Communications to the Editor

3-Alkyl-4-aminobutyric Acids: The First Class of Anticonvulsant Agents That Activates L-Glutamic Acid Decarboxylase

Two important neurotransmitters involved in the regulation of brain neuronal activity are γ-aminobutyric acid (GABA), one of the most widely distributed inhibitory neurotransmitters, and L-glutamic acid, an excitatory neurotransmitter.[1] The concentration of GABA is regulated by two pyridoxal 5'-phosphate dependent enzymes, L-glutamic acid decarboxylase (GAD; EC 4.1.1.15), which catalyzes the conversion of L-glutamate to GABA and GABA aminotransferase, which degrades GABA to succinic semialdehyde.[2] When the concentration of GABA diminishes below a threshold level in the brain, convulsions result;[3] raising the brain GABA levels appears to terminate the seizure.[4] A reduction in the concentrations of GABA and of GAD has been implicated in the symptoms associated with epilepsy[5,6] as well as several other neurological diseases such as Huntington's chorea,[7,8] Parkinson's disease,[9,10] Alzheimer's disease,[11] and tardive dyskinesia.[12] Administration of GABA peripherally is not effective because GABA, under normal conditions, cannot cross the blood–brain barrier, presumably as a result of its lipophobicity;[13] however, several other approaches have been taken to increase the brain concentrations of GABA, including to make prodrugs of GABA[14,15] and to inactivate

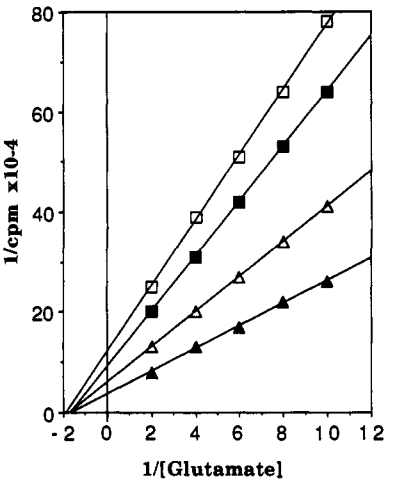

Figure 1. Activation of GAD by (*R*)-3-methyl GABA. The GAD assay[29] was run in the absence (□) and presence (0.25 mM (■), 1.0 mM (△), 2.5 mM (▲) of (*R*)-3-methyl GABA.

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Table I. Activation of GAD by 3-Alkyl GABA Analogues

$H_3\overset{+}{N}CH_2\underset{\underset{R}{|}}{CH}CH_2COO^-$

R	2.5 mM	1.0 mM	500 μM	250 μM	100 μM	50 μM
(*R,S*)-methyl	239[a]	168	142	128	118	107
(*R*)-methyl	327	202	185	135	128	109
(*S*)-methyl	170	118	–[b]	103	–	–
3,3-dimethyl	174	125	–	109	–	–
(*R,S*)-ethyl	172	128	–	108	–	–
(*R,S*)-*n*-propyl	156	112	–	105	–	–
(*R,S*)-isopropyl	140	108	–	104	–	–
(*R,S*)-*n*-butyl	178	117	–	105	–	–
(*R,S*)-isobutyl	143	113	–	109	–	–
(*R,S*)-*sec*-butyl	169	119	–	105	–	–
(*R,S*)-*tert*-butyl	295	174	147	121	117	108
(*R,S*)-neopentyl	279	181	–	130	–	–
(*R,S*)-isopentyl	142	118	–	109	–	–
(*R,S*)-cyclohexyl	125	100	–	100	–	–
sodium valproate	207	138	124	119	115	105
gabapentin	178	145	–	105	–	–
milacemide	230	170		126		

[a] The amount of activation is expressed as the ratio of the V_{max} in the presence of the activators to the V_{max} in the absence of the activators times 100%. Assays were carried out as described in ref 26. Duplicate measurements on one of the compounds gave values within 5% of each other; these values represent single determinations at each concentration. [b] Not determined.

