Comparative Inhibition of Thrombin, Plasmin, Trypsin, and Complement by Benzamidines Using Substituent Constants and Regression Analysis

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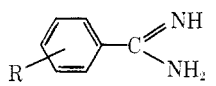
Data on inhibition of thrombin, plasmin, trypsin, and complement by similar series of 3- and 4-substituted benzamidines have been evaluated using substituent constants and regression analysis. The correlations derived suggest that both lipophilic and electronic factors contribute to binding interaction for each enzyme system but to varying degrees. Thrombin and complement appear to possess similar binding sites while differing from plasmin and and trypsin. Support for this hypothesis was obtained by deriving a comparative correlation of binding to thrombin *vs.* trypsin.

Several peptide hydrolases exhibit a preference for hydrolyzing peptide linkages in which the carbonyl portion is contributed by the basic amino acids lysine or arginine. Among these are the enzymes thrombin (3.4.4.13)[1] and plasmin (3.4.4.14)[1] which are of pharmacological significance as a result of their involvement in the formation and dissolution of blood clots. The digestive enzyme, trypsin (3.4.4.4), appears to exert a similar specificity,[1] and complement, a mixture of 11 serum proteins,[2] is also known to possess hydrolytic activity similar to thrombin and plasmin.[2]

Markwardt[3-5] has conducted several studies on inhibition of thrombin, plasmin, and trypsin using congeneric series of meta- and para-substituted benzamidines (I). Also, the inhibition of complement by meta-substituted benzamidines has been investigated by Baker.[6] The extensive data in these reports have served as the basis for the present attempt to compare the active site binding characteristics of thrombin, plasmin, trypsin, and complement in a quantitative manner using the extrathermodynamic approach developed by Hansch.[7]



I

Method

Inhibition of thrombin, plasmin, and trypsin by benzamidines was reported as competitive inhibition constants, K_i, using *N*-α-benzoyl-DL-arginine-4-nitroanilide as substrate.[3-5] The K_i is assumed to be an estimate of the enzyme-inhibitor dissociation constant. The inhibition of complement by benzamidines was reported as per cent inhibition of sheep red blood cell lysis at several concentrations of inhibitor.[6] Not all benzamidines gave an increase in lysis inhibition with increased concentration. Only those compounds which did show an increase in inhibition with increased concentration are included here. The concentration required for 50% inhibition (I_{50}) has been estimated graphically from the reported data and is assumed to be related to the binding affinity for some site within complement. In accordance with the procedures developed by Hansch,[7] the K_i and I_{50} are taken as measures of the concentration of molecules with substituent X required to exert a standard biological response. The structural contributions to this response are then factored in the form of the Hammett-type free-energy relationship

$$\log 1/C_X = k\pi_X + k'\sigma_X + k''E_s(X) + k''' \quad (1)$$

In eq 1, C_X is the molar concentration of the benzamidine with substituent X which produces an equivalent biological response such as K_i or I_{50}. The constants k, k', k'', and k''' are fixed for a given system and are determined by computerized multiparameter regression analysis. The π values for substituents were estimated from previously determined values as measured in model systems[7,8] where $\pi = \log P_X - \log P_H$ and P_X is the octanol-water partition coefficient for nitrobenzene with substituent X and P_H is the partition coefficient for unsubstituted nitrobenzene. The nitrobenzene model system was selected for its ability to approximate the electron-withdrawing effect of the positively charged amidine on partition coefficients of substituted benzamidines. Except where noted, π values were calculated from measured nitrobenzene partition coefficients for methyl, methoxy, nitro, and halo substituents[7,8] by taking advantage of the additive nature of π. For the few substituents that have not been measured in the nitrobenzene system, the following relationship derived by Hansch was utilized to estimate corrections to be applied to π values measured in benzene model systems.[8]

$$\Delta\pi = \pi_{\text{nitrobenzene}} - \pi_{\text{benzene}} = -0.51\sigma + 0.28$$

