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Perspective

Anxioselective Anxiolytics

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Introduction

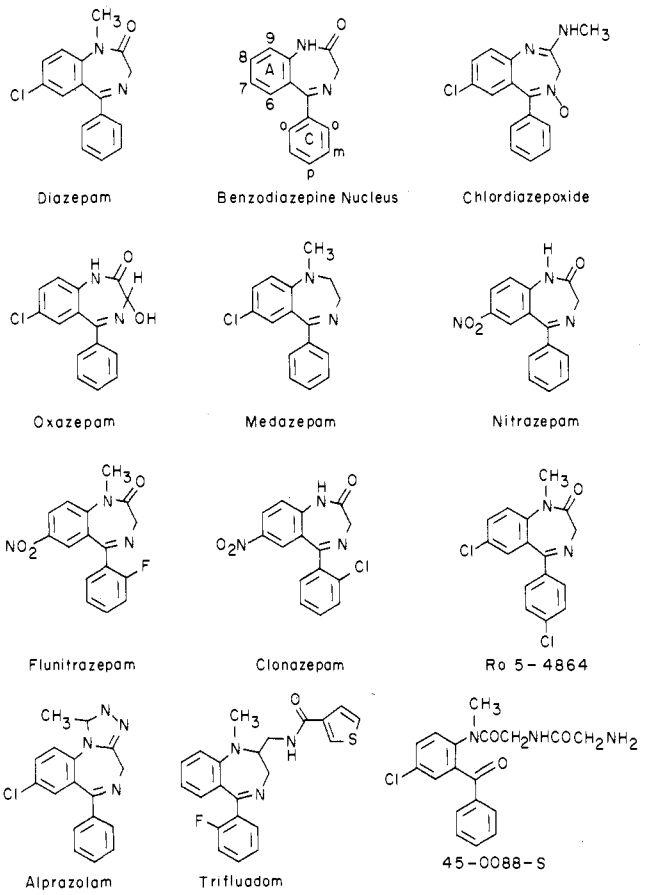
Anxiety is a normal functional state in mammals often conducive to environmental awareness and, consequently, survival. The ever increasing complexity of human life has, however, given rise to anxiety states, i.e., fear in the absence of appropriate stimuli, that sometimes preclude "normal" functioning and, as such, can lead to incapacitation and a reduced involvement with the external environment. While alcohol and other centrally active drugs, such as the barbiturates, neuroleptics, and meprobamate, have been used with varying degrees of success to alleviate anxiety,[1] the benzodiazepines (BZs), because of their low toxicity and clinical effectiveness, are the most widely used anxiolytics (antianxiety agents), some 8000 tons being consumed in the United States in a single year.[2]

In addition to being anxiolytics, the BZs are also potent hypnotics and possess sedative, muscle relaxant, and anticonvulsant properties. While these ancillary pharmacological activities are well tolerated by most individuals, they have led to extensive efforts to elucidate the molecular mechanism of action of these agents in order to provide further information to aid in the design and synthesis of more anxioselective compounds.

Pharmacology of the Benzodiazepines

General. In vivo assessment of the pharmacological activity of several BZs has resulted in the use of chlordiazepoxide, diazepam, and oxazepam (Chart I) as anxiolytics, while clonazepam and nitrazepam are used as a anticonvulsant and hypnotic, respectively. From a structure–activity standpoint,[3] electron-withdrawing substituents at position 7 of ring A (Chart I) and at both ortho positions in ring C enhance central activity (flunitrazepam, clonazepam), while similar substitutions at positions 8 or 9 or electron-donating groups at position 7 of ring A reduce activity. Meta or para (Ro 5-4864) substitutions in ring C can also reduce central activity. While the in vivo antianxiety activity of the dipeptidoaminobenzophenone, 45-0088-S[4] (Chart I), would suggest that an intact B ring is not essential for activity, the compound undergoes biotransformation to a closed 1,4 ring structure in vivo.[5]

Chart I



Biochemical approaches to the mechanism of action of the BZs have focused, by virtue of existing technology, on

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Table I. Benzodiazepine Receptor Subtypes

receptor designation	localization	approx M_r	affinity for [^{3}H]BZ	pharmacological characteristics
BZ_1	brain: primarily cerebellar neurons[a]	51K[b]	10^{-9} M	high affinity (10^{-8} M) for CL 218872[c] high affinity for clonazepam (10^{-9} M) negligible affinity ($>10^{-5}$ M) for Ro 5-4864[d] binding enhanced by etazolate; bicuculline sensitive binding enhanced by GABA; bicuculline sensitive[e] ?selectively labeled by β-CCM[f]
BZ_2	brain: primarily hippocampus[c]	55K[b]	10^{-9} M	low affinity (10^{-7} M) for CL 218872[c] high affinity for clonazepam (10^{-9} M)[d] ?selectively labeled by DMCM[f] binding enhanced by etazolate, pentobarbital, GABA; bicuculline sensitive low affinity for β-CCE[g]
BZ_3	brain		10^{-9} M	low affinity for CL 218872 insensitive to GABA/bicuculline and etazolate binding enhanced by pentobarbital high affinity for β-CCE[g]
anticonvulsant	brain synaptosomal		10^{-5} M	low affinity for BZ's good correlation with BZ antagonism of electroshock-induced convulsions[h]
peripheral	peripheral tissues: liver, kidney, mast cells, platelets, lymphocytes, heart[i] brain: nonneuronal and nonsynaptosomal cellular elements[j]		10^{-9} M	no correlations with clinical or physiological actions of BZ's[d] low affinity (10^{-5} M) for clonazepam high affinity for Ro 5-4864 (10^{-9} M)[d] can be labeled with [^{3}H]Ro 5-4864 binding not GABA stimulated[k] ?acceptors[l]

[a] References 27–29. [b] Reference 38. [c] References 33–35. [d] References 19–22. [e] References 48 and 68–70. [f] Reference 58. [g] Reference 36. [h] Reference 37. [i] Reference 21. [j] References 30 and 52. [k] Reference 52. [l] Reference 31.

the effects of these compounds on the functioning of classical neurotransmitter systems. Acetylcholine,[6] serotonin,[7] norepinephrine,[8] and dopamine,[9,10] as well as the intracellular messenger, cyclic AMP,[11] have all been impoicated in the mechanisms of action of the BZs. The septohippocampal pathway has also been suggested to have a dominant role in physiological function during anxiety.[12] Electrophysiological[13,14] and biochemical studies[15] have, however, shown that the primary action of the BZs is to enhance presynaptic inhibitory processes in both the brain and spinal cord, a finding indicative of an interaction with GABA or glycine, the major inhibitory transmitters in the mammalian nervous system. In one of the first applications of the technique of radioligand binding, a correlation was found for a series of BZs between their ability to inhibit the binding of [^{3}H]strychnine, a glycine antagonist, to glycine receptors and their clinical efficacy.[16] In subsequent electrophysiological studies,[17] however, while flurazepam, GABA, and glycine were found to inhibit spontaneous cell firing in rat medulla, strychnine only antagonized the effect of glycine. Conversely, the GABA antagonist bicuculline blocked the effects of both GABA and flurazepam, not affecting the response to glycine. These data, together with other studies,[13-15] provided evidence that GABA, rather than glycine, was the neurotransmitter involved in the effects of the BZs. From a molecular standpoint, there is no compelling evidence to explain how the BZs facilitate GABAergic neurotransmission.[18] These compounds are not GABAmimetics nor do they inhibit GABA uptake or promote its release from brain tissue.[2]

The BZ Receptor. Radioligand binding has become an increasingly important tool in defining the potential pharmacological activity of novel chemical entities and in providing structure–activity relationships for various classes of therapeutic agents. It may be noted, however, that binding assays, by virtue of their direct measurement of membrane recognition sites, may not always measure efficacy.

The discovery of the endogenous ligands for the opiate receptor, the enkephalins and endorphins, has raised the possibility that therapeutic agents, instead of directly affecting the cellular actions of classical neurotransmitters, might, in fact, be mimicking or antagonizing the actions of previously unidentified neurohumoral agents in mammalian tissue. The description[19,20] of high-affinity, saturable binding sites for the BZs in mammalian brain and peripheral tissues[19-21] raised the possibility that there

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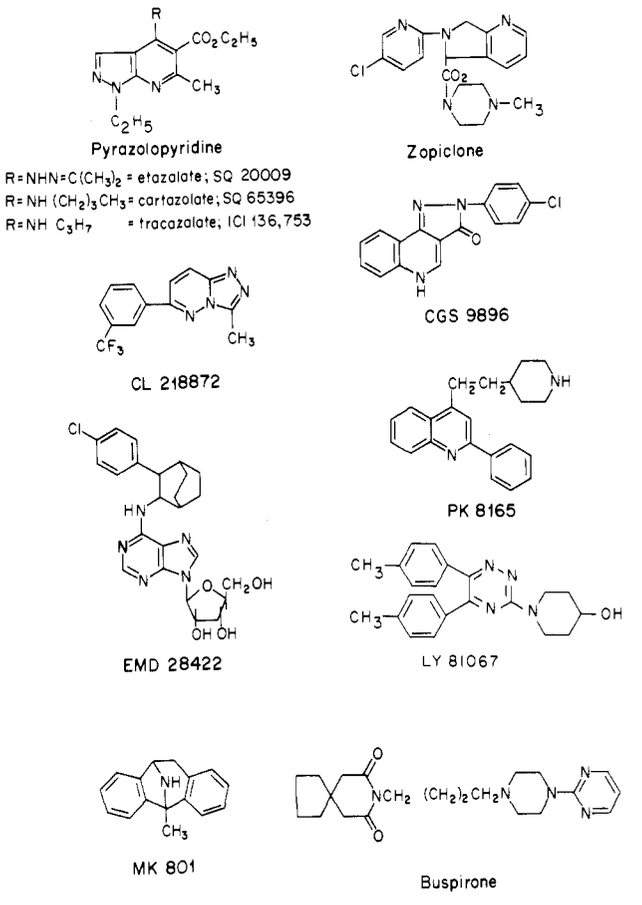
might exist an endogenous ligand for the BZ receptor that could be either anxiolytic or anxiogenic (anxiety inducing). Binding of the BZs to brain recognition sites is regionally distributed[22] and located synaptically,[23] findings consistent with the BZs being involved in some aspect of neurotransmission. Displacement of specific [^{3}H]BZ binding by a series of BZs showed a good correlation with their clinical efficacy.[24] A similar correlation was also found for their ability to increase responding in a behavioral conflict paradigm.[25] Examination of BZ binding in lesioned animals and mutant mice and by autoradiographic techniques[26-29] has indicated that central BZ receptors are neuronally located. Binding of [^{3}H]BZs in peripheral tissues is pharmacologically distinct from that occurring in the CNS,[21,22] the centrally active BZ clonazepam being essentially inactive at peripheral sites (Table I), while Ro 5-4864 (Chart I), a BZ with low affinity for central sites, has high affinity for peripheral sites. Rather confusingly, peripheral recognition sites are also found in the CNS,[30] being nonneuronally located. The physiological function of peripheral sites is unknown,[21] they have, however, been classified as "acceptors" rather than putative receptors.[31] It is pertinent to note that for present purposes, the term receptor is used to describe sites to which BZ radioligands bind. While some physiological and pharmacological correlations have been made for such binding, these recognition sites have not been conclusively identified as receptors.

BZ Receptor Heterogeneity. Initial observations in mammalian brain indicated the presence of a single central binding site. Subsequent analysis of specific [^{3}H]BZ binding in the brains of other species,[32] especially the codfish, yielded curvilinear Scatchard plots, a possible indication of BZ receptor multiplicity. Manipulation of central [^{3}H]BZ binding by heat inactivation of the receptor and examination of binding in the presence of the triazolopyridazine, CL 218872 (Chart II), indicated the presence of two distinct BZ receptor subtypes.[33] The BZ_1 receptor had a high affinity for CL 218872 and was preferentially located in the cerebellum. The BZ_2 receptor had a low affinity for the triazolopyridazine and was located in the hippocampus. The reported minimal ataxic and anticonvulsant activity of CL 218872 in animal models[34]

Chart II



led to the suggestion that the BZ_1 receptor might mediate the anxiolytic effects of putative antianxiety agents, while the BZ_2 receptor might be involved in the ancillary pharmacological actions of the BZs.[35] A third type of high-affinity receptor[36] and a low-affinity "anticonvulsant" BZ receptor[37] have also been described, the binding of BZs to the latter correlating with their efficacy in preventing electroshock-induced convulsions (Table I).

It is unclear at the present time whether BZ receptor heterogeneity is the result of distinct protein entities[33,34,38] or of different association/allosteric states of similar protein subunits. Examination of central BZ receptors by radiation inactivation[39] and photoaffinity labeling[40] indicated the presence of multimeric forms of the receptor. However, other studies have provided evidence for the existence of low- and high-affinity states of a single receptor[41,42] that can be subjected to kinetic analysis.[43] Pharmacological examination of brain [^{3}H]BZ binding has led to the concept of a receptor complex. This is thought

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to be comprised of a GABA recognition site, a high-affinity BZ receptor, and a chloride anion ionophore,[44-47] this latter entity being the physiological mechanism by which the inhibitory neurotransmitter produces its effects. It is possible, therefore, that molecular and allosteric differences in BZ receptor properties may reflect differential interactions between the components of this complex.

GABA Interactions with the BZ Receptor: Enhancement of Radioligand Binding

At micromolar concentrations, GABA can enhance the binding of [^{3}H]BZs to central receptors, an effect reflected as an increase in affinity rather than a change in the number of binding sites.[48] This effect of GABA occurs in all brain regions thus far studied[49,50] and is bicuculline sensitive, concentration dependent, and stereoselective, the $S(-)$ isomer of the GABAmimetic 5-methylmuscimol being more active than its $R(+)$ enantiomer.[51] BZ binding to peripheral BZ recognition sites is not sensitive to GABA modulation.[52]

The high concentrations of GABA required to enhance BZ binding and the fact that the potent GABAmimetics isoguavacine and THIP (4,5,6,7-tetrahydroisooxalo[4,5-*c*]pyridin-3-ol) were unable to stimulate such binding[53] led to the suggestion[53,54] that a novel type of BZ receptor, distinct from the high-affinity sites classified as $GABA_A$[54] (bicuculline sensitive) and $GABA_B$[55] (baclofen sensitive), was involved in this phenomenon. Further evidence to support this hypothesis came from the observations that chemical modulation of $GABA_A$ binding[56,57] and the abolition of low-affinity GABA binding in cerebellum by tissue preincubation[54] did not attenuate the effects of the amino acid on BZ binding. The identity and the characterization of the low-affinity GABA component of the BZ receptor complex remains to be resolved and, as discussed below, may be a potential target for novel, GABA-like anxiolytics.

The increase in binding of BZ-like compounds (BZ agonists) by GABA is termed as the "GABA shift",[58] the ratio of the affinity of an agonist in the absence of GABA to that in its presence being greater than unity. In contrast, compounds that antagonize the effects of anxiolytics, the BZ antagonists (see below), have GABA shift ratios of 1.0 or less. Thus, this ratio offers a convenient in vitro method by which to determine whether compounds interacting with the BZ receptor are agonist or antagonist in nature. GABA can also enhance BZ binding in vivo.[59]

BZs have also been reported to modulate GABA radioligand binding[60] via antagonism of the effects of a noncompetitive peptide inhibitor of GABA binding termed GABAmodulin. Chronic treatment with clonazepam, which decreases BZ receptor numbers in mouse forebrain,[61] can reciprocally increase the number of high-affinity GABA binding sites in forebrain and cerebellum.[62] These effects of the BZs on GABA binding have not, however, been a reproducible phenomemon.[63,64] Thus, while antagonism of the effects of GABAmodulin may explain the physiological actions of the BZs, the evidence in support of the existence of this protein entity is equivocal at the present time.

Other Agents That Facilitate BZ Radioligand Binding

In addition to GABA, several other agents facilitate the binding of BZ agonists to central BZ receptor sites. These include the barbiturates,[65] which, like the BZs, are CNS depressants with sedative/hypnotic properties and can enhance postsynaptic responses to GABA;[66] the avermectins, a group of novel anthelmintic agents;[67] and several putative non-BZ anxiolytic agents, including the pyrazolopyridines,[68-70] EMD 28422,[71] LY 81067,[72] and MK 801[73] (Chart II). The actions of this latter group of compounds, as discussed further below, may be related to their potential as antianxiety agents, while the effects of the barbiturates may relate to the ancillary pharmacological properties of the BZs. The effect of avermectin is irreversible,[74] bicuculline sensitive, and reflected as an increase in both the affinity and number of BZ binding sites.[67,74] While the avermectins can enhance the muscle-relaxant effects of diazepam,[67] no evidence has yet been obtained to indicate that they have any putative anxiolytic activity.

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The anthelmintics can also enhance [^{3}H]GABA binding.[75]

The anticonvulsant diphenylhydantoin (DPH), which enhances the electrophysiological actions of the BZs,[45] also increases the number of BZ binding sites following in vivo treatment,[76] an effect additive with that of GABA. In vitro, however, DPH inhibits [^{3}H]BZ binding,[77] an effect enhanced by GABA, suggesting that DPH may function as a BZ agonist.[58] Whether the low-affinity "anticonvulsant" BZ receptor is involved in the in vivo DPH effects is not known. Increases in the number (B_{max}) of BZ binding sites by in vivo and in vitro manipulations with DPH,[77] avermectin,[67] EMD 28422,[71] MK 801,[73] seizure induction,[78] acute BZ administration,[79] or amygdala kindling[80] have given rise to the concept of so-called "cryptic" receptors. These may be allosterically induced high-affinity states of a normally occurring low-affinity BZ receptor.[18] At the present time, it is not certain whether these effects occur by a common mechanism, irrespective of whether the actions of these various compounds, like those of GABA, are antagonized by bicuculline.

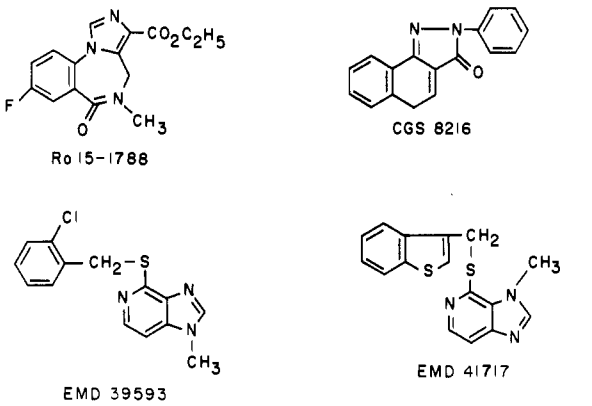
Non-BZ Putative Anxiolytics

The availability of radioligand binding assays for the BZs has, in conjunction with relevant in vivo behavioral avoidance paradigms,[81-83] led to the identification of at least nine classes of non-BZs that may have anxiolytic potential (Chart II). Certain of these compounds are termed anxioselective inasmuch as they have less tendency to elicit the sedative, anticonvulsant, and ataxic side effects associated with BZ usage.

The **pyrazolopyridines** etazolate (SQ 20009; Chart II) and cartazolate (SQ 65396) are active in the rat conflict procedures predictive of anxiolytic potential, an action initially ascribed to their being phosphodiesterase inhibitors.[11] Both compounds can, however, enhance BZ binding via a bicuculline/picrotoxin sensitive increase in receptor affinity.[68,69,84] Cartazolate[85] and tracazolate (ICI 136753)[70] also enhance [^{3}H]GABA binding in mammalian brain membranes by increasing the number of binding sites, suggesting an interaction with the GABA–BZ complex. The diaryltriazine **LY 81067** can also enhance BZ and GABA binding by mechanisms similar to the pyrazolopyridines. The compound is, however, more active and causes a greater enhancement of binding than the latter group.[72] **Zopiclone** (RP 27267), a pyrollopyrazine (Chart II) has high affinity for central BZ receptors (IC_{50} = 30–60 nM)[86] and is a potent hypnotic. The quinoline derivative **PK 8165** also has high affinity for central BZ binding sites (IC_{50} = 10^{-9} M).[87] It is active in the Vogel rat conflict procedure and at doses up to 50 mg/kg ip has no anticonvulsant, sedative, or ataxic activity and may thus be anxioselective. The pyrazoloquinoline **CGS 9896** (Chart II) and its analogue **CGS 8216** (Chart III) are, respectively, an agonist and antagonist at the BZ receptor. Both compounds have ususually high in vitro activity in the BZ binding assay (IC_{50} = 400 pM),[88] and like PK 8165, CGS 9896 is apparently anxioselective, having no muscle-relaxant, cataleptic, or sedative activity. It is, however, an anticonvulsant. CGS 8216 was discovered as a direct consequence of its in vitro binding activity. It was inactive in the animal behavorial conflict test procedures[89] but because of its binding activity was examined further in combination with the BZs and found to antagonize their pharmacological activity. The lipophilic adenosine analogue **EMD 28422** (Chart II) enhances BZ binding in vivo and in vitro by an increase in receptor density that is bicuculline sensitive.[71] The purine analogue is also active in animal conflict procedures and can also potentiate the effects of subthreshold doses of diazepam. The triazolopyridine **CL 218872**, in addition to having putative anxiolytic potential,[34,35] has proven to be an important research tool in delineating BZ receptor subclasses in the mammalian CNS. The low propensity of CL 218872 to elicit the ataxia and depression usually associated with anxiolytic usage led to the suggestion that the compound might be anxioselective. Its preferential interaction with the cerebellar BZ_1 receptor subtype further led to the hypothesis that this receptor might be specifically involved in the anxiolytic actions of antianxiety agents.[35] The BZ_2 receptor has, however, also been considered as the focal point of the anxiolytic effects of the BZs,[47] which tends to confuse the issue somewhat. A related series of compounds, the triazolopyrimidines,[90] are also being evaluated as putative anxiolytics.

Two other non-BZs, **MK 801** (a dibenzocycloheptenimine)[73] and **buspirone** (an azaspirodecanedione)[91] (Chart II), may also be putative anxiolytics, although neither has

Chart III



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any direct interaction with central BZ recognition sites. They may thus be describd as atypical. MK 801 is active in rat conflict procedures and is a potent anticonvulsant, 10 and 100 times more active than clonazepam and pentobarbital, respectively.[92] The compound neither displaces nor enhances BZ binding and has no apparent interaction with the high-affinity $GABA_A$ receptor. Acute but not chronic in vivo treatment with MK 801 can increase BZ receptor site density, indicating a possible interaction with cryptic BZ recognition sites.[73] The anticonflict, but not the anticonvulsant, actions of MK 801 have a delayed onset of approximately 1 h,[92] suggesting the possible involvement of a metabolite in the former response. The anxiolytic activity of buspirone was found as a consequence of its calming action in rhesus monkeys,[93] the compound being originally developed as a neuroleptic. Buspirone is anxioselective, having no apparent anticonvulsant or sedative/hypnotic properties. It does not interact with BZ receptors in vitro but has pronounced dopaminergic agonist activity,[91] a finding that when taken together with evidence implicating this monoamine in the etiology of anxiety,[9,10] has led to the suggestion[91] that its anxioselective anxiolytic activity is a result of its effects on central dopaminergic pathways. In addition, buspirone has been reported to be a selective dopamine autoreceptor antagonist,[94] although the importance of this dopaminergic component has been questioned in light of the compound's central serotoninomimetic and α-adrenolytic activity.[95] Preliminary data[96] has indicated that buspirone can increase in vitro GABA binding and in vivo BZ binding in CNS membrane preparations. An arylpiperazine derivative of buspirone, MJ 13805-1, which has apparent anxiolytic activity, is devoid of both BZ and dopaminergic activity.[97] Further studies on the mechanism of action of these "atypical" anxiolytics are required before any discrete mechanisms can either be ascribed to, or excluded from, their mode of action.

BZ Antagonists

In addition to the pyrazoloquinoline, CGS 8216 (Chart III), four other classes of BZ antagonist have been described. The benzodiazepine Ro 15-1788 (Chart III), like CGS 8216, can antagonize the anxiolytic, anticonvulsant, muscle-relaxant, and hypnotic/sedative actions of the BZs.[98] This BZ can also antagonize the effects of zopiclone but has no effect on the anxiolytic or anticonvulsant actions of MK 801.[99] Ro 15-1788 may also have some agonist activity.[100] Two imidazopyridines, EMD 39593 and EMD 41717 (Chart III), have been reported[101] to be selective anxiolytic antagonists having no significant activity against the anticonvulsant and muscle-relaxant actions of the BZs. When compared to CGS 8216 and Ro 15-1788, these imidazopyridines have a relatively low affinity for the BZ receptor (IC_{50} = 10^{-6} M vs. 10^{-10}–10^{-9} M). The purine inosine may also be a BZ antagonist,[102] although, like Ro 15-1788, it may be a partial agonist. The final group of compounds that have been identified as antagonists of the pharmacological action of the BZs are the β-carbolines,[103] which are discussed further below.

The binding of the BZ antagonists is, in general, unaffected by GABA. These compounds, therefore, have GABA shift ratios near unity.[58] Also, unlike BZ agonists, they are able to bind with high affinity to photoaffinity inactivated BZ–receptor complexes,[104] indicating that antagonists bind to the BZ receptor in a manner different from that of agonists. The concept of agonist and antagonist "domains" on the receptor has been suggested.[18,105,106]

Candidates for the Endogenous BZ Ligand

The discovery of a high-affinity, specific recognition site for the BZs in mammalian brain has led to an intensive search for naturally occurring compounds that displace [^{3}H]BZ binding and also have some in vivo pharmacological actions consistent with a interaction with central anxiolytic systems.

Early studies[44] on the anion dependence of BZ binding indicated, by analogy with similar studies on the effects of halide anions on the activity of compounds at opiate, GABA, and α-adrenergic receptors, that the endogenous ligand for the BZ receptor might be a BZ antagonist and, therefore, an anxiogenic factor. This would suggest that the BZs are actually antagonists with respect to normal CNS function. A list of candidates for the endogenous ligand is shown in Table II.

Peptides and Proteins. Several peptides and proteins have been isolated from mammalian brain that are competitive inhibitors of central [^{3}H]BZ binding. These include BCF (BZ competitive factor) I and II,[107] DBI (diazepam binding inhibitor),[108] a protease-labile protein of M_r 3K[109] and several endogenous factors isolated from human cerebrospinal fluid.[110] The best characterized of these candidates is nepenthin, a M_r 16K protein isolated from rat bile duct and small intestine.[111] This protein has a K_i of 46 nM for the central BZ receptor and has no significant interactions with central receptors for GABA, norepinephrine, dopamine, serotonin, opiates, or acetylcholine. Although this peptide has not been isolated from brain, immunoreactivity to nepenthin antibodies is found on neurons in the deep cortical regions of rat forebrain. A peptide of similar molecular weight has been isolated from mammalian plasma.[112]

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Table II. Putative Endogenous Ligands for Central BZ Receptors

compound	M_r	affinity for BZ receptor: IC_{50}, M	comments
		A. Proteins and Peptides	
BCF-I (benzodiazepine competitive factor)	40–70K		isolated from porcine neocortex[a]
BCF-II			
DBI (diazepam binding inhibitor)	11K		no effect on GABA binding[b]
protease labile peptide	3K		like BZ's can induce slow rhythmic brain waves[c]
CSF factors	0.7–3.6K		isolated from human CSF; effects on [^{3}H]BZ binding enhanced by GABA[d]
nepenthin	16K	4.6×10^{-8} [e]	isolated from rat bile duct and small intestine; immunoreactivity to nepthin antibodies in cortex
			effects on binding not GABA sensitive[f]
serum protein	16K		GABA enhances effect on BZ binding[g]
		B. Low M Factors (M < 400)	
1. Purines			
inosine, hypoxanthine[h]		$1\text{–}2 \times 10^{-3}$	
1-methylisoguanosine[i]		1.9×10^{-5}	low-affinity ligands
2-chloroadenosine[j]		7.5×10^{-4}	?related to some as yet unidentified
isobutylmethylxanthine[j]		2.7×10^{-4}	purine-like compound
nicotinamide[k]		3.9×10^{-3}	
2. Miscellaneous			
thromboxane A_2[l]			prostaglandin endoperoxide intermediate
tribulin[m]			isolated from human urine
			stress-induced increase reversed by BZ's MAO inhibitor
prostaglandins A_1 and A_2[n]		$7\text{–}15 \times 10^{-6}$	
melatonin[o]		5.4×10^{-4}	
D-thyroxine[p]		5.0×10^{-7}	
brain aqueous extracts[q]			
β-carbolines[r]		$1\text{–}5 \times 10^{-9}$	highest affinity of all endogenously derived compounds

[a] Reference 107. [b] Reference 108. [c] Reference 109. [d] Reference 110. [e] K_i. [f] Reference 111. [g] Reference 112. [h] References 113 and 114. [i] Reference 117. [j] Reference 116. [k] Reference 115. [l] Reference 127. [m] Reference 126. [n] Reference 128. [o] Reference 129. [p] Reference 130. [q] Reference 131. [r] References 58, 103, 132, and 144.