GABA aminotransferase.[16,17] Another, yet untapped, approach to increase brain GABA levels would be to design a class of *activators* of GAD. GABA in brain presynaptic endings (synaptosomes) appears to exist in at least two separate pools, one that is preferentially formed from GABA taken up from the extracellular space (after its utilization in neurotransmission) and one that is newly synthesized from glutamate by GAD.[18] Synaptosomal studies indicate that the newly synthesized pool is more readily released than the uptake pool in a calcium-sensitive manner. Conversely, the uptake pool is acted upon by the degradative enzyme GABA aminotransferase more so than is the synthetic pool.[18] Therefore, the synaptosomal results suggest that pharmacological activation of GAD activity should be a particularly effective way to increase synaptic release of GABA and that activation of GAD should be a particularly effective way to produce anticonvulsant effects. This approach would increase the GABA pool that is poised for utilization in neurotransmission. Furthermore, this is the only approach that not only would increase the GABA levels, but also would increase the effective activity of GAD, an enzyme in diminished concentration in a variety of neurological disorders.[5-12] There already are two anticonvulsant agents, milacemide[19] and sodium valproate,[20] that have been reported to activate GAD to a small extent in vivo. Milacemide (100 mg/kg po) was shown to raise the GAD levels 11% and to increase GABA levels by 28–38%. Sodium valproate (400 mg/kg) increased GAD activity by up to 28%, depending upon which brain region was measured. However, these compounds were not investigated in vitro with purified GAD to determine their direct effect on that enzyme. During our investigations of the effects of 3-alkyl GABA analogues as alternative substrates for GABA aminotransferase,[21] we tested these compounds with GAD and were surprised to find that they caused activation. Here we communicate our initial studies with these analogues, which constitute the first *class* of activators of GAD in vitro, and show that they also have anticonvulsant activity.

Incubation of purified pig brain GAD[22] with a series of 3-alkyl GABA analogues[23] gave activation plots with in-

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Table II. Prevention of Tonic Extensor Seizures in Mice by 3-Alkyl GABA Analogues

3-substituent	dose, mg/kg	effect:[a] no. protected/ no. tested	3-substituent	dose, mg/kg	effect:[a] no. protected/ no. tested
(*R*,*S*)-methyl	100	3/5	*n*-butyl	100	2/10
(*R*)-methyl	100	5/10	isobutyl	14.4	9/10
(*S*)-methyl	100	5/10	*sec*-butyl	30	2/10
3,3-dimethyl	100	8/10	*tert*-butyl	100	5/10
ethyl	100	5/5	neopentyl	100	4/10
n-propyl	100	3/10	isopentyl	100	0/10
isopropyl	100	6/10			

[a] The compounds were tested for anticonvulsant activity in male CF-1 mice (20–28 g) by intravenous administration followed 120 min later with low-intensity corneal electroshock at 17 mA base-to-peak sinusoidal current for 0.2 s.[29] Anticonvulsant activity was determined by prevention of tonic extensor seizures of the hindlimbs from electroshock application.

creasing concentrations of the analogues; Figure 1 shows the results with (*R*)-3-methyl GABA. All of the other analogues gave similar plots. The activation results for all of the 3-alkyl GABA analogues and the known anticonvulsant drugs sodium valproate,[24] gabapentin,[25] and milacemide[19] are compiled in Table I. By Lineweaver–Burk and nonlinear regression[27] analyses of the data, all of the compounds activated GAD by increasing V_{max} without affecting K_m of L-glutamate (nonessential activation),[28] suggesting that they act at an allosteric site on the enzyme. There does not appear to be an obvious structure–activity relationship to the activation event, but the activation is stereoselective for the *R* isomer of 3-methyl GABA relative to the corresponding *S* isomer. The known anticonvulsant agents, sodium valproate, gabapentin, and milacemide are only 55–70% as active in the GAD activation assay as is (*R*)-3-methyl GABA. With 100 μM (*R*)-3-methyl GABA the GAD activity rose 28%; this is the same rise in GAD levels that is observed in vivo by a 400 mg/kg dose of sodium valproate.[20] All of the 3-alkyl GABA analogues also exhibited anticonvulsant activity against low-intensity electroshock treatment without producing ataxia (Table II). 3-Isobutyl GABA is, by far, the most potent of the analogues tested.

We do not know if the 3-alkyl GABA analogues increase the concentration of GABA in vivo, although they should be capable of crossing the blood–brain barrier, because simple alkyl analogues of GABA, such as γ-vinyl GABA,[30] are known to cross this membrane. Also, there are specific transport mechanisms for certain amino acids.[31] There does not appear to be a definite structure–activity relationship between the GAD activation and anticonvulsant activity; for example, 3-isobutyl GABA is the most potent of the compounds in the anticonvulsant activity test, but is one of the weaker activators of GAD. However, the differences in potency between the compounds in vitro was not particularly marked, and there also may be significant differences in the permeability of these drugs to the blood–brain barrier. Delivery of these compounds to their presumed site of action might be further complicated by differing metabolic stabilities, differences in distribution within the brain, or different abilities of compounds to enter the intracellular compartment where GAD is localized. These factors may account for the relatively weak anticonvulsant action of several of the compounds when given intravenously to mice. An alternative explanation for why there is little correlation between the in vitro and in vivo results is that these compounds may cause anticonvulsant effects by a mechanism unrelated to GAD. At present the mechanism of action of these anticonvulsant agents is unknown, but the data in Tables I and II support a possible mechanism that involves GAD activation.