In the equation σ is the Hammett substituent constant for the substituent in question. It should be noted that the equation only provides a very rough estimate for the direction and magnitude of change in π. The equations derived using nitrobenzene π values do not differ significantly from those derived using benzene as a model system. The Hammett σ values[9] were taken from the sources indicated. Since reliable Taft E_s values[10] are not available for many of the substituents employed, they have not been included in the correlations. Another parameter which has been successfully utilized in quantitative correlations is polarizability, P_E.[11-15] Tables I–III contain the data on substituent changes and biological activities for 3- and 4-substituted benzamidines. The correlations were derived using an IBM 360/65 computer with a multiple parameter regression analysis program.

Results and Discussion

Initial attempts to correlate inhibition of thrombin, plasmin, and trypsin by combined sets of 3- and 4-substituted benzamidines met with limited success. Only thrombin inhibition could be correlated successfully in this manner affording eq 2 where π and σ are the sum for the substituents. The sets of benzamidines were then divided into 3-substituted and 4-substituted subsets. These subsets were analyzed separately in an effort to achieve meaningful comparisons of the binding of similar molecules to the active sites of the respective enzyme systems. Correlations of the inhibition of thrombin, plasmin, trypsin, and complement by 3-substituted benzamidines are presented in Table IV.

$$\log 1/K_i = 0.30\ (\pm 0.07)\pi - 0.82\ (\pm 0.31)\sigma + 0.28\ (\pm 0.15) \quad (2)$$
$$n = 39;\ s = 0.29;\ r = 0.92$$

In Table IV, n is the number of data points in the set, s is the standard deviation, and r is the correlation coefficient. The numbers in parentheses are the 95% confidence intervals for the coefficients. In all cases, the correlations are improved by combination of σ_m and π in the same equation and the improvements are significant at the 0.90 level or better as determined in the F test (Table IV, footnote *a*). The positive sign of the coefficients associated with the π term in eq 3–10 indicates a dependence on lipophilic character of the substituents where an increase in lipophilicity should result in stronger inhibition of each

Table I. Inhibition of Thrombin, Plasmin, and Trypsin 3-Substituted Benzamidines

R	π[a]	σ_m[c]	P_E[e]	Thrombin log $1/K_i$ Obsd[f]	Thrombin log $1/K_i$ Calcd[h]	Plasmin log $1/K_i$ Obsd[f]	Plasmin log $1/K_i$ Calcd[i]	Trypsin log $1/K_i$ Obsd[f]	Trypsin log $1/K_i$ Calcd[i]
H	0.0	0.0	1.10	0.66	0.51	0.46	0.60	1.46	1.44
CH_3	0.52	−0.07	5.72	0.39	0.73	0.64	0.54	1.48	1.48
OCH_3	0.28	0.12	7.36	0.57	0.48	0.96	0.81	1.66	1.60
OC_2H_5	0.78	0.10	11.98	0.74	0.64	0.77	0.84	1.57	1.68
OC_4H_9	1.78	0.10	21.22	1.03	0.94	0.85	0.94	1.92	1.88
OC_5H_{11}	2.28	0.10	25.84	1.28	1.08	1.00	0.99	1.96	1.98
OC_6H_{13}	2.78	0.10[d]	30.46	1.24	1.23	1.10	1.04	2.10	2.08
OC_7H_{15}	3.28	0.10[d]	35.08	1.15	1.37	1.07	1.09	2.19	2.18
Cl	0.58	0.37	5.96	0.10[g]	0.33				
$O(CH_2)_3OC_6H_5$	2.29[b]	0.10[d]	42.35	1.22[g]	1.09				
NO_2	−0.39	0.71	7.30	−0.26[g]	−0.28				

[a] Calculated from measured log P values (ref 7 and 8). [b] Corrected for folding interaction (ref 8). [c] From D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23,** 420 (1958). [d] Estimated values. [e] Calculated from atomic refractivity (ref 12 and 14). [f] From ref 4 except as noted. K_i is in mM. [g] From ref 3. [h] Calculated *via* eq 7. [i] Calculated *via* eq 8. [j] Calculated *via* eq 9.

of the systems. While the relative size of the coefficients suggests a greater nonpolar character in that area of the binding site for thrombin, the size of the 95% confidence intervals makes all four systems essentially equivalent.

