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Naturally occurring benzodiazepines: current status of research and clinical implications

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Abstract Naturally occurring benzodiazepines (BZDs) were first detected in mammalian tissues in 1986. They comprise a variety of 1,4-benzodiazepines corresponding to drugs commercially available for the treatment of anxiety disorders, sleep disturbances and epileptic seizures. Several biosynthetic pathways leading to the formation of BZDs are currently being discussed and have led to the proposition of possible precursor molecules.

For years, the identification of naturally occurring BZDs in mammalian organisms was mostly confined to *post mortem* CNS material for sensitivity reasons. While radioimmunoassay and radioreceptorassay techniques have been tentatively applied to quantitations of genuine BZDs from human milk and cerebrospinal fluid, accurate measurements in peripheral blood have only recently become accessible, e. g., by gas chromatography/selected ion monitoring-mass spectrometry (GC/SIM-MS). This review summarizes existing evidence of benzodiazepines' occurrence in nature and discusses implications for neuropsychiatric disorders.

Key words Benzodiazepines · Benzodiazepine receptor ligands · Natural products · Neurotransmitters

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History

Benzodiazepines were first introduced into clinical practice in 1960 and are today the most widely used psychotropic drugs. Their pharmacological profile covers anxiolytic, sedative-hypnotic, muscle relaxant and anticonvulsive effects (Beckmann 1984) which have made BZDs indispensable to neuropsychiatric therapy. Rapid onset of action and good acceptance lend BZDs to first line treatment of acute anxiety, insomnia, psychomotor agitation, epileptic seizures and alcohol withdrawal with a choice of approximately 50 agents commercialized worldwide for intramuscular, rectal, intravenous, oral and intranasal administration.

The discovery of high affinity BZD binding sites in cerebral cellular membranes (Möhler & Okada 1977) marked the beginning of a search for putative endogenous receptor ligands. Eventually, in 1986 two 1,4-benzodiazepines were purified from bovine brain (Sangameswaran *et al.* 1986). This finding was subsequently confirmed by other groups (Wildmann *et al.* 1987, Medina *et al.* 1988) and shown not to result from environmental contamination with pharmaceutical BZDs: In 1990, proof was given of genuine BZDs in human brains stored in paraffin since the 1940s, i. e., well before the era of industrial BZD synthesis (Klotz 1990). Moreover, several rare BZDs not generally available for therapeutic use have been isolated from plants (Wildmann *et al.* 1988) in support of a biosynthetic pathway yet to be traced.

Benzodiazepines in nature

Chemical structures

Natural BZDs are found in soil, plant, animal and human tissues; they are virtually indistinguishable from BZDs of industrial origin in terms of chemical structure and pharmacological activity. In the past ten years, the natural occurrence of at least nine different 1,4-benzodiazepines has

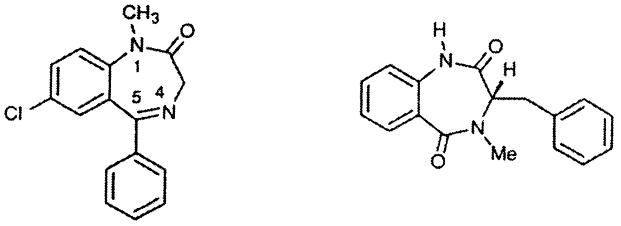

Fig. 1 Diazepam (**a**), a widely used anxiolytic and cyclopeptine (**b**), a benzodiazepine devoid of anxiolytic properties produced by *Penicillium verrucosum* var. *cyclopium*.

been established and their chemical structure confirmed by mass spectrometric analyses. Two frequently identified agents, diazepam and N-desmethyldiazepam are known to be common metabolites of various other BZDs such as medazepam or ketazolam and may in part result from biodegradation of other still undetected natural BZDs. Among the compounds already identified are also several BZDs not currently in therapeutic use, e. g., deschlorodiazepam and isodiazepam.

A common characteristic of pharmacologically active BZDs is their typical heterocyclic framework with a phenyl ring in the 5-position forming a characteristic carbon skeleton. The search for natural analogs has led to cyclopeptine, a benzodiazepine without diazepam-like activity synthesized from anthranilic acid by the mold *Penicillium verrucosum* var. *cyclopium* (Fig. 1). While the phenyl substituent in cyclopeptine is not in the 5-position, this molecule has been considered a possible intermediate product in the non-industrial *de novo* synthesis of 1,4-benzodiazepines (Bringmann 1992).