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(26) The enzyme was assayed in 10-mL vials sealed with serum caps through which a center well (Kontes catalogue no. 882320-000) was inserted. The center well was charged with 200 μL of freshly prepared 8% KOH solution. Various concentrations of L-glutamic acid (0.5, 0.25, 0.166, 0.125, 0.10 mM) containing [1-^{14}C]-L-glutamate (10 μCi/mmol) in 50 mM potassium phosphate buffer, pH 7.2, were shaken at 37 °C in separate vials with purified L-glutamic acid decarboxylase (57 μg; sp act. 7.8 nmol/min·mg) in a total volume of 2.0 mL. After the vials were shaken for 60 min, the enzyme reactions were quenched by the addition of 200 μL of 6 M sulfuric acid to the contents of each of the vials. The vials were shaken for an additional 60 min at 37 °C. The center wells were removed and placed in scintillation vials with 10 mL of scintillation fluid for radioactivity determination. The same assays were repeated except in the presence of various concentrations of the activators (2.5, 1.0, 0.5, 0.25, 0.1, 0.05 mM). The V_{max} values were determined from plots of 1/dpm versus 1/[glutamate] at various concentrations of activators.

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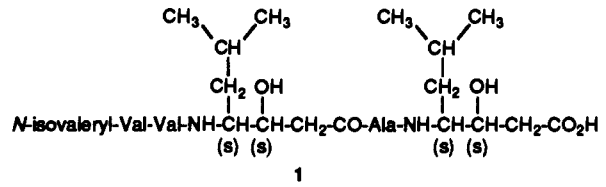
New Pepstatin Analogues: Synthesis and Pepsin Inhibition

Since the isolation of pepstatin, isovaleryl-L-valyl-L-valyl-(3*S*,4*S*)-statyl-L-alanyl-(3*S*,4*S*)-statine (1), by Umezawa,[1,2] investigators have pursued the development and biochemical study of proteinase inhibitors similar in structure to pepstatin.[3-11] The recent discovery of the aspartyl proteinase of the human immunodeficiency virus 1, (HIV-1), and its inhibition by pepstatin, has increased the search for synthetic analogues of pepstatin with increased substrate specificities.[12-16] However, the therapeutic value of pepstatin is limited.



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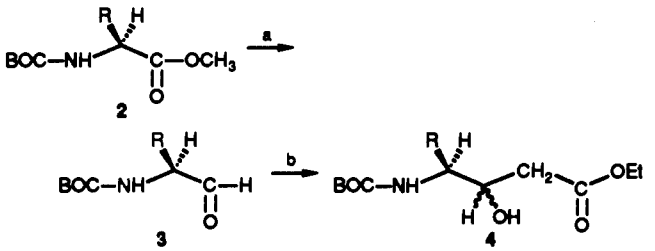
Scheme I[a]



R = $CH_2CH(CH_3)_2$, $CH_2C_6H_5$, $CH_2C_6H_4OH$, $CH_2CH_2CH_2CH_3$

[a] (a) Diisobutylaluminum hydride, toluene, −78 °C; (b) ethyl acetate is pretreated with lithium diisopropylamide in THF at −78 °C for 30 min followed by addition of aldehyde in THF, 2 h at −78 °C.

For a proteinase inhibitor to have therapeutic value, it should be chemically stable, active as low concentrations, selective for a particular proteinase in the presence of other proteinases with similar substrate specificities, and should readily penetrate cell membranes. Pepstatin satisfies the first two of these criteria. The third criterion, specificity, is satisfied only at the level of the major mechanistic class. Pepstatin, while a very effective inhibitor of aspartyl proteinases, is not very selective within this class of enzymes. Pepstatin is also known not to penetrate well cell membranes due to its size and lipophilicity.

The exact mode of inhibition of many aspartyl proteinases by pepstatin has not been well documented. However, crystal structure studies of the HIV-1 aspartyl proteinase inhibited by pepstatin show that the statine residue occupies both the P_1 and P_1' sites, acting as a dipeptide in the bound enzyme.[17] Several studies have also been conducted concerning the nature of inhibition of other

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