If the intercepts of eq 7–10 are taken as measures of the sensitivity of these systems to inhibition by benzamidines, they can be ranked in the following manner. Trypsin is clearly the most susceptible with plasmin and thrombin being essentially equivalent. The inhibition of complement is reported in a different context so that the intercepts of the complement equations are not relative to the other systems.

The most interesting result is the coefficient for the electronic parameter σ_m. Thrombin and complement inhibition increases with electron-donating substituents while plasmin and trypsin inhibition appears to increase with electron withdrawal. Increased binding with electron donation through the ring would be anticipated since this would tend to stabilize the positive charge on the protonated amidine, apparently the major requirement for binding to these systems. The large 95% confidence intervals for the coefficients associated with σ_m in eq 8 and 9 indicates that these terms are very unreliable. Since the inclusion of the σ_m term in eq 8 for plasmin and eq 9 for

Table II. Inhibition of Thrombin, Plasmin, and Trypsin 4-Substituted Benzamidines

R	π[b]	σ_p[d]	P_E[j]	Thrombin log $1/K_i$ Obsd[k]	Thrombin log $1/K_i$ Calcd[n]	Plasmin log $1/K_i$ Obsd[k]	Plasmin log $1/K_i$ Calcd[o]	Trypsin log $1/K_i$ Obsd[k]	Trypsin log $1/K_i$ Calcd[p]
H	0.0	0.0	1.10	0.66	0.21	0.46	0.29	1.46	1.16
CH_3	0.49	−0.17	5.72	0.40[l]	0.52	0.52[l]	0.37	1.52[l]	1.36
Cl	0.51	0.23	5.96	0.40[l]	0.13	0.60[l]	0.38	1.40[l]	1.13
OCH_3	0.15	−0.27	7.36	0.13[l]	0.52	0.35[l]	0.40	1.16[l]	1.35
NH_2	−0.49	−0.66	5.41	1.10[l]	0.71	0.89[l]	0.37	1.92[l]	1.45
CO_2H	0.01	0.39[e]	7.25	−0.30[l]	−0.18	−0.18[l]	0.40	0.70[l]	0.94
C_2H_5	0.99	−0.15	10.34	0.25	0.65	0.38	0.46	1.16	1.44
C_3H_7	1.49	−0.13[f]	14.96	0.50	0.78	0.40	0.54	1.42	1.53
C_4H_9	1.99	−0.16[f]	19.58	1.14	0.96	0.52	0.62	1.55	1.64
C_5H_{11}	2.49	−0.15[g]	24.20	1.72	1.10	0.46	0.71	2.10	1.74
OC_2H_5	0.65	−0.24	11.98	0.33	0.64	0.35	0.49	1.00	1.43
OC_3H_7	1.15	−0.25	16.60	0.38	0.80	0.30	0.57	1.05	1.53
OC_4H_9	1.65	−0.32	21.22	0.92	1.02	0.40	0.65	1.42	1.67
OC_5H_{11}	2.15	−0.34	25.84	1.26	1.19	0.60	0.74	1.70	1.78
OC_6H_{13}	2.65	−0.34[h]	30.46	1.46	1.34	0.70	0.82	1.77	1.88
OC_7H_{15}	3.15	−0.34[h]	35.08	1.40	1.48	1.10	0.90	2.00	1.98
OC_8H_{17}	3.65	−0.34[h]	39.70	1.62	1.64	1.40	0.99	2.22	2.07
OC_9H_{19}	4.15	−0.34[h]	44.32	1.92	1.78	1.60	1.07	2.40	2.17
$OC_{10}H_{21}$	4.65	−0.34[h]	48.94	1.96	1.93	1.46	1.16	2.30	2.27
$OC_{11}H_{23}$	5.15	−0.34[h]	53.56	2.00	2.08	0.92	1.24	2.40	2.36
$OC_{12}H_{25}$	5.65	−0.34[h]	58.18	1.82	2.23	0.85	1.32	2.16	2.46
$CO_2C_2H_5$	0.54	0.45	16.61	−0.36[l]	−0.08	0.30[l]	0.57	0.70[l]	1.01
$CO_2CH_2C_6H_5$	2.17	0.45[h]	36.10	0.82[l]	0.41	1.10[l]	0.92	1.70[l]	1.34
NO_2	−0.39	0.78	7.30	−0.85[m]	−0.69				
$OCH_2C_6H_5$	2.28	−0.42[f]	31.47	1.46	1.30	0.60	0.84	2.05	1.85
$O(CH_2)_3C_6H_5$	2.68[c]	−0.24	40.71	1.44	1.24	1.35	1.01		
Br	1.03	0.23	8.86	0.42[l]	0.28	0.82[l]	0.43	1.40[l]	1.23
CH_2COCO_2H	−0.09	0.05[i]	16.50	1.70[l]		1.40[l]		1.22[l]	1.12
3,4-Cl_2[a]	1.09	0.60	11.92	−0.02[m]					
3-NO_2, 4-CH_3[a]	0.10	0.54	13.02	−0.04[m]					

[a] These two derivatives were included in the derivation of eq 2; the substituent parameters are the sum for both substituents. [b] Calculated from measured log P values (ref 7 and 8). [c] Corrected for folding interaction (ref 8). [d] From D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23,** 420 (1958). [e] From M. G. Hogben and W. A. Graham, *J. Amer. Chem. Soc.*, **91,** 283 (1969). [f] From H. H. Jaffe, *Chem. Rev.*, **53,** 191 (1953). [g] From M. Charton, *J. Org. Chem.*, **30,** 552 (1965). [h] Estimated value. [i] Estimated from ethyl acetate value: M. Charton, *J. Org. Chem.*, **30,** 969 (1965). [j] Calculated from atomic refractivity (ref 12 and 14). [k] From ref 4 except as noted. K_i is in mM. [l] From ref 5. [m] From ref 3. [n] Calculated *via* eq 17. [o] Calculated *via* eq 19. [p] Calculated *via* eq 18.

Table III. Inhibition of Complement by 3-Substituted Benzamidines

R	π[a]	σ_m[c]	P_E[b]	Log $1/I_{50}$ Obsd[f]	Log $1/I_{50}$ Calcd[n]
H	0.0	0.0	1.10	−0.61	−0.51
CF_3	1.11[b]	0.48[f]	6.08	−0.78	−0.94
NO_2	−0.39	0.71	7.30	−1.62	−1.17
OC_3H_7	1.28	0.10	16.60	−0.16	−0.28
OC_4H_9	1.78	0.10	21.22	−0.15	−0.18
OC_5H_{11}	2.28	0.10	25.84	0.13	−0.08
O-*i*-C_5H_{11}	2.08	0.10[g]	25.84	0.14	−0.08
$OCH_2C_6H_5$	2.41	0.10[g]	31.47	0.02	0.04
$O(CH_2)_3OC_6H_5$	2.29[c]	0.10[g]	42.35	0.34	0.28
C_4H_9	2.02	−0.08[h]	19.58	−0.05	−0.01
i-C_5H_{11}	2.32	−0.08[g]	24.20	−0.19	0.09
C_6H_5	2.38[b]	0.06	25.21	0.10	−0.05
$CH_2C_6H_5$	2.65	−0.01[i]	29.83	−0.34	0.13
$(CH_2)_2C_6H_5$	3.15	−0.05[i]	34.45	0.03	0.28
$CH{=}CHC_6H_5$	3.06[b]	0.14[j]	33.98	−0.06	0.05
$(CH_2)_2$-4-pyridyl	1.67	−0.05[g]	32.24	0.34	0.23
OCH_3	0.28	0.12	7.36	−0.48	−0.51
Br	0.76	0.39	8.86	−0.48[m]	−0.78
$O(CH_2)_4C_6H_5$	3.31[c]	0.10[g]	45.33	0.62	0.35
$(CH_2)_4$(4-$CH_3CONHC_6H_4$)	2.58[c]	−0.08[g]	55.21	0.40	0.76
$(CH_2)_2$-2-pyridyl	1.67	−0.05[g]	32.24	0.46	0.23
$(CH_2)_2$-3-pyridyl	1.67	−0.05[g]	32.24	0.39	0.23
$(CH_2)_4$-3-pyridyl	2.07[c]	−0.08[g]	41.48	0.62	0.46
3-CH_3, 4-CH_3	0.52[d]	−0.07[d]	5.72[d]	−0.54	−0.33
3-CH_3, 5-CH_3	0.52[d]	−0.07[d]	5.72[d]	−0.22	−0.33