Occurrence in plants

Pharmacologically active BZDs have been detected in a number of plants and nutritive plant products commonly used for human consumption, e. g., potato tuber, wheat, rice, soy beans, cherries, maize, mushrooms, lentils and grapes (Wildmann *et al.* 1988a, Unseld *et al.* 1989, Klotz 1991). Following different extraction and purification procedures, the authors obtained BZDs in amounts varying from 10 to 600 ng/kg. Agents chemically identified by mass spectrometric analysis include diazepam, N-desmethyldiazepam, isodiazepam, lorazepam, delorazepam, deschlorodiazepam, lormetazepam, 2′-chlorodiazepam and, most recently, temazepam (Kavvadias *et al.* 2000). Interestingly, BZD-like molecules with both RIA and RRA binding activities have also been found in aqueous herbal extracts from medicinal South American/Caribbean plants which has led to speculations that some natural BZDs may participate in sedative effects of certain traditional tea preparations, e. g., from *Tilia* spp. (Medina *et al.* 1989, Sand *et al.* 1998a). Awareness of dietary uptake of BZDs from a variety of foodstuffs has already sparked interest in customized agricultural applications (Grassi *et al.* 1998).

Occurrence in mammalian tissues

Following the original discovery by Sangameswaran *et al.* (see above), traces of BZD have been reported in different mammalian tissues with concentrations mostly in the low ng range (per g wet tissue). Results refer to bovine brain and cow milk (Medina *et al.* 1988), human brain (Klotz 1990), human serum (Duthel *et al.* 1992, Sand *et al.* 1998b), human milk (Pena *et al.* 1991), rat brain and adrenals (Wildmann *et al.* 1987), rat serum (Wildmann & Ranalder 1988) and horse serum (Sand *et al.*, unpublished data). Among the agents determined are diazepam, N-desmethyldiazepam, oxazepam and lorazepam; their identity has unequivocally been confirmed by mass spectrometric analyses.

In rat brain, quantitative mapping of immunoreactivity has revealed variations in regional distribution of BZD-like molecules with peak concentrations localized in the medial septum and the striatum (Wolfman *et al.* 1991). By light and electron microscopy of both rat and human brains, immunoprecipitates were traced to neuronal perikarya and dendritic processes as opposed to axons and axonal terminals (De Blas 1993). These studies have supplemented earlier subcellular fractionations of rat cortices which had indicated the presence of BZD-like molecules in the synaptosomal cytosol and synaptic vesicle fractions (Medina *et al.* 1988). However, the storage of natural BZDs in synaptic vesicles subsequently to be released at the synapse remains hypothetical in that identification of BZDs at a neuronal level has so far only been conducted by immunocytochemical analyses.

Detection and quantitation

BZDs are stable in biological media when stored at –20° C for several weeks or months (Sioufi & Dubois 1990) but for analytical procedures the time lapse between sampling, centrifuging (if necessary) and storage at –20° C should be kept short. As a rule, only polypropylene vials or coated glass recipients should be used in order to avoid the adsorption of BZDs to glassware. In the analysis of blood samples, protein binding (70 to 99 %) must be suppressed.

Immunoassays

The majority of analytical techniques for the isolation and quantitation of BZDs have been published mostly with regard to emergency toxicology screenings and forensic medicine examinations (Lambert *et al.* 1995). Among these, the immunoassays are often chosen to either precede or confirm chromatographic screening procedures. They may be subdivided into homogenous and heterogenous immunoassays:

Homogenous non-quantitative assays, such as the widely used enzyme-multiplied immuno technique (EMIT), the cloned enzyme donor immuno assay (CEDIA) or fluorescent-polarization immunoassay (FPIA) are aimed

at rapid BZD identification in the therapeutic or toxic ranges; even then, the formation of conjugates may lead to false negative results (Schütz 1989). This may be overcome by the incorporation of enzymatic hydrolysis at the price of reduced specificity (Beck *et al.* 1997), yet test kits do not qualify for identification of naturally occurring BZDs. Cross-reactivity has been observed with various household chemicals and oxaprozin (Warner 1989, Fraser & Howell 1998).