Miscellaneous Low-Molecular-Weight Factors. Several purines, including inosine, hypoxanthine,[113,114] and nicotinamide,[115] displace [^{3}H]BZ binding with IC_{50} values in the 10^{-5}–10^{-3} M range. The phosphodiesterase inhibitor IBMX (isobutylmethylxanthine) and 2-chloroadenosine can also displace [^{3}H]BZs from rat brain membrane sites.[116] The most active purine thus far examined is the marine natural product 1-methylisoguanosine (MIG),[117] which has an IC_{50} of 19 μM. The "purinergic" aspect of BZ action while being controversial, inasmuch as the compounds are weak inhibitors of BZ binding, is intriguing, since the purines have become a recurring theme in the area of anxiolytic research. The pyrazolopyridines were originally thought to produce their anxiolytic effects by increasing cyclic AMP levels via their inhibition of cyclic nucleotide phosphodiesterase,[11] while etazolate is reported to be more active in antagonizing adenosine-stimulated cyclic AMP formation in mammalian brain tissue than theophylline.[118,119] Likewise, the BZ antagonist CGS 8216 can also antagonize the effects of adenosine on cyclic AMP production.[89] Furthermore, the adenosine analogue EMD 28422 has putative anxiolytic activity,[71] while the imidazopyridine anxiolytic antagonists EMD 39593 and EMD 41717[101] can be considered as structurally related to the purines. The well-defined sedative actions of adenosine[120] have also been ascribed to BZ inhibition of synaptosomal adenosine uptake.[121] However, such effects require micromolar concentrations of the purine. Furthermore, clinically effective BZs, such as clonazepam and chlordiazepoxide, are essentially inactive in displacing [^{3}H]-nitrobenzylthioinosine, a ligand specific for adenosine uptake sites.[122] There is also no correlation between the effects of a series of putative anxiolytics in displacing [^{3}H]diazepam from rat brain synaptic membranes and their ability to displace adenosine A-1 radioligand binding.[123,124] GABA-stimulated BZ binding is however, more

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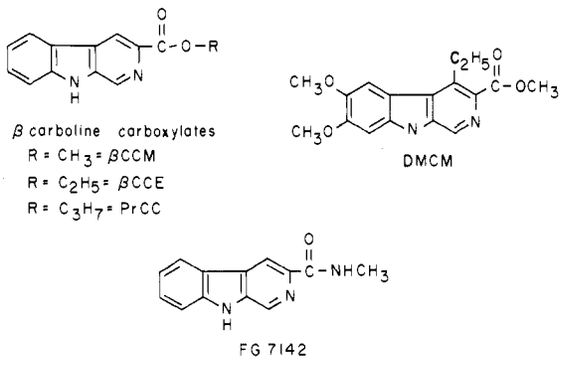
Chart IV



sensitive to purine inhibition than that occurring in the absence of the inhibitory amino acid,[125] indicating that despite their weak activity, the purines do have some BZ agonist-like activity. At the present time, therefore, while there are conflicting viewpoints as to the mode of action and physiological importance of the purines in relation to the BZs, the possible existence of a purine-related entity that has thus far escaped detection cannot be discounted.

A factor termed tribulin,[126] isolated from human urine, can displace [^{3}H]BZs binding in brain, as can thromboxane A_2,[127] prostaglandins of the A series,[128] melatonin and related structural analogues,[129] thyroid hormones and their derivatives,[130] and aqueous extracts from bovine brain.[131] As with the purines, however, the affinity of these compounds, where known, is, with the exception of D-thyroxine (IC_{50} = 0.5 μM[130]), in the micromolar range. It is of interest, however, that tribulin secretion is increased during stress, a phenomenon that can be reversed by BZ treatment.[126]

β-Carbolines. Like tribulin, the ethyl carboxylate ester of β-carboline (β-CCE) was isolated from human urine[132] and, together with other members of this well-known chemical group, is the most active, endogenously derived inhibitor of BZ radioligand binding (K_i = 4–7 nM[103]). The β-carbolines are found in platelets[103] and in brain.[133] Since the isolation procedure for β-CCE involved prolonged high temperatures and low pH conditions,[132] it was thought likely that β-CCE might be an aldehyde condensation product of a peptide or protein containing tryptophan or tryptamine. While this possibility remains to be explored, the β-carbolines as a group (Chart IV), irrespective of origin, have provided important information regarding BZ receptor function. The compounds are BZ antagonists and as such have given some insights into the putative physiological function of an endogenous ligand of this type,[44] namely that of an anxiogenic or anxiety-inducing factor.[58] As antagonists, the β-carbolines can be divided into two groups based on their pharmacological activity and their GABA shift properties. The binding of β-CCE and that of the corresponding propyl ester, PrCC, is not significantly affected by GABA.[134,135] The binding of the methyl ester β-CCM and the 6,7-dimethoxy-4-ethyl analogue of β-CCM, DMCM (Chart IV), is decreased by the amino acid.[58,136]

The β-carbolines, as a group, have an interesting spectrum of pharmacological properties related to convulsant activity. β-CCM and DMCM are convulsants, both compounds evoking metrazole-like cloniotonic convulsions in mice.[58,136] β-CCE has proconvulsant activity; thus, while being devoid of any overt convulsant activity on its own, it can potentiate the convulsant effects of metrazole.[137-139] PrCC is neither convulsant nor proconvulsant. It can, however, like Ro 15-1788 and diazepam, antagonize the convulsant actions of β-CCM.[140] Since, by definition, antagonists are devoid of intrinsic efficacy, the effects of β-CCM and DMCM, and perhaps that of β-CCE, may indicate a degree of interaction with an agonist domain on the BZ–receptor complex that has yet to be characterized. On the basis of their GABA shifts and convulsant profiles, ligands for the BZ receptor have been separated into three groups.[58]

Group 1 comprises BZ-like agents whose binding is enhanced in the presence of GABA; group 2 includes Ro 15-1788 and PrCC, BZ antagonists lacking overt intrinsic activity; and group 3 includes β-CCE, DMCM, β-CCM, and FG 7142, the methyl amide of β-CCE, BZ antagonists with convulsant/potential anxiogenic activity. This latter group of compounds has been provisionally described as "inverse" antagonists,[141] a term that, while somewhat antithetical, may be adequate until such time as the recognition site(s) for the β-carbolines on the BZ–receptor complex are better defined.