[a] Calculated from measured log P values (ref 7 and 8). [b] Estimated from benzene log P *via* $\Delta\pi = -0.51\sigma + 0.28$; ref 8. [c] Corrected for folding interactions (ref 8). [d] Values used were for 3 substituent only. [e] From D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, **23,** 420 (1958), except as noted. [f] From M. G. Hogben and W. A. Graham, *J. Amer. Chem. Soc.*, **91,** 283 (1969). [g] Estimated value. [h] From M. Charton, *J. Org. Chem.*, **30,** 552 (1965). [i] From M. Charton, *ibid.*, **30,** 969 (1965). [j] From H. H. Jaffe, *Chem. Rev.*, **53,** 191 (1953). [k] Calculated from atomic refractivity (ref 12 and 14). [l] I_{50} values estimated graphically from plots of per cent inhibition *vs.* concentration in mM reported in ref 6. Only 3-substituted molecules for which 50% inhibition could be estimated are included. [m] Value from 44% inhibition. [n] Calculated *via* eq 16.

trypsin does afford a significant improvement in correlation, it is possible that electronic effects are involved in direct binding interaction between the substituents or the aromatic ring and that portion of the enzyme binding site. It is obvious that variation in the electronic character of these substituents is not nearly enough to properly assess this type of contribution to binding.

The differences in binding sites are emphasized to a greater extent on examination of correlations involving polarizability, P_E, in place of π (Table IV, eq 11–16). In all cases, P_E varies in a similar manner to π; however, P_E does give a better correlation of complement inhibition. The two 3,5-disubstituted molecules were accommodated fairly well by eq 16, suggesting that the second substituent exerted little influence on the binding interaction. The correlations for trypsin and plasmin with P_E and σ_m did not result in statistically significant improvements over those with P_E alone while those for thrombin and complement, eq 15 and 16, are significant at the 0.995 level of the F test (Table IV, footnote *a*). Since P_E does encompass more of an electronic character than π, it is very interesting that the σ_m terms drop out in the trypsin and plasmin correlations but remain virtually unchanged in correlations of thrombin and complement. This may definitely be a result of the much greater range in electronic character of the substituents included in the thrombin and complement correlations but also reinforces the concept that there may be a different electronic environment in that portion of the trypsin and plasmin binding sites interacting with the 3 substituents.

The effects of 3-nitro- and 3-halogen-substituted benzamidines on plasmin and trypsin could help to clarify the influence of electronic character on inhibition of these systems.