Heterogenous immunoassays, e. g., radioimmunoassay (RIA) or radioreceptor assay (RRA) which require a separation step feature high sensitivity. From the pioneering research on natural BZDs in the mid 1980s, both RIA and RRA have retained their importance as valuable and cost-effective analytical instruments at a quantitation limit of ~0.3 ng/ml. An integral part of many extraction and purification protocols (e. g., Rothstein *et al.* 1993), they provide estimates of BZD content of a given matrix in the form of diazepam equivalents (DE) or binding indices. As with homogenous assays, discrimination between parent drug and metabolites is not possible; while affinities for BZDs are generally in the low nanomolar range, occasional cross-reactions with similar molecules may occur.

Chromatography

The chromatographic identification of BZDs can be achieved by quick, non-quantitative testing as in thin-layer chromatography (TLC) via aminobenzophenones and Bratton-Marshall detection. However, TLC does not reliably identify all BZDs and is notably less effective in screening for tetracyclic agents (e. g., alprazolam, midazolam, triazolam) (Schütz 1990). For quantitative testing and improved specificity, high-performance liquid chromatography (HPLC) is an attractive option. HPLC may be performed in conjunction with UV detection or with photodiode array detection (DAD) for additional spectral information (Lambert et al. 1995); quantitation limits vary between 10 and 50 ng/ml (Sioufi & Dubois 1990) and equipment is available in most laboratories. Another highly valuable, non-planar analytical approach is by gas chromatography (GC) with quantitation limits of 2 to 20 ng/ml. The best results are obtained with capillary column GC and electron-capture-detection (ECD); however, analytical GC may be hampered by decomposition of thermolabile BZDs such as oxazepam or chlorodiazepoxide unless an initial derivatization step, e. g., by trimethylsilylation, is included. Identification problems in the analyses of substances with identical retention times on HPLC or GC can be solved by coupling of either procedure with mass spectrometry (MS) (see below). Finally, BZDs may also be determined by capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC, Jinno *et al.* 1996).

Mass spectrometry

Referred to as the state-of-the-art in ultra-sensitive identification of BZDs, mass spectrometry (MS) holds significant analytical potential. Selective quantitations by mass analysis depend on complex equipment; however, they can provide a reliable means of calibrating or verifying less complicated methods which will accommodate larger numbers of samples per day. By monitoring only characteristic ions from a given BZD, the compound-of-interest undergoes a 'tailor-made' analysis in MS. The elimination of impurities prior to the introduction of samples into the ion source is an essential requirement in fully exploiting the sensitivity of the method. An internal standard increases the reliability of the assay and will generally correct for any losses related to purification procedures. Recent optimizations in on-line HPLC/electrospray tandem mass spectrometry (HPLC/ESI-MS-MS) and gas chromatography/negative chemical ionization mode (GC/NCI-MS) have broadened the range of applications. Detection limits of 0.5 pg/ml and 1.0 pg/ml have been reported for HPLC/ESI-MS-MS and GC/NCI-MS, respectively (Zeneroli *et al.* 1997, Cirimele *et al.* 1997). Again, successful coupling with GC depends on thermal stability and volatility of the compounds analyzed.

As has been observed, MS studies tend to yield lower amounts of BZDs than quantitations by conventional assay techniques (RRA/RIA). In similar matrices, this phenomenon is best explained by a net gain in specificity over RRA and RIA (Duthel *et al.* 1992): expression of BZD content in DE, as in RRA or RIA, is liable to include cumulated activities from a variety of BZDs and additionally, a certain proportion of non-BZD related activity. With MS, analyses are targeted toward a limited number of defined BZDs and quantitation is restricted to these compounds. This feature has allowed selective monitoring of a single benzodiazepine in serum over time (Sand *et al.* 1998b).

Origin of naturally occurring benzodiazepines

Formation in plants

Recent compelling evidence from sterile cultures has shown that plants produce pharmacologically active BZD molecules (see Kavvadias *et al.* 2000). The authors have demonstrated the existence of biosynthetic pathways resulting in the *de novo* formation of delorazepam, diazepam and temazepam, a hypnotic agent hitherto not detected in nature. This major advancement follows earlier findings on BZR binding activity in extracts of sterile potato plants (Sand *et al.* 1998a) and the identification of several BZDs (diazepam, N-desmethyldiazepam, delorazepam, lorazepam and delormetazepam) in potato marrow, a medium considered to be close-to-sterile (Wildmann *et al.* 1988c). The synthesis of naturally occurring BZDs by vegetal cells had originally been suggested in 1987 (Wildmann *et al.* 1987) and had been supported by a significant increase in vegetal BZD content observed during germina-

tion of wheat and potato cultures (Wildmann 1988a). It is to be assumed that biosynthetic formation in plants refers to a variety of 1,4-benzodiazepines with diazepam-like properties, more of which are going to emerge in the near future.

