the distribution and varied between 110 and 150 nm. At pH 8 no colloidal particles were detected, indicating that at this pH, the molecules were present as ionized micelles and monomers since the CMC for this compound is 30 μM. U-35401 was present as precipitates over pH's from 4 to 5.3. Over the pH range from 4 to 5, the compound was tested as 100 μM solutions. Above that pH, the concentration of the compound was reduced to 50 μM to keep the instrument in an accurate range. From pH 5.7 to 6.5 a single population of particles was observed at each pH with a mean size ranging from 120 to 180 nm. No colloidal particles of U-35401 were detected at pH 8.

The results obtained demonstrate that all of the novenamines tested form particles in solution when studied over a pH range from pH 4 to 6.5. The formation of particles was pH-dependent and was only observed when the pH was near the pK_a for U-35122 and U-35401. In the case of U-34445, the particle size remained essentially constant regardless of the pH. The particles are colloidal in nature and range in size from 100 to 150 nm. Each of the compounds studied contains an ionizing hydroxyl group. We propose that these colloidal particles are charged negatively at their surface and that they consist of a core of unhydrated, protonated monomers while some of the monomers located at the surface are ionized, thus generating a negatively charged surface. The negatively charged surfaces prevent the particles from coalescing with each other and keep the colloid system in a self-induced emulsified state. The existence of such self-emulsifying colloidal systems represents a very unique and unanticipated discovery and further demonstrates the strong colligative properties of the compounds.

Inhibition of RNase H. All of the compounds studied possess a very high specificity for RNase H in that they are completely ineffective as inhibitors of the HIV-1 DNA polymerase function when tested at concentrations of 100 μM. They do, however, inhibit the RNase H activity of other RT species such as the ones associated with HIV-2 and MLV and their potency is similar to the one observed with HIV-1 RNase H. Using equation (3) for the analysis of the results, the dose-response

curve against the HIV-1 RNase H enzyme activity for U-34445 is not hyperbolic nor does it increase over a 100-fold concentration range as one would expect for a simple monomeric and competitive inhibitor (fig. 2). Instead the compound shows no inhibition at concentrations up to 20 μM whereupon the inhibition increases from none to complete inhibition over the 20 to 50 μM concentration range. The IC_{50} of this compound is, thus, approximately 25 μM and coincides with its CMC. The inhibition pattern is consistent with the interpretation that U-34445 is inactive as an RNase H inhibitor when it is a monomer and it is only active in the form of micelles. Likewise, inhibition of the nuclease activity by U-35122 is not consistent with simple, competitive inhibition by the monomer. Its IC_{50} is 75 μM but since its CMC is 30 μM, U-35122 inhibits the nuclease only when it is present in micellar form (fig. 2). U-35401 has an IC_{50} of 50 μM in the nuclease assay and its dose-response curve is also not hyperbolic. The CMC is 47 μM and coincides with the IC_{50} (fig. 2) which indicates that this compound inhibits the nuclease also only in its micellar form.

The micelles formed appear spherical in shape with a mean diameter of ~100 Å as observed with the electron microscope. The lengths of the fully extended molecules are 31.99 Å for U-34445, 27.92 Å for U-35122, and 27.30 Å for U-35401. Hence, the radii of the spherical micelles as observed under the electron microscope are somewhat larger than the molecular dimensions suggest, although these size estimates do not include water space. Moreover, their shapes and sizes might have been altered during the fixation process.