Examination of available data on inhibition of thrombin, plasmin, and trypsin by 4-substituted benzamidines

Table IV[a]

	n	s	r	Eq
Thrombin log $1/K_i = 0.35\ (\pm0.16)\pi + 0.28\ (\pm0.28)$	11	0.28	0.86	3
Plasmin log $1/K_i = 0.14\ (\pm0.11)\pi + 0.64\ (\pm0.20)$	8	0.14	0.80	4
Trypsin log $1/K_i = 0.22\ (\pm0.06)\pi + 1.46\ (\pm0.11)$	8	0.08	0.97	5
Complement log $1/I_{50} = 0.35\ (\pm0.15)\pi - 0.70\ (\pm0.30)$	25	0.36	0.71	6
Thrombin log $1/K_i = -0.96\ (\pm0.75)\sigma_m + 0.29\ (\pm0.13)\pi + 0.51\ (\pm0.27)$	11	0.20	0.94	7
Plasmin log $1/K_i = 1.57\ (\pm1.90)\sigma_m + 0.10\ (\pm0.10)\pi + 0.60\ (\pm0.18)$	8	0.11	0.90	8
Trypsin log $1/K_i = 0.85\ (\pm1.02)\sigma_m + 0.20\ (\pm0.06)\pi + 1.44\ (\pm0.10)$	8	0.06	0.98	9
Complement log $1/I_{50} = -1.23\ (\pm0.70)\sigma_m + 0.26\ (\pm0.13)\pi - 0.44\ (\pm0.29)$	25	0.29	0.83	10
Thrombin log $1/K_i = 0.03\ (\pm0.02)P_E + 0.23\ (\pm0.38)$	11	0.33	0.78	11
Plasmin log $1/K_i = 0.02\ (\pm0.01)P_E + 0.60\ (\pm0.20)$	8	0.13	0.85	12
Trypsin log $1/K_i = 0.02\ (\pm0.00)P_E + 1.41\ (\pm0.10)$	8	0.06	0.98	13
Complement log $1/I_{50} = 0.03\ (\pm0.01)P_E - 0.76\ (\pm0.25)$	25	0.31	0.80	14
Thrombin log $1/K_i = -1.27\ (\pm0.68)\sigma_m + 0.02\ (\pm0.01)P_E + 0.50\ (\pm0.26)$	11	0.19	0.94	15
Complement log $1/I_{50} = -1.13\ (\pm0.56)\sigma_m + 0.02\ (\pm0.01)P_E - 0.53\ (\pm0.23)$	25	0.24	0.89	16

[a] F tests: eq 3 *vs.* 7, $F_{1,8} = 8.8736$; eq 11 *vs.* 15, $F_{1,8} = 18.5944$; eq 5 *vs.* 9, $F_{1,5} = 4.5525$; eq 4 *vs.* 8, $F_{1,5} = 4.4830$; eq 6 *vs.* 10, $F_{1,22} = 13.3518$; eq 14 *vs.* 16, $F_{1,22} = 17.2700$.

(Table II) afforded useful correlations of thrombin and trypsin inhibition with σ_p and π, eq 17 and 18. Both equations are significant improvements over the equations in π alone at the 0.975 level of the F test (thrombin $F_{1,24}$ = 25.6344; trypsin $F_{1,23}$ = 7.8660). The smaller intercept values in eq 17 and 18 as compared to eq 7 and 9 indicate that thrombin and trypsin are less susceptible to inhibition by 4-substituted benzamidines. The negative coefficients associated with σ_p in eq 17 and 18 support the assumption that inhibition of both enzymes is dependent upon stabilization of the positively charged amidine by electron-donating substituents. The relative size of these coefficients does suggest that an increase in electron-donating character of the 4 substituent would result in a much larger increase in inhibition of thrombin than of trypsin. The small but significant positive coefficients associated with π imply that a similar degree of nonpolar character is important in the binding interaction with both enzymes. It should be noted here that the substituent where R equals $CH_2COCOOH$, while an excellent inhibitor in both systems, was very poorly fit by the thrombin correlation. This substituent which would be ionized at the pH of the inhibition studies may be involved in ionic interactions at the thrombin active site. Substitution of P_E for π in these correlations did not give any improvement. The best equation for plasmin inhibition by 4-substituted benzamidines has a correlation coefficient of 0.70 (eq 19). Unfortunately, no data are available on inhibition of complement by 4-substituted benzamidines.

