oncogene products exhibit protein tyrosine kinase activity.[63]

Drugs that modulate gene transcription are already known, of course, since steroid hormone analogues and some antitumor drugs function in this manner. Other drugs of this type could be sought, with knowledge of how hormones or other physiological signals control eukaryotic gene expression. These new drugs could then be used to regulate the synthesis of important receptors or enzymes. For agents that directly affect gene transcription, sequence-specific DNA binding will be the most difficult aspect of development since, without this property, such drugs would be too toxic for use against non-life-threatening diseases. Synthetic agents with selective DNA binding ability are now being developed, as demonstrated by the work of Dervan[66] and Hurley[67] and their co-workers, so it is likely that this will soon lead to new drugs that control gene transcription.

Summary and Prognosis

The exciting developments reviewed show the positive value of the interplay between medicinal chemistry, biochemistry, and molecular biology to antibiotic discovery and production. Considerable progress is being made by the empirical approaches currently necessitated due to the complexity of the biological systems involved. Much more can be done by rational approaches whose importance will be more forcefully felt as our knowledge about how microorganisms make antibiotics matures. Genetically engineered microorganisms certainly will play an active role in the production of known metabolites, and when enough knowledge about the workings of antibiotic production genes is acquired, recombinant organisms will be designed to produce new biologically active secondary metabolites with potential drug use. It also is increasingly clear that the areas of drug discovery and development involving the study of enzyme inhibitors and drug–receptor interaction will greatly benefit from the ability to alter protein structures by genetic engineering.[68] Thus, even if a medicinal chemist has only enough knowledge about molecular biology to communicate intelligently with biologists and biochemists, this will open avenues for fruitful research otherwise hidden in the complexity of biological systems modification.

Acknowledgment. I am grateful for the enthusiastic efforts of John Anderson, Sandor Biro, Sophia Deli, Stefano Donadio, Janice Duncan, Mary Jo Donovan, Pat Guilfoile, Osamu Hara, Haruyasu Kinashi, Marlena Skarbek, Karl Maurer, Haideh Motamedi, Hiroshi Nakayama, Rosa Navarette, Naryan Punekar, Ali Shafiee, Toshio Takatsu, Jesus Vara, Mark Weber, Bill Wessel, Connie Wierman, Hirokazu Yamamoto, and especially Evelyn Wendt-Pienkowski, who have made the transition into molecular biology research possible in my laboratory. The unpublished research described here was supported by grants from the National Institutes of Health (GM 25799) and Xechem, Inc.

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Communications to the Editor

A Multisubstrate Adduct Inhibitor of a Purine Biosynthetic Enzyme with a Picomolar Dissociation Constant

Sir:

Glycinamide ribonucleotide transformylase (GAR TFase; EC 2.1.2.2) is a crucial, reduced folate requiring enzyme involved early in de novo purine biosynthesis, catalyzing the formyl transfer from (6*R*,α*S*)-10-formyl tetrahydrofolate, **3**, to glycinamide ribonucleotide (GAR, **2a**) shown in Figure 1.[1] It has thus attracted some interest as a target enzyme for rational drug design of antineoplastic agents.[2] Published data showed these agents to be modest inhibitors of GAR TFase, but they were not specific.[3,4] An in vivo test of 5,10-dideazatetrahydroaminopterin,[5-7] which demonstrated potency against solid tumors in mice, also provided indirect evidence that the site of action was GAR TFase. Interestingly, these types of tumors are resistant to methotrexate therapy.

A number of potent specific inhibitors of enzymes have been designed[8] with use of the concept of multisubstrate adduct inhibition (MAI).[9,10] Tying together both sub-