Microbial biosynthesis

Precursors of benzodiazepine receptor (BZR) ligands have successfully been obtained from gut bacteria of rodents, such as a strain of *Acinetobacter lwoffii* (Yurdaydin *et al.* 1995). In rats, these active compounds are found to induce a significant increase in BZR ligands which may subsequently cross the blood-brain barrier and accumulate in the event of hepatic failure. Earlier, the isolation of BZD-like agents from both bovine rumen and incubates of ruminal contents with several common grasses had pointed at a microbial participation in BZD biosynthesis (Medina *et al.* 1991). To date, evidence of microbial contribution to BZD formation is incomplete; however, a number of presumptive precursor molecules are currently being investigated.

A rational concept for the *in vivo* formation of the diazepam-like 1,4-benzodiazepine deschloronordiazepam established in recent years focuses on the quinoline alcaloid viridicatin, itself a well-known metabolite of cyclopeptine in different mould species, as a biogenetic intermediate (Bringmann 1992). Its strong structural resemblance with diazepam-like BZDs counts in favor of a microbial origin of naturally occurring BZDs. The appealing feature of this biosynthetic model resides in its analogy to the well-established and optimized industrial BZD synthesis, starting with 2-aminobenzophenone (2-ABP) amidated with glycine (see Overwil & Meyer 1973). In support of the rationale, *in vivo* formation of pharmacologically active halogenated 1,4-benzodiazepines such as N-desmethyldiazepam by *P. verrucosum* has been observed in the presence of halogenated derivatives of 2-ABP in what appears to be an enzymatic condensation reaction (Bringmann & Mader 1995). Halogenation of unsubstituted 2-ABP is in turn thought to be mediated by special bacterial haloperoxidases which are able to introduce halogen atoms into organic molecules from halide ions and hydrogen peroxide. Feeding experiments are currently under way to ascertain the remaining postulated reaction steps which all constitute biochemical standard transformations. An alternative pathway with viridicatin as the main molecular building stone, implies reduction and reductive amination reactions (Fig. 2, Bringmann & Mader 1995).

Neuronal or glial biosynthetic pathway

It has been argued that formation of BZDs may take place within the CNS in the presence of a suitable substrate. Indeed, active low molecular weight BZR ligands are generated from radiolabeled tryptophan within the rat CNS (Medina *et al.* 1993). The authors emphasized the close similarity of these substances to BZD molecules as indicated by specific binding to an anti-BZD monoclonal antibody (MAb 21–7F9). In agreement with the findings on *in vivo* CNS utilization of BZD-precursors from gut flora (see above), the *in vitro* incubation of rat brain homogenates and slices has yielded compounds with BZR binding ac-