GABA modulation of [^{3}H]Ro 15-1788 binding differs from that described for [^{3}H]BZs.[142] With this antagonist radioligand, binding of BZ agonists is increased, while that of β-CCM is decreased. However, the binding of purines is either unaffected by GABA (inosine, hypoxanthine) or decreased (MIG), a finding in contrast to the reported GABA-elicited increase in activity of these compounds by using an agonist ligand.[125] β-CCM may preferentially label BZ_1 receptors,[136] while DMCM may be selective for BZ_2 receptors.[58] The synthetic β-carboline FG 7142 in preliminary clinical evaluation was found to be anxiogenic, evoking acute anxiety and fear in the absence of appropriate stimuli, a response that was rapidly attenuated by BZ administration.[47,58] β-CCE can also product behavioral and physiological symptoms of anxiety in primates.[143]

An extensive structure–activity profile has been generated for the β-carbolines[58,136-144] at the BZ receptor. It is

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important to bear in mind, however, that his group of compounds can also alter several processes involved in serotoninergic neurotransmission,[145] as can diazepam.[146] Their potent interactions with the central BZ receptor, therefore, do not indicate an absolute degree of specificity.

The BZ-Receptor Complex

As mentioned briefly above, there is a considerable amount of experimental evidence to support the concept of a BZ–receptor complex. This comprises a low-affinity GABA recognition site, a high-affinity BZ receptor, and a chloride ionophore. The effects of chloride and GABA on BZ radioligand binding have led to the conceptualization of various models of this complex,[15,18,30,46,47,147] which have also been extended to include the effects of the barbiturates, pyrazolopyridines, and other agents that alter BZ binding in brain tissue.

This GABA/BZ relationship is also supported by studies using solubilized BZ receptors, binding of BZ ligands to which is susceptible to modulation by both GABA and chloride.[148,149] While it is apparent that such theoretical approaches to an as yet unisolated entity do not delineate between the various receptor subtypes previously described and the various pharmacological and physiological properties of the therapeutic agents involved, they have provided a basis for the consolidation of research efforts in this area and have also prompted new approaches distinct from, but complimentary to, the biochemical ones that have formed the major contribution to this Perspective. By using an electrophysiological technique termed fluctuation analysis, clinically active BZs have been shown to have no significant effect on the elementary ion conductance of GABA-activated chloride channels in embryonic mouse spinal neuron cultures but instead potentiate the effects of the amino acid by increasing the frequency of channel opening.[150] In contrast, barbiturate potentation of GABA responses[66] occurs via an increase in the time that chloride channels are open with a corresponding decrease in their open frequency. The effects of GABA on the three groups of BZ ligand described above have been suggested[58] to reflect the activation state of the chloride ionophore component of the receptor complex. Ionophore opening is a GABA-mediated effect. BZs with a preferential affinity for the GABA-activated ionophore, the BZ agonists, might act to stabilize this conformation. Conversely, the "inverse" or convulsant β-carboline BZ antagonists that have a higher affinity (GABA ratio < 1.0) for the nonactivated form of the BZ–receptor complex may stabilize the ionophore in a conformation with reduced chloride conductance and, consequently, reduce GABAergic neurotransmission. DMCM can, in fact, reduce the electrophysiological actions of GABA,[151] while in the mouse neuroblastoma cell line NB_2A, a GABAmimetic-induced increase in chloride anion influx is facilitated by diazepam.[152]

Are the BZ Receptor and Anxiety Synonymous?

The findings described above, the discovery of the BZ receptor, the search, still continuing, for endogenous ligands, and the interactions at the molecular level between GABA and the BZs have focused attention on the BZ receptor and its subtypes as the site at which anxiolysis may take place. However, as already noted, it is unclear which of the two major receptor subtypes, BZ_1 or BZ_2, may be designated anxioselective.[35,47]

The BZs have been termed second-generation psychotropic agents,[153] since their actions do not reflect simple agonism or antagonism but rather a facilitation of the actions of a neurotransmitter, GABA. This conceptualization of possible mechanisms of drug action, which will no doubt be extended to other drug, as opposed to neurotransmitter, receptors,[47] has important ramifications in terms of new drug design. Indeed, if the BZ receptor may be taken as prototypic, the systems involved may be considered as more akin to multimeric allosteric enzyme complexes than simple recognition sites for bioactive agents.

It is therefore perhaps worthwhile considering whether a unitary approach, i.e., the BZ receptor, to antianxiety drug design is the only one available. That is, are compounds that interact with the BZ–receptor complex the most likely to have anxiolytic potential? In addition, are the pharmacological actions of the BZs restricted to the pathophysiology of anxiety?

Several non-BZs (CGS 9896, zopiclone, CL 218872) can interact directly with the BZ receptor (see above), while others (the pyrazolopyridines, LY 81067) enhance radioligand binding to both the BZ and GABA components of the receptor complex, suggesting the induction of a lowered affinity state for any endogenous BZ antagonist[58] and a potential facilitation of GABAergic neurotransmission. On the other hand, buspirone and MK 801 have no in vitro affinity for BZ recognition sites but can modulate BZ receptor function in vivo.[73,96] The effects of buspirone on monoamine function[91,95] and the central sympathomimetic actions of MK 801[92] raise the possibility that BZ receptor related processes may be only one facet of the etiology and anxiety. If this is so, then restricting drug-evaluation efforts solely to the BZ receptor may preclude the characterization of other neuronal systems that may be more directly and intimately involved in the anxioselective actions of the BZs and other antianxiety agents.

A further complication is that newer BZs have been described that have primary putative therapeutic actions not normally associated with the BZs as a chemical series. Trifluadom (Chart I)[154] is an analgesic with low affinity for central BZ reactors, while alprazolam (Chart I)[155] and related BZs, such as U 43,465F,[156] are putative antidepressants, as is CGS 7525A, a BZ that is structurally related to mianserin.[157] Like the latter compound, CGS 7525A is a selective α_2-adrenoceptor antagonist. The relationship between antidepressants and anxiolytics is well know,[158] the former being used for the treatment of anx-

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iety,[8] while anxiolytics are prescribed as adjunct therapy during depression. Interestingly, while the anxiolytic component of the action of U 43,465F is antagonized by Ro 15-1788, its putative antidepressant effects are not.[156]

The analgesic actions of trifluadom, while somewhat expected, may be considered in light of the fact that the potent GABAmimetic THIP can reduce pain perception.[159] Thus, the analgesic BZ may interact in some unique manner with, and facilitate, central GABAergic systems involved in pain perception. The opiate antagonist naloxone has been reported to reverse the anxiolytic actions of both chlordiazepoxide and alcohol, a possible indication of the involvement of endorphins in anxiety.[160]

In view of these findings, it is possible that BZs may be considered as general modulators of GABAergic function. Accordingly, it is pertinent to consider whether there might be some specificity in regard to which systems are modulated by different BZ-related structures and, also, whether the ancillary pharmacological properties associated with the BZs are inevitable and make the likelihood of the discovery of anxioselective anxiolytics interacting with the BZ receptor a goal with limited feasibility.

New Anxiolytic Strategies?

The area of anxiolytic research, as evidenced by the fact that the majority of papers cited in this Perspective cover the period of the last 4 years, is an exciting, expanding one. In attempting to distill the advances that have been made, it is apparent that there is a constant need to incorporate new findings within a framework that is itself in its infancy and that, along with the enkephalin/endorphin concept, has radically changed our whole perception as to how drugs may act. This has consequently led to new approaches to drug design, and nowhere more than in the area of anxiolytic research has medicinal chemistry made such a rapid, relevant contribution to the understanding of some of the molecular mechanisms involved. Whether some of the non-BZ putative anxiolytics are indeed anxioselective will not be known until they have been throughly evaluated in the clinic. Nonetheless, the search for drugs that may have less tendency to elicit the side effects associated with BZ usage is an active one.

Compounds such as buspirone and the BZs alprazolam and trifluadom underline the need, however, for a holistic approach to the evaluation of the pharmacological properties of newly synthesized chemical entities rather than a restriction to the receptor-based approach. Only by testing new compounds in relevant animal models, and eventually in the clinic, can subtle nuances of receptor function be separated from in vitro artifacts.

While evidence from the molecular level to support the BZ facilitation of GABAergic neurotransmission has yet to be validated, it has been suggested[153] that GABAmimetics may be anxiolytics. The potent GABA agonist muscimol has been reported to have actions similar to diazepam,[153] while the GABAmimetic progabide can mimic the effects of diazepam in certain animal test systems.[161]

In this context it may be hypothesized that the BZ drug receptor lies between the GABA site and the chloride ionophore. The GABA-activated form of the BZ receptor[58] may have less affinity for any endogenous anxiogenic factor. However, in chronic anxiety, this GABA activation may not be sufficient, in and of itself, to faciliate chloride ion conductance. Thus, the BZs and other putative anxiolytic agents may act to stabilize the GABA-activated form of the BZ receptor and thus prevent reassociation of the endogenous ligand. Although this concept is speculative, it would not be too farfetched to think that more potent GABAmimetics might be anxioselective anxiolytics. If this is a feasible approach, it will, however, be necessary to better characterize the GABA recognition site involved in such phenomena.

Of further interest in regard to the development of novel antianxiety agents is the possibility that the excitatory neurotransmitter glutamate may be anxiogenic.[162] Anxiety may therefore be an imbalance between the activities of the inhibitory, anxiolytic (GABA), and excitatory, anxiogenic (glutamate), systems in mammalian brain. In this regard, it is of interest that diazepam can inhibit glutamate release in vitro.[163]

In considering a simplistic molecular approach to anxiolytic drug action, it is worthwhile noting that there are discrepancies between the degree of receptor occupancy and the overt pharmacological actions of the BZs.[47] Thus, the antimetrazole effects of a series of clinically effective BZs, irrespective of potency, occur when the receptors are only 25% occupied. This observation, together with the cryptic receptor concept, would indicate that there is much yet to be understood about the intrinsic properties of BZ recognition sites. Indeed, from the observations made with trifluadom, it seems likely that there may be BZ receptors, as yet uncharacterized, that are not involved in the mechanisms of anxiety. In this context, one might also consider what the functional significance of peripheral BZ recognition sites may be.[21] The effects of BZs on peripheral function appear to be minor.[1] This may, however, be a reflection of the BZs and anxiety being treated as synonymous entitities, and perhaps studies with Ro 5-4864 and related "peripheral" BZs may open up new therapeutic areas in which this class of compound may be used.

The BZ antagonists are also a potential new area for BZ research. In addition to the possibility that compounds related to FG 7142 may be used as central stimulants, a longer acting analogue of Ro 15-1788, Ro 15-3505, has been used to prevent the sedative side effects associated with the use of the antischistosomal BZ Ro 11-3128.[164] The use of these compounds in BZ overdose has also been suggested.[88]

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