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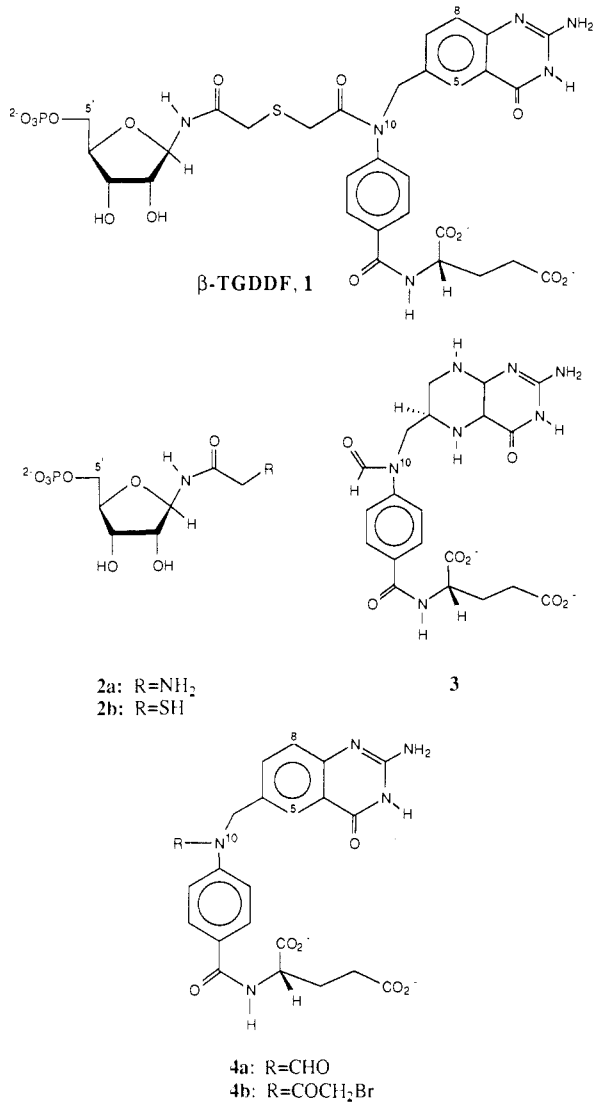

Figure 1. Multisubstrate adduct inhibitor TGDDF (ThioGAR dideazafolate, 1) and substrates (**2a** and **3**) for the reaction catalyzed by GAR TFase. Compound **4a** is an alternate substrate for the transformylase (Smith, G. K.; Mueller, W. T.; Benkovic, P. A.; Benkovic, S. J. *Biochemistry* **1981**, *20*, 1241-5); compound **4b** acts as an irreversible inactivator of the enzyme.

strates of a bimolecular, enzyme-catalyzed reaction gives a resultant molecule possessing the binding stabilization of both individual substrates, in addition to the entropic advantage of reduced molecularity.[11] One should note that a multisubstrate adduct inhibitor is not intended to mimic the transition state of a catalyzed reaction.

We describe here the first successful multisubstrate adduct inhibitor (1, in Figure 1, TGDDF = ThioGarDi-DeazaFolate) for GAR TFase which includes nearly all of the structural features of the two substrates and provides the molecule with a very high specific affinity for the enzyme. The adduct has been synthesized by a convergent synthesis employing a directed coupling as the last step.[12] The full functionality of the 5′-phosphoribose portion undoubtedly accounts for the high specificity for GAR TFase, particularly in light of the relatively modest activity and low specificity of structurally simpler adducts containing folate, but not 5′-phosphoribose moieties.[13]

Chemistry[14]

The synthesis of the MAI 1 (Figure 2) was based on the previously described compound N^{10}-(bromoacetyl)-5,8-dideazafolate,[15] **4b**, an electrophilic irreversible inactivator of the enzyme. This incorporates the structural features of the alternate substrate **4a**. Design and synthesis of a complimentary nucleophilic GAR analogue (ThioGAR, **2b**) allowed a convergent and regiospecific synthesis under mild conditions providing a product with inherent hydrolytic and oxidative stability. The mercapto analogue of GAR allowed the coupling reaction to be carried out at neutral pH in an aqueous buffered medium. Compound **4b** was conveniently available for use after HPLC purification; the description of the synthesis of ThioGAR, **2b**, follows.

ThioGAR, **2b**, was prepared by the route outlined in Figure 2, the centerpiece of which was the DCC coupling of tribenzoylribosylamine **5** with the S-protected mercaptoacetic acid.[16] (Tritylthio)acetic acid was formed (93%) by the condensation of equimolar amounts of triphenylmethanol with mercaptoacetic acid in excess trifluoroacetic acid. Coupling of the acid with tribenzoylribosamine **5** was promoted by DCC. The yield after flash chromatography was 60% of a mixture of anomers. The tribenzoyl ribonucleoside, **6**, was deprotected with NaOMe in MeOH, giving an 86% yield of the water-insoluble tritylthio ribonucleoside **7**. The crude riboside was phosphorylated with a 10-fold molar excess of phosphoryl chloride at 0 °C in trimethyl phosphate.[17] After hydrolytic workup, the product could be purified either by Sephadex A-25 ion-exchange chromatography or by preparative RP-HPLC to give a 35% yield. The latter allowed separation of anomers.[18]