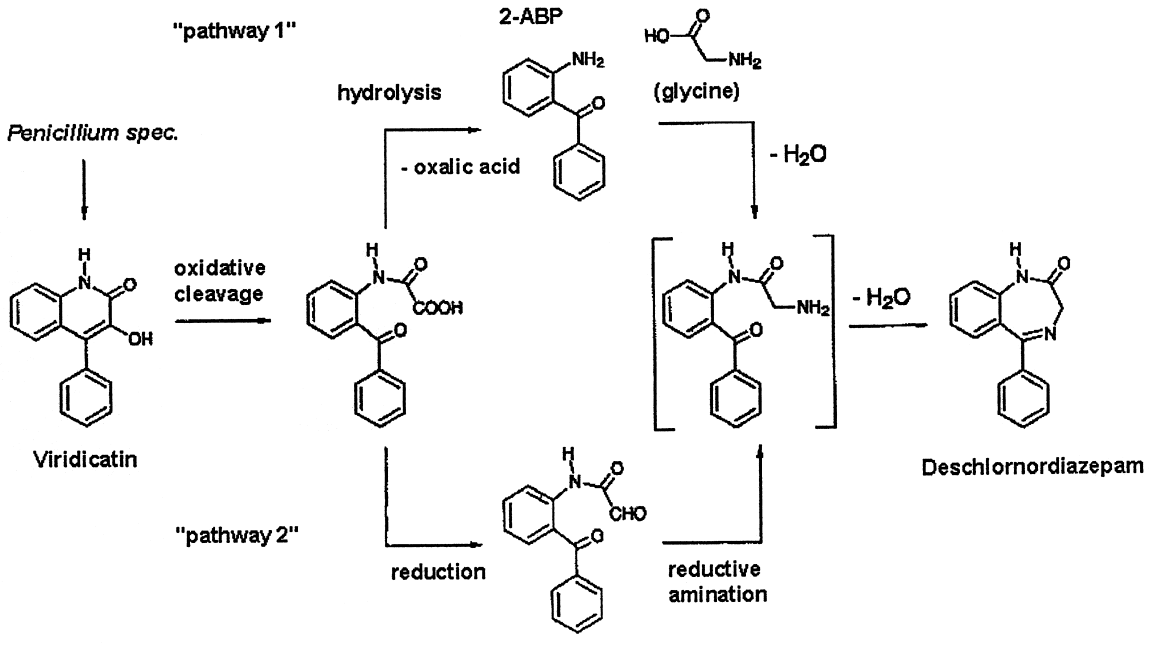

Fig. 2 Proposed biosynthetic pathway for biosynthesis of naturally occurring diazepam-like BZDs from the quinoline alkaloid viridicatin, a metabolite of cyclopeptine in *Penicillium* strains; *2-ABP* 2-aminobenzophenone

tivity (Piva *et al.* 1991). Likewise, BZD-like immunoreactivity is described in neuroblastoma x glioma NG 108–15 cells incubated with a selection of amino acids (De Blas 1993). While these studies provide preliminary evidence, demonstration of *in vivo* enzymatic halogenation, a key issue in neuronal or glial formation of BZDs, is object of ongoing research.

Clinical implications

Benzodiazepines are known to interact with the benzodiazepine allosteric modulatory site of the γ-aminobutyric acid (GABAA) receptor in the CNS (Braestrup & Squires 1978). The GABAA receptor is a chloride channel complex that mediates fast inhibitory transmission in the brain. By increasing the frequency of chloride channel opening, BZDs enhance the effects of GABA and hyperpolarize the cell: as a result, responsiveness to incoming stimuli is decreased. Dysfunction of the GABAergic system has been implicated in a number of neuropsychiatric disorders and has also served as the rationale for a number of clinical observations and studies into the functional role of natural BZDs.

In a dual approach, research has concentrated both on direct identification of naturally occurring BZDs and on metabolic or behavioral correlates of BZD-related neuromodulation. While the latter have proved valuable in providing clinical hypotheses, their limitations in terms of specificity need to be considered.

Hepatic encephalopathy

Hepatic encephalopathy (HE) is a complication of acute or chronic hepatocellular failure and characterized by a generalized depression of CNS function progressing from mild confusion to coma (Conn 1987, Schafer & Jones 1990). Preliminary investigations had revealed a positive correlation of cognitive impairments and severity of liver dysfunction with excess concentrations of endogenous BZR ligands in blood of cirrhotic animals and humans (Mullen *et al.* 1990, Yurdaydin *et al.* 1993, Basile *et al.* 1994, Kapczinski et al. 1996). Likewise, in a recent study on 113 patients with liver cirrhosis, severity of the disease as measured by electroencephalographic recordings, and functional liver status was found to correlate with serum levels of BZD-like compounds expressed in DE (Avallone *et al.* 1998). Interestingly, levels were found to match those encountered in BZD-medicated, non-cirrhotic individuals with normal states of consciousness. Among the agents involved, diazepam and N-desmethyldiazepam were detected by MS; as in earlier studies, serum BZD levels in HE reached several hundred ng/ml whereas levels in healthy controls were consistently below 1 ng/ml. In the setting of HE due to fulminant hepatic failure (FHF), however, elevations in plasma levels of diazepam, N-desmethyldiazepam or BZD-like activity were limited to a subsample of patients with advanced HE (Zeneroli *et al.* 1998). It may be concluded that manifestation of BZD accumulation as HE depends on additional factors, e. g., the degree of pre-existing ammonia-induced brain dysfunction (Avallone *et al.* 1998).

Clinical trials with flumazenil, a BZR antagonist, have led to a significant improvement of HE symptoms in approximately 60 % of patients treated with the drug (Scollo-Lavizzari & Steinmann 1985, Grimm *et al.* 1988, Bansky *et al.* 1989, Ferenci *et al.* 1990, Basile *et al.* 1991). It has been assumed that the nature of the underlying liver failure codetermines responsiveness to flumazenil and accounts for considerable variations in clinical outