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Inhibition of Sleep and Benzodiazepine Receptor Binding by a β-Carboline Derivative

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MARTIN, J V , J M COOK, T J HAGEN AND W B MENDELSON *Inhibition of sleep and benzodiazepine receptor binding by a β-carboline derivative* PHARMACOL BIOCHEM BEHAV **34**(1) 37–42, 1989 —The effects of systemic injections of β-carboline-3-carboxylate-*t*-butyl ester (β-CCtB) were investigated with regard to normally occurring sleep and several measures of benzodiazepine receptor occupancy in rats A dose of 30 mg/kg of β-CCtB was found to have a long time-course of action as measured by an in vivo assay for benzodiazepine binding, with an 84% depletion of [^{3}H]diazepam binding at one hour after the intraperitoneal injection This dose of β-CCtB was shown to delay sleep onset, decrease non-REM and total sleep in the first two hours after the injection, and to delay the appearance of REM sleep after the sleep onset The dose- and time-dependence of the effects on sleep approximated the dose- and time-dependence of inhibitory effects of an IP injection of β-CCtB on in vitro measures of benzodiazepine receptor affinity and number

β-Carboline-3-carboxylate-*t*-butyl ester Sleep REM Inverse agonist Benzodiazepine

THE demonstration and characterization of neuronal binding sites for benzodiazepines have enhanced the understanding of the mechanisms of action underlying the behavioral effects of these drugs. Early studies of the central benzodiazepine recognition site indicated a close correlation between the binding affinities of a variety of benzodiazepines and their potencies as muscle relaxants, anxiolytics and anticonvulsants (16,27). Subsequent investigations described the effects of β-carboline derivatives, such as β-carboline-3-carboxylic acid ethyl ester (β-CCE), which was found to bind to benzodiazepine receptors with extremely high affinity (2,17) and to reverse the anticonvulsant and anxiolytic effects of benzodiazepines in vivo (6, 19,21). In addition, some β-carboline derivatives were found to possess intrinsic activities opposite in nature to the effects of benzodiazepines, including anxiogenic (5) and proconvulsant or convulsant (4,24) qualities. The intrinsic activities of these "inverse agonists" could be reversed by competitive antagonists of the benzodiazepine receptor site (4, 5, 18, 21) Newer β-carboline derivatives have been shown to possess each of the possible types of activity with regard to the benzodiazepine receptor, including agonist, inverse agonist, and antagonist properties (1,8)

While the endogenous ligand for the benzodiazepine receptor is still a controversial subject, recent studies demonstrated the presence of the n-butyl ester of β-carboline-3-carboxylic acid (β-CCnB) in brain extracts (23) and that levels of this compound are increased in response to stressful manipulations (10) Since the extraction and isolation procedures were unlikely to lead to the artifactual de novo formation of β-CCnB (23), the possibility exists that a derivative of β-carboline-3-carboxylic acid might serve a role as an endogenous ligand for the benzodiazepine receptor

The study of the behavioral effects of β-carboline derivatives has been restricted by the short half-lives of most of the available compounds in plasma (7,24). This limitation is particularly evident in the investigation of the role of the benzodiazepine receptor in sleep, a process with a long time-course Earlier studies of sleep utilized 3-hydroxymethyl-β-carboline (3HMC), an inverse agonist with a relatively slow degradation in rat plasma (24), but with a low affinity for the benzodiazepine receptor ($K_i \approx 1$ 5 μM) By itself, 3HMC was found to decrease normal sleep, and, at a lower dose, to inhibit the sleep-inducing effects of flurazepam (14,15) Other studies, using enantiomers of an optically active benzodiazepine (B_{10}), indicated that the hypnotic effects of benzodiazepines are stereospecific (11). These findings, therefore, suggested

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a role for the benzodiazepine receptor in the induction of sleep

In order to more fully delineate the involvement of the benzodiazepine receptor in sleep mechanisms, the present studies examined the effects of β-carboline-3-carboxylate-*t*-butyl ester (β-CCtB), a relatively stable β-carboline derivative with a high affinity ($K_i \approx 10$ nM) for the benzodiazepine receptor site (25)

METHOD

Animals

Male 200–250 g Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY) The rats were housed in groups of 3–5 (and after surgery, individually) in plastic tubs having solid bottoms lined with cedar bedding Temperature was maintained at 25 0 to 28 3°C with lights on from 8 00 a m to 8 00 p m Food and water were available ad lib

Drug Injections

β-CCtB was synthesized as described previously (25)