Deprotection of 8 to ThioGAR, **2b**, and coupling with compound **4b** were accomplished in one step, with oxygen-free reagents. TritylthioGAR, 8, was treated with 80% aqueous TFA and then neutralized to produce a buffered pH 7.5 solution. Addition of bromoacetyl folate derivative **4b** and reaction at 60 °C for 1 h gave an adduct which could be purified on RP-HPLC using gradient elution (CH_3CN in H_2O; both solvents contained 0.1% TFA). Repurification using an isocratic mixture of the same solvents (12% at 0.7 mL/min) gave pure single anomers of the adduct, with the β anomer eluting before the α.[19]

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(18) NMR spectral data for the pure β-anomer: δ 7.31 (d, 7.2 Hz, 6 H), 7.21 (m, 9 H), 4.92 (d, $J_{H1'-H2'}$ = 4.4 Hz, 1 H), 3.97 (t, 5.0 Hz, 1 H), 3.85 (q, 3.7 Hz, 1 H), 3.75 (m, 3 H), 3.08, 2.99 (two second-order doublets, $J_{apparent}$ = 15.73 Hz, 2 H). The anomeric proton of the α-anomer has a chemical shift of δ 5.1 and a $J_{H1'-H2'}$ = 4.3 Hz.
(19) An approximately equimolar mixture of α- and β-anomers results as judged by HPLC and NMR. The adduct has been obtained in yields ranging from 50% to 100% when a 5-fold excess of 8 was employed. The solution of pure anomer must be neutralized (aqueous NH_3) before concentration (Speed-Vac); in the presence of TFA, anomerization occurs. Assignments of anomers were made by NMR.

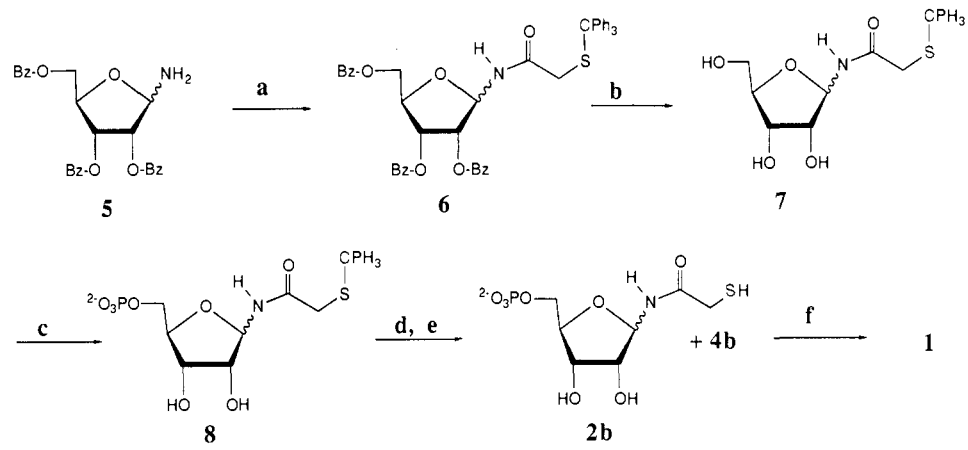

Figure 2. Synthetic scheme for TGDDF (**1**) via ThioGAR (**2b**) and N^{10}-(bromoacetyl)-5,8-dideazafolate (**4b**). Reagents and conditions are as follows: (a) DCC, $Ph_3CSCH_2CO_2H$, acetone, room temperature, 14 h; (b) NaOMe, room temperature, 45 min; (c) 10 equiv of $POCl_3$, $(MeO)_3PO$, 0 °C, 2 h; (d) 80% trifluoroacetic acid/H_2O, room temperature, 45 min; (e) aq NH_3 to pH 7.0, 100 mM HEPES, pH 7.0; (f) 50 mM HEPES, pH 7.0, 60 °C, 1 h.

The molecular weight of 780 (M + 1 = 781 in FAB-MS) and the NMR spectrum[20] (containing an aromatic region and a glutamyl pattern at ca. 2.5 ppm characteristic of the folate analogue, **4b**, and three multiplets at ca. 4 ppm matching a phosphoribose moiety) show that the molecule is a 1:1 conjugate of folate and GAR analogue. Further analysis of the NMR spectrum showed anomeric protons (the α anomer gave a doublet at δ 5.44, and the β anomer gave a doublet at δ 5.24, in agreement with literature values[16]) and overlapping resonances at δ 3.3 that correspond to two bridging methylenethio groups. The electronic spectrum of TGDDF[21] is identical with that of N^{10}-acetylated DDF.[15]

Biochemistry

The interaction of β-TGDDF with GAR TFase was characterized by the effect of the inhibitor on the activity of the enzyme as well as independent measures of its affinity for GAR TFase. The thermodynamic dissociation constant, K_D, for the E·β-TGDDF complex was measured by following the enhancement of the inhibitor's 395-nm fluorescence (excitation at 275 nm) upon binding to GAR TFase (Figure 3). A concentrated *E. coli* GAR TFase solution was added to an 11 nM solution of purified β-TGDDF; for each addition, the fluorescence at three different wavelengths (395, 400, and 405 nm) was measured. Fluorescence titration data was analyzed by the method of Taira and Benkovic.[22] The data were fit to an equation describing the fluorescence in terms of measurable quantities:

$$F_{Tot} = F_I - \frac{(F_I - F_{EI})\{(E_T + I_T + K_D) - [(E_T + I_T + K_D)^2 - 4E_T \cdot I_T]\}^{1/2}}{2I_T} \quad (1)$$

using the NLIN procedure from the SAS statistical package.[23] The data are initially fit to three parameters (I_T, the total inhibitor added; F_{EI}, the fluorescence of the enzyme–inhibitor complex; and K_D), using the measured F_I (fluorescence of the inhibitor), F_{Tot} (measured fluorescence), and E_T (cumulative enzyme added at each point). When coincidence of I_T and F_{EI} with measured values show the data to be acceptable, the fit is done to K_D alone. Figure 3 shows the data for the 395-nm emission, with a line calculated from eq 1 superimposed. The average value for K_D calculated from the three wavelengths is 250 pM, with a standard error of 50 pM. The α-anomer has not been thoroughly characterized, but its binding affinity for GAR TFase is significantly lower then the β-anomer.

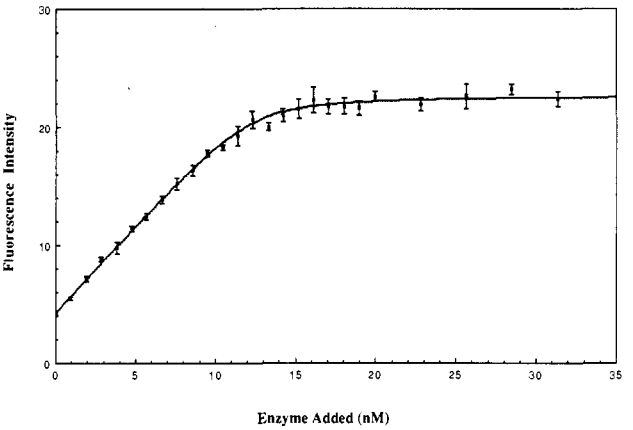

Figure 3. Fluorescence titration (excitation at 275 nm, emission at 395 nm) of 11 nM β-TGDDF with 0.95 nM aliquots of GAR TFase. The error bars show the upper and lower limits of the 10 measurements made at each concentration. Superimposed on the data is a line calculated for K_D = 250 pM.

β-TGDDF acts as a slow, tight-binding[24] inhibitor against four species of GAR TFase[25] (*E. coli*,[26] Avian,[27]

(20) NMR spectrum of the anomeric mixture (D_2O): δ 7.7–7.5 (m, 3, *p*-phenylene and H-5), 7.42 (t, 1, H-7), 7.2–7.14 (m, 3, *p*-phenylene and H-8), 5.44 (d, 1, $J_{H1'-H2'}$ = 4.4 Hz, α-anomeric C1′-H), 5.24 (d, 1, $J_{H1'-H2'}$ = 5.3 Hz, β-anomeric C1′-H), 4.87 (s, 2, C9-CH_2), 4.36 (m, 1, glutamic acid C_α-H), 4.2–3.7 (4, C5′-CH_2, C3′-CH, C2′-CH), 3.21 (m, 4, CH_2SCH_2), 2.16 (t, 2, $J_{\beta-\gamma}$ = 7.4 Hz, glutamic acid C_γ-H), 1.95 (two multiplets, 2, glutamic acid C_β-H).

(21) UV (50 mM HEPES, pH = 7.5): λ_{max} 230 (ϵ = 54.5 cm^{-1} mM^{-1}), λ_{sh} 255 (ϵ = 26.1 cm^{-1} mM^{-1}), λ_{max} 310 (ϵ = 4.19 cm^{-1} mM^{-1}).

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(25) All assays were carried out by following the increase of 5,8-dideazafolate absorbance at 295 nm in buffered medium at 26 °C. To initiate the reaction, enzyme (1 nM final concentration) was added to a mixture of saturating substrates and variable amounts of inhibitor. A characteristic family of curves was obtained, showing slow, tight binding inhibition. The onset of steady-state inhibition was ca. 3 min for 3.48 nM inhibitor and ca. 14 min for 1.16 nM inhibitor.

HeLa O,[28] and L1210[28]). Furthermore, the binding and inhibition stoichiometries are 1:1.[29] As expected, neither 1 nM AICAR TFase[30] nor DHFR[31] is affected by 10 nM β-TGDDF (K_i > 100 nM), a concentration which completely abolishes GAR TFase activity. The slow, tight-binding characteristics of β-TGDDF preclude a straightforward assessment of the competition of either substrate with TGDDF inhibition. That the addition of high levels of GAR to an E·TGDDF complex[32] causes release of TGDDF suggests (in the absence of evidence for any allosteric effect of GAR) that the two molecules are competing for the same site. The release of β-TGDDF from GAR TFase in the absence of substrates was shown by dialysis of a 10 nM solution of the complex against 50 mM Tris buffer (pH 7.5, 100 mM KCl) at 4 °C, which showed >90% recovery of activity (compared to control enzyme) after 24 h.

Conclusion

The first successful multisubstrate adduct inhibitor of GAR TFase, β-TGDDF is also the most potent inhibitor reported for that enzyme (K_D = 250 ± 50 pM). This compound shares with other MAI's high potency (typically 10^3–10^6 times the binding affinity of either substrate), high specificity, and reversibility. The binding affinity of β-TGDDF is approximately 3-fold higher then the product of the K_m values of the two substrates,[33] which is comparable to other potent MAI's.

The synthesis of the compound is simplified by convergent design and by a relatively small number of stereocenters, concentrated in portions of the molecule that have natural origins. The only stereocontrol problem lies at the ribose C-1 position, giving two interconvertible diastereomers at the end. The choice of the planar, oxidized 5,8-dideazafolate moiety solves problems that might stem from stereochemical control or oxidation at the heterocycle. The selection of thioGAR and a bromoacetyl folate derivative allow the coupling of fully deprotected compounds and the production of a hydrolytically stable thioether linkage.

The preference of GAR TFase for binding β-TGDDF vs α-TGDDF follows from the presumption that only the β anomer of GAR is recognized as a substrate by the enzyme.[34] This is further evidence that β-TGDDF binds in the active site and likely binds in a conformation very similar to that adopted by the substrates. The adduct's strong resemblance to substrates gives it unique selectivity and high potency for a clinically important target enzyme.

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Registry No. α-**1**, 119655-67-3; β-1, 119637-95-5; α-**2b**, 119619-77-1; β-**2b**, 119619-78-2; **4b**, 101375-70-6; α-**5**, 116500-78-8; β-**5**, 58368-68-6; α-**6**, 119619-71-5; β-**6**, 119619-72-6; α-**7**, 119619-73-7; β-**7**, 119619-74-8; α-**8**, 119619-75-9; β-**8**, 119619-76-0; EC 2.1.2.2, 9032-02-4; Ph_3CSCH_2COOH, 34914-36-8; $HSCH_2COOH$, 68-11-1.

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(32) After a solution 1 nM in both enzyme and inhibitor was incubated for 10 min, sufficient GAR was added to produce a 100 μM solution, and the fluorescence intensity at 395 nm (excitation at 275) was monitored over time. Simple exponential curve fitting on the data suggests a k_{off} of <0.007 s^{-1}, with approximately 25% of the complex dissociating.
(33) K_m values for GAR (**2a**: ca. 23 μM), and N^{10}-formyl-5,8-dideazafolate (**4a**: ca. 36 μM) were measured at pH 8.5 for *E. coli* GAR TFase.
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