thrombin

$$\log 1/K_i = 0.30\ (\pm 0.08)\pi - 0.99\ (\pm 0.41)\sigma_p + 0.21\ (\pm 0.18) \quad (17)$$
$$n = 27;\ s = 0.30;\ r = 0.93$$

trypsin

$$\log 1/K_i = 0.20\ (\pm 0.07)\pi - 0.58\ (\pm 0.43)\sigma_p + 1.16\ (\pm 0.17) \quad (18)$$
$$n = 26;\ s = 0.27;\ r = 0.85$$

plasmin

$$\log 1/K_i = 0.02\ (\pm 0.01)P_{E4} + 0.27\ (\pm 0.22) \quad (19)$$
$$n = 26;\ s = 0.31;\ r = 0.70$$

The quality of all the correlations is perhaps better than should be expected, as the K_i values are only accurate to 15–25%. The least reliable values are in the plasmin test results which may account for a large part of the difficulty encountered in correlation. The long aliphatic ether substituents in the 4 position (Table II) probably exceed the limits of hydrophobic interaction in all three enzyme binding sites since log $1/K_i$ values do level off or drop slightly where R is nonyloxy. Attempts to analyze this observation further by deriving parabolic relationships in π were unsuccessful. That P_E and π were virtually indistinguishable and thus covariant in some of the correlations is, again, due to the types of substituents examined. A clear choice could be made in the correlations of complement inhibition where the substituent selections were such that P_E and π did not vary in the same manner.

The correlations of 3- and 4-substituted molecules, taken together, do suggest that the benzamidine binding sites of thrombin and complement are quantitatively similar to each other and that the benzamidine binding sites of plasmin and trypsin are quantitatively similar to each other. Thus, the four test systems appear to split into two divisions with regard to the physicochemical characteristics of their binding sites. In an attempt to substantiate these indications, correlations of the differences in log $1/K_i$ were conducted. This was accomplished by substracting the log $1/K_i$ for one enzyme from the log $1/K_i$ for another for each substituted benzamidine tested against both systems in question. The result is then a correlation of the log of the ratio of $1/K_i$'s with the physicochemical differences in binding sites. It can also be used to predict the structural modifications which might increase inhibition of one system at the expense of the other, *i.e.*, increase selectivity. A similar procedure was employed in derivation of Taft σ^* parameter[16,17] and by Kutter[18] to compare intravenous with intraventricular activities of analgetics. This treatment is in effect a subtraction of regression equations which is here conducted by computer in order to obtain 95% confidence intervals for coefficients as well as standard deviations and correlation coefficients. Correlation of the difference in log $1/K_i$ for thrombin–trypsin and thrombin–plasmin for 3-substituted benzamidines proved to be inconclusive. The few molecules tested against all three enzymes contained only very small variations in the electronic parameter while the more significant variations in π appear to affect binding to the three enzymes in a very similar manner. Correlation of binding differences of 4-substituted benzamidines (Table III) afforded eq 20 for thrombin–trypsin. The equation does substantiate binding

$$\log 1/K_i\ (\text{thrombin}) - \log 1/K_i\ (\text{trypsin}) = 0.10\ (\pm 0.04)\pi - 0.33\ (\pm 0.24)\sigma_p - 0.93\ (\pm 0.10) \quad (20)$$
$$n = 25;\ s = 0.15;\ r = 0.83$$

site differences between thrombin and trypsin in terms of electronic character and lipophilic character. The two-parameter equation is a significant improvement over the one-parameter equation at the 0.995 level ($F_{1,18}$ = 14.9724). Only those substituents which are included in both eq 17 and 18 were also included in eq 20. Complement could not be included in the comparative analysis since the measure of inhibition was on a different basis and since most of the substituent variations were also different from the enzyme data. These results do support the findings of others who compared thrombin to trypsin and plasmin on a kinetic basis.[19,20]

It should now be possible to extend these studies to quantitative comparisons of systems which possess similar binding specificities such as kallikrein.[21] Recent reports of very strong inhibition of thrombin, trypsin, plasmin, and kallikrein by diamidines could lead to extremely intriguing comparative quantitative correlations when the data on appropriate congeneric series become available.[21,22] Perhaps the most important conclusion to be reached from the correlation results in this report is that a *single*, carefully designed, set of congeners can and should be used to analyze binding to or inhibition of a number of biological systems. Data generated in this manner could then be extremely useful in the design of more selective, site specific, inhibitors for each of the biological systems.

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In Vivo Metabolite Condensations. Formation of N^1-Ethyl-2-methyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone from the Reaction of a Metabolite of Alcohol with a Metabolite of Lidocaine†

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Isotopic labeling is used to demonstrate that N^1-ethyl-2-methyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone (2) is generated *in vivo* in the Rhesus monkey by condensation of ω-ethylamino-2,6-dimethylacetanilide (4), a metabolite of lidocaine, with acetaldehyde formed by ingestion of alcohol. This substance (2) was previously reported as a metabolite obtained from the urine of man after ingestion of lidocaine. Evidence now suggests that, under normal physiological conditions, the major portion of 2 originally determined was an artifact arising from the condensation of 4 with trace amounts of acetaldehyde present in the solvent used in the isolation procedure. The extreme ease, under a variety of mild nonenzymatic conditions, with which the condensation takes place demonstrates the care that must be exercised in interpreting data that potentially involve similar *in vivo* condensation reactions. The generality and potential importance of such reactions between aldehydes and amines are pointed out.

In the course of a study concerning the biotransformation of the potent antiarrhythmic agent lidocaine (1) in man, we discovered[1] a new metabolite, N^1-ethyl-2-methyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone (2). Further investigation also revealed the presence of a second new metabolite and it was subsequently shown to be N^1-ethyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone (3). Although the formation of 2 could readily be rationalized as proceeding *via* an intramolecular cyclization following initial oxidation (see Discussion), it was not apparent how 3 could be obtained from either 1 or 2 in any simple fashion.

In a series of papers in 1960, Hollunger[2] demonstrated that in rabbit, liver enzymes were largely responsible for the biotransformation of lidocaine and that a key metabolite was ω-ethylamino-2,6-dimethylacetanilide (4). Later Beckett and Boyes[3] demonstrated that 4 was a major biotransformation product of lidocaine in man and their report was subsequently confirmed by others[4] (Chart I).

Since 4 was known to be a major metabolic product in man, it seemed possible that 3 might be arising *via* an *in vivo* intermolecular condensation between 4 and some one carbon fragment at the oxidation level of formaldehyde. Similarly, the formation of 2 could be viewed as arising from an intermolecular condensation between 4 and acetaldehyde. To investigate the origin of these metabolites and the feasibility of such reactions *in vivo*, a series of labeling experiments was undertaken.

As will be shown, the results of these experiments clearly demonstrate the ease of such condensation reactions under very mild reaction conditions and, hence, the care that must be taken in interpretation of results. Further, they demonstrate that while the formation of 2 under normal physiological conditions cannot be unambiguously established at this time, 2 can be generated *in vivo* after lidocaine administration when the plasma level concentration of acetaldehyde is artificially increased by concomitant administration of ethanol.

Results

Human Studies. (a) Isolation of Metabolites. After oral administration of randomly tritiated lidocaine hydrochloride to three normal human volunteers, urine and feces were collected, frozen, and stored over a period of 72 hr. No attempt was made to control urinary pH during these studies. Of the radioactivity present in the urine 5–10% could be accounted for as extractable organic bases while the total radioactivity present represented 50% of the administered dose. Homogenization of the feces followed by oxidation of an aliquot and scintillation counting indicated that this route of elimination is of minor impor-

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