In all studies β-CCtB was prepared and administered in the same way Initially, β-CCtB was dissolved in a 1 1 solution of ethanol and Emulphore polyoxyethylated vegetable oil (G A F Corp , New York, NY) This solution was diluted with nine volumes of phosphate buffered saline (PBS, 154 mM NaCl, 5 6 mM Na_2HPO_4, 1 0 mM KH_2PO_4, pH 7 2) just prior to use The drug (or vehicle) was administered intraperitoneally (IP) in a final volume of 5 cc/kg

Sleep Studies

For each of two doses of β-CCtB (5 or 30 mg/kg), a group of 7–8 rats was implanted with electrodes Prior to surgery, rats were anesthetized with an intramuscular injection of 70 mg/kg ketamine and 6 mg/kg xylazine Four 0–80 stainless steel machine screws were implanted to act as dural electroencephalographic (EEG) electrodes, and two 0 010-inch Teflon-coated stainless steel wires were inserted into the nuchal musculature for electromyographic (EMG) recording Further lengths of the stainless steel wire connected the electrodes to a connector plug (Amphenol Corp , Salem, NH), and the entire assemblage was cemented in place using dental acrylic (Kerr Corp , Romulus, MI) (12) During a one-week recovery period the rats were accustomed to handling, and for the night preceding a study they were housed in the chambers in which they would be tested The next morning, starting at approximately 9 30 a m , each rat was given an IP injection of either β-CCtB or vehicle and an eight-hour sleep recording was immediately begun On a subsequent week, the rats were given the alternative injection of either β-CCtB or vehicle, and retested

EEG and EMG were recorded on a Grass Model 78 polygraph (Grass Instrument Co , Quincy, MA) with a vertical calibration of 50 μV/cm and a paper speed of 10 mm/sec The resulting records were scored by a single investigator who was unaware of the treatment condition Each 30-sec epoch was designated as a) waking, b) nonrapid eye movement (REM) sleep or c) REM sleep according to standard criteria (12,13) Data were analyzed in four successive two-hour blocks by a two-way analysis of variance (ANOVA) for repeated measures, examining the effects of drug treatment and time In the case of a significant effect of drug treatment, individual post hoc comparisons were made by a Fisher's Least Significant Difference test

In Vivo [^{3}H]Diazepam Binding

Inhibition of in vivo binding of [^{3}H]diazepam was used as a measure of the occupancy of the benzodiazepine receptor by the unlabeled β-CCtB (7,29) At varying times after the IP injection of β-CCtB or vehicle, groups of three rats were injected intravenously (IV) with 50 μCi of [^{3}H]diazepam (specific activity = 86 6 Ci/mmol, New England Nuclear, Boston, MA) in 200 μl of 1 1 ethanol/PBS Animals were decapitated at 90 sec after the IV injection, their brains dissected, weighed, and frozen on dry ice To determine total membrane binding of [^{3}H]diazepam, one-half of each brain was homogenized in 25 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7 4) for 20 sec at full speed using a polytron (Brinkmann Instruments, Westbury, NY) The other half of each brain was homogenized in the same way in a solution additionally containing 3 μM unlabeled diazepam, to determine nonspecific binding Each homogenate was then incubated for 30 min at 0–4°C Aliquots of 250 μl of the homogenates were filtered through Whatman (GF/B) glass fiber filters, followed by two washes with 5 ml of ice-cold Tris-HCl buffer The radioactivity on the filters was determined using Ready-Solv (New England Nuclear) scintillation cocktail in a Beckman LS 8100 (Fullerton, CA) counter Protein concentration was measured using a Bio-Rad (Richmond, CA) dye binding assay (3) Specific binding of [^{3}H]diazepam was determined as the difference between the binding for the two brain halves Data were analyzed by a one-way ANOVA Individual post hoc comparisons were made by a Fisher's Least-Significant Difference test

In Vitro [^{3}H]Diazepam Binding

At each time point after an IP injection of β-CCtB or vehicle, separate binding analyses were performed on the brain tissue from each of 3–6 rats, using a modification of previously described methods (22,26) Each brain was homogenized in 100 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7 4) using a Brinkmann polytron at setting 7 for 30 sec Homogenates were frozen on dry ice until use After thawing and resuspending, 0 5 ml of the membrane suspension was added to triplicate incubation tubes with a final concentration of 0 5 to 25 nM [^{3}H]diazepam (specific activity = 85 3 Ci/mmol, New England Nuclear) in a total volume of 1 0 ml Parallel duplicate incubation mixtures included, additionally, 6 μM unlabeled flunitrazepam to determine nonspecific binding The mixtures were incubated on ice for 45 min with gentle shaking, collected on Whatman GF/B filters, and rapidly washed with two five-ml aliquots of Tris-HCl buffer, using a filtration manifold (Brandel, Gaithersburg, MD) Radioactivity on the filters was determined by liquid scintillation photometry Protein concentration was measured using a dye binding assay (3) A binding affinity (K_D) and a total number of binding sites per mg protein (B_{max}) were determined, then, by a separate graphical analysis (22,26) for each individual rat Data were analyzed by a one-way ANOVA, followed by a Fisher's Least Significant Difference test to compare individual dose treatments against the control treatment

RESULTS

Sleep Studies

The high dose of β-CCtB (30 mg/kg) had a significant effect on total sleep, $F(1,7) = 10\ 13$, $p < 0\ 02$ As shown in Table 1, post hoc comparisons indicated that the effect of β-CCtB was to significantly decrease total sleep in the first two-hour interval after the injection (see Fig 2) Similarly, the sleep latency (time between the injection and the first 90 sec of uninterrupted sleep) was significantly greater after injection of 30 mg/kg β-CCtB as compared to the vehicle condition The effect of β-CCtB on total sleep was reflected in a significant drug effect on non-REM sleep,

TABLE 1

EFFECTS OF 5 AND 30 mg/kg β-CCtB ON SLEEP PARAMETERS OVER AN EIGHT-HOUR PERIOD IN RATS*

	0–2 Hours		2–4 Hours		4–6 Hours		6–8 Hours	
	Vehicle	β-CCtB	Vehicle	β-CCtB	Vehicle	β-CCtB	Vehicle	β-CCtB
5 mg/kg β-CCtB†								
Sleep Latency‡	27 1	27 1						
	±6 2	±3 9						
REM Latency§	66 0	80 4						
	±14 7	±20 1						
Non-REM Sleep	50 3	47 1	67 6	66 1	64 1	67 5	56 8	54 1
	±3 7	±4 6	±2 0	±4 3	±1 6	±3 4	±5 1	±4 2
Intermittent	38 3	41 4	38 4	40 7	36 1	32 3	44 7	49 1
Waking Time¶	±8 3	±3 1	±3 3	±6 6	±1 6	±3 2	±6 7	±6 4
REM Sleep	4 3	4 4	12 3	13 1	19 5	20 1	16 4	15 7
	±1 1	±1 2	±2 8	±3 2	±1 5	±2 5	±3 7	±3 6
Total Sleep	54 6	51 4	79 9	79 2	83 6	87 6	73 1	69 9
	±4 6	±5 2	±3 3	±6 6	±1 7	±3 2	±7 7	±6 9
30 mg/kg β-CCtB#								
Sleep Latency	16 6	40 6**						
	±3 6	±7 4						
REM Latency	55 2	146 4**						
	±9 2	±25 6						
Non-REM Sleep	48 1	20 8††	65 6	57 1	57 1	59 7	54 2	58 4
	±2 4	±2 2	±5 6	±7 1	±4 7	±4 2	±3 1	±5 0
Intermittent	47 9	57 4	40 4	54 2	46 7	40 2	47 9	46 1
Waking	±2 3	±7 6	±6 0	±8 9	±5 2	±6 4	±4 9	±6 3
REM Sleep	4 7	0 6	12 8	8 1	14 9	14 1	16 3	15 1
	±0 7	±0 5	±1 1	±2 6	±2 1	±2 5	±2 3	±2 5
Total Sleep	52 8	21 4††	78 4	65 1	72 1	73 8	70 5	73 6
	±2 3	±2 5	±6 2	±9 1	±5 5	±4 2	±4 6	±6 3

*Polygraph tracings were scored in 30-sec epochs for eight hours after the injection of drug or vehicle Results are presented as the mean number of minutes ± S E M of each EEG-defined stage of consciousness for four consecutive two-hour intervals Sleep parameters are defined in detail in (13)

†n = 7

‡Sleep latency was defined as the time from the injection until the first 90 uninterrupted seconds of sleep (sleep onset)

§REM latency was defined as the time after the sleep onset until the occurrence of 60 seconds of REM sleep, interrupted by no more than 60 seconds of another stage of consciousness

¶Intermittent waking time was defined as the time scored as waking after the sleep onset

#n = 8

**$p<0$ 01 as compared to vehicle by Student's t-test

††$p<0$ 01 as compared to vehicle by post hoc comparison using Fisher's Least Significant Difference Test

$F(1,7) = 7$ 96, $p<0$ 05 Again, as seen in Table 1, non-REM sleep was significantly inhibited by the drug treatment as compared to control values in the first two-hour period The inhibitory effect of 30 mg/kg β-CCtB on non-REM sleep appeared to lessen over time, as is reflected in a significant drug-by-time interaction term in the ANOVA, $F(3,21) = 3$ 67, $p<0$ 05 In contrast, there was no significant effect of drug treatment on REM sleep or intermittent waking time, nor a significant drug-by-time interaction REM latency (the time from the onset of sleep until the onset of REM sleep) was significantly lengthened by 30 mg/kg β-CCtB (see Table 1)

No significant effects of drug treatment (or drug-by-time interactions) were evident in those rats treated with 5 mg/kg β-CCtB, though changes in sleep parameters tended to occur in the same directions as with the higher dose of drug (see Fig. 1 and Table 1) As was also true for the experiment with 30 mg/kg β-CCtB, well-known circadian influences were apparent as a significant main effect of time in the ANOVA for each sleep parameter, for total sleep, for example, $F(3,18) = 22.13$, $p<0$ 00001

In Vivo [^{3}H]Diazepam Binding

A significant effect of treatment condition was evident in the ANOVA of the in vivo binding of [^{3}H]diazepam, $F(4,2) = 17$ 89, $p<0$ 02 (see Fig 2B) This difference was due to a dramatic 84% decrease in the available binding at 1 hr after the injection of 30 mg/kg β-CCtB as compared to the vehicle-injected control group ($p<0$ 01 by post hoc Least Significant Difference test) By six hours after the injection of the β-CCtB, the specific in vivo binding had returned to approximately control levels

In Vitro [^{3}H]Diazepam Binding

At one hour after injection, the dose of β-CCtB significantly affected the apparent K_D (Fig 1B) measured in brain tissue

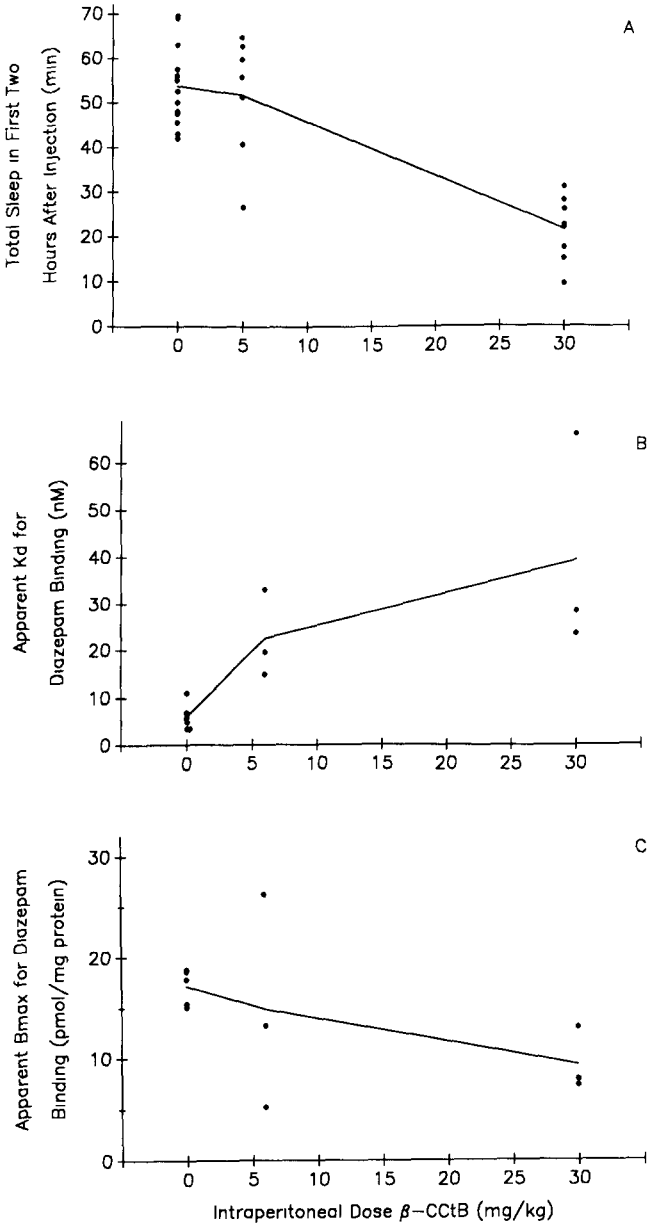

FIG 1 Comparison of the effects of various doses of β-CCtB after an IP injection in rats All values represent data from individual rats (A) Total sleep in the first two hours following the injection (n = 7–8) (B and C) Apparent K_d and B_{max} for [^{3}H]diazepam binding to brain membranes from rats killed at one hour postinjection (n = 3–6)

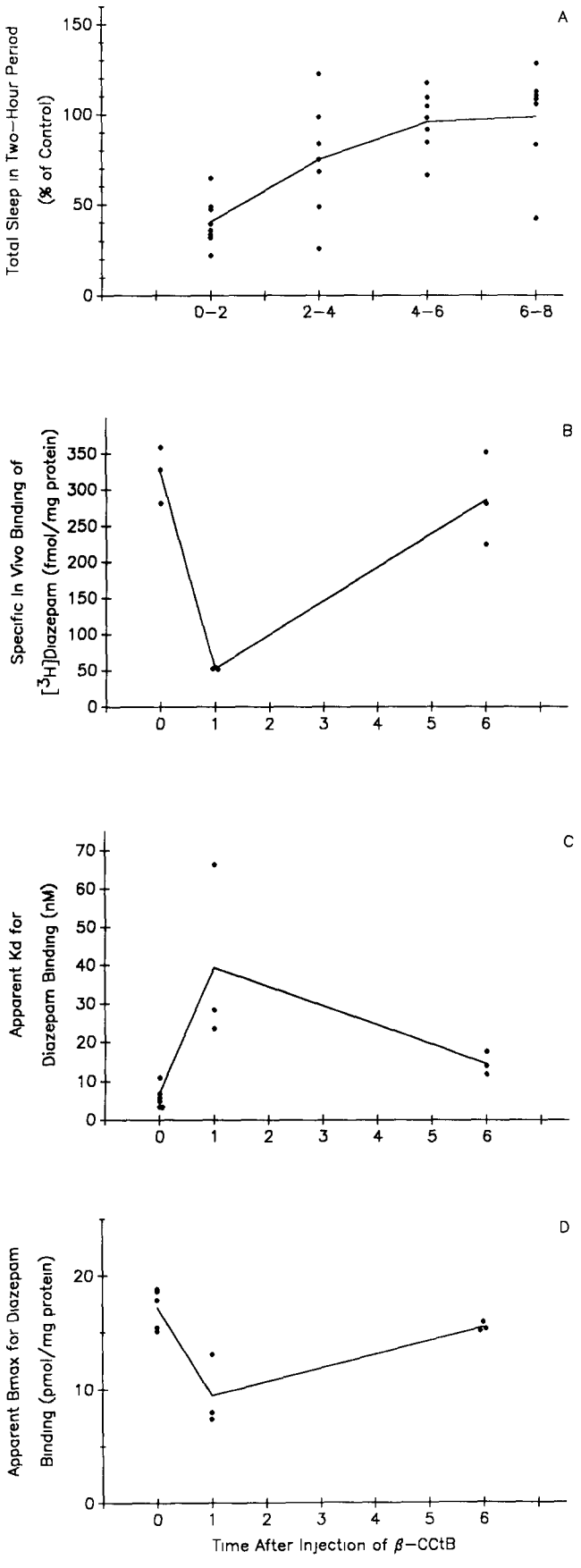

FIG 2 Comparison of the time courses of the effects of 30 mg/kg IP β-CCtB All values represent data from individual rats (A) Total sleep in successive two-hour intervals following the injection (n = 8) (B) In vivo binding of [^{3}H]diazepam at various times after injection (n = 3–6) Combined values for vehicle-injected controls are plotted as Hour 0 (C and D) Apparent K_d and B_{max} for in vitro [^{3}H]diazepam binding to brain membranes from rats killed at various times after injection (n = 3–6) Combined values for vehicle-injected controls are plotted as Hour 0

derived from the injected rats, $F(2,9) = 7\ 89$, $p<0\ 01$ By post hoc Least Significant Difference test, the group of rats injected with 30 mg/kg β-CCtB showed a significantly higher measured K_D than the rats injected with vehicle ($p<0.005$). B_{max} was not significantly altered by treatment condition in this dose-response experiment, $F(2,9) = 2\ 35$, $p<0\ 15$