Discussion

The novenamines studied here are amphiphiles with well-segregated hydrophilic and lipophilic domains and each molecule possesses one ionizing hydroxyl group. At pH = 4, in their protonated form, the compounds form very stable liquid-expanded insoluble monolayers at the air/water interface where they stabilize each other by hydrophobic interactions. All three novenamines still formed stable insoluble monolayers at a pH of 8, although they were less stable at that pH. This implies that they lay more flat on the water surface at high pH than at low pH. The structures are consistent with compounds that have colligative or colloidal properties. At pH = 4, in their protonated form, all three compounds formed precipitates characterized by highly diverse particle sizes. At pH's near their pK_a, the compounds formed small particles of a uniform size measuring approximately 150 nm, consistent with an aggregate of many molecules. These particle sizes conform to a narrow Gaussian distribution, indicating a highly organized structure, e.g. an emulsion with a charged outer shell and an inner core of uncharged molecules. Although the self-emulsifying particles are formed at a non-physiological pH, they may serve as vehicles for storage and delivery of the inhibitors, especially if, when transferred to a solution at physiological pH, the particles slowly dissolve to form biologically active monomers or micelles. In alkaline solutions at pH 8, no particles large enough to be detected by quasi-elastic light scattering were observed. However, surface tension measurements revealed the presence of micelles at this pH. Electron micrographs indicate that the micelles are spherical with a diameter of approximately 10 nm. The length of the fully extended monomers range from 27 to 32 Å, consistent with micelles with a low aggregation number.

The novenamines studied yielded anomalous dose-response curves when tested against RNase H that are not consistent with a simple competitive inhibitor. At low concentrations, when monomers predominate in solution, no inhibition of the reaction occurred. As the inhibitor concentrations were raised to the CMC, inhibitory activity was observed which indicates that these compounds inhibit the enzyme only when they are in the form of micelles. We realize that the IC_{50} values are rather high as they represent the sum of the inactive monomers and the active micelles and the specific compounds studied here are thus not suitable for clinical applications. However, the compounds can serve as templates for the synthesis of improved versions such as the generation of covalently linked dimers, trimers, oligomers, etc., or related monomers having lower CMC's.

It is somewhat surprising that these inhibitors are only active as micelles. The substrate for RNase H is an RNA:DNA hybrid, and the estimated distances between the negatively charged phosphate groups on the sugar-phosphate backbone of the double helix range from 5.9 to 6.9 Å[9]. It is reasonable to predict that the molecules in the micelles are in a compacted state with areas of similar magnitude to the areas calculated at the collapse pressure in insoluble monolayers. In this case the negative charges present at the surface of the micelles are separated by approximately the same distance as the ones for the phosphate groups on the RNA:DNA double helix. Thus, we propose that the inhibitors in their micellar form interact with the substrate binding domain of the enzyme normally recognizing the phosphate groups on the backbone of the RNA:DNA substrate. This interaction prevents binding of the RNA:DNA substrate and thereby blocks the action of the enzyme.

It is of interest to note the differences of the spacers connecting the lipophilic side chain to the novenamine residue present in each of these compounds. U-34445 and U-35122 inhibit the RNase H enzyme only in micellar form. The spacer in U-34445 is a carboxyl residue and the one in U-35122 as a carbamoyl moiety. The molecular dimensions of both these linkers are

similar. Conversely, U-35401 contains a benzoyl spacer which increases the lipophilicity of its lipid end, compared to U-34445 or U-35122. Additions or truncations of the lipophilic carbon chains by one carbon atom significantly reduce the potency of the compounds as RNase H inhibitors. Moreover, additions or deletions involving two or more carbon atoms abolish the inhibitory activity. Hence, the length of the carbon chain has a profound effect on the potency of the novenamines.

Specific RNase H inhibitors are rare, and only a few examples of such inhibitors are cited in the literature. N-ethylmaleimide[10], uranyl pentafluoride salts, metal phthalocyanines[11], vanadyl complexes, dAMP[12,13], illimaquinone[14] and the cephalosporin degradation product HP 0.35[15] inhibit the RNase H function preferentially, as compared to the polymerase function. The polyethylenesulfonic acid U-9843[8], ammonium trichloro(dioxyethylene-O,O'-)tellurate[16], azidothymidylate[10,17] and sulfated polyanions[18] inhibit both the polymerase and nuclease functions. The amphiphilic inhibitors discussed in this report differ significantly from all these inhibitors in terms of their chemical structures and in terms of their unusual physical properties and inhibition patterns.

The results presented in this paper illustrate that an understanding of the surface properties of a pharmacologically active agent is crucial for the design and delivery of drugs.

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