In Fig 2, the time course of the effects of an injection of 30 mg/kg β-CCtB on in vitro [^{3}H]diazepam binding parameters are compared to vehicle-injected controls (representing the binding at Hour 0) In this experiment, treatment condition significantly altered both K_D, $F(2,9) = 8\ 79$, $p<0\ 01$, and B_{max}, $F(2,9) = 6\ 66$, $p<0\ 02$ Post hoc analyses indicated that K_D was significantly elevated ($p<0\ 005$) and B_{max} significantly decreased ($p<0\ 01$) at 1 hr after injection of the drug, as compared to vehicle-injected controls. By 6 hr, both K_D and B_{max} were not significantly different from controls in the post hoc Least Significant Difference test

DISCUSSION

Our results show that an IP injection of β-CCtB strongly inhibits normally occurring sleep in rats The dose- and time-dependence of the effects on sleep tended to parallel the dose- and time-dependence of inhibitory effects of an IP injection of β-CCtB on several measures of benzodiazepine receptor occupancy in brain A secondary finding was the inhibition of the initiation of REM sleep by β-CCtB, over and above the inhibition of the sleep initiation per se This result implies that β-CCtB may have effects on the normal progression of sleep stages during sleep onset, in addition to a gross inhibition of sleep

Our finding of an intrinsic inhibitory effect of β-CCtB on sleep is in keeping with the previous classification of most of the esters of β-carboline-3-carboxylic acid as inverse agonists for the benzodiazepine receptor (5,8) However, to date the evidence for classification of β-CCtB as agonist, antagonist, or inverse agonist is not decisive For example, in cultured central neurons, β-CCtB increases Cl^- conductance in a manner characteristic of the effects of benzodiazepine receptor agonists (20) In earlier behavioral studies, β-CCtB antagonized the anticonvulsant and anticonflict, but not ataxic, effects of diazepam (25) No intrinsic proconvulsant or proconflict actions of β-CCtB were demonstrated (25), leading the authors to conclude that β-CCtB is not an inverse agonist but is instead a selective antagonist for the benzodiazepine receptor (BZ_1 subtype) It is unclear whether our present evidence for an intrinsic action of β-CCtB on normally occurring sleep represents a quantitative difference in pharmacological sensitivities or a qualitative difference in the mechanisms for effects of benzodiazepines on sleep as compared to other behaviors In any case, research using β-CCtB may be useful in dissecting apart the various behavioral and physiological effects of benzodiazepines

The time-course of inhibition by β-CCtB of in vivo benzodiazepine receptor binding shows marked differences as compared to previously published effects of other β-carboline derivatives The effects of peripherally injected ethyl and propyl esters of β-carboline-3-carboxylic acid (β-CCE and β-CCP) on in vivo benzodiazepine receptor binding are extremely rapid, with peak effects in minutes and essentially complete time-courses of action in twenty minutes (7) In contrast, the long-lasting inhibition of benzodiazepine receptor binding by β-CCtB, measured in hours instead of minutes, may reflect a slowing of the metabolism of this ester by the *t*-butyl moiety (25) Behavioral studies may therefore benefit from a longer duration of action of β-CCtB as compared to other esters of β-carboline-3-carboxylic acid

The effects of an IP injection of β-CCtB on the brain membrane binding affinity for [^{3}H]diazepam determined later in vitro are consistent with circulating β-CCtB acting as a simple competitive inhibitor for the benzodiazepine binding site However, the effects on the total receptor binding per mg protein (B_{max}) may require a more complex explanation In this regard, it may be of significance that both stress and anxiogenic β-carbolines decrease the numbers of low-affinity cortical GABA receptors in rats (1) Similarly, short-term stress has been reported to rapidly and reversibly decrease numbers of benzodiazepine receptors in cortex and hippocampus (9) In addition, acute stress was found to increase levels of presumably endogenous β-CCnB measured in rat cerebral cortex (10) It is conceivable, then, that the effects of an injection of β-CCtB on the B_{max} for [^{3}H]diazepam binding result from actions of the β-CCtB on processes other than the direct interaction of the injected ligand with the benzodiazepine receptor

In conclusion, our results demonstrate a clear inhibition of normally occurring sleep by β-CCtB, a novel β-carboline derivative having not only a high affinity for the benzodiazepine receptor but also a long time course of action in vivo The dose- and time-dependence of the effects on sleep tended to parallel the inhibitory effects of an IP injection of β-CCtB on several measures of benzodiazepine receptor occupancy in brain β-CCtB may therefore be useful in examining the role of the benzodiazepine receptor in such long-term processes as the physiological control of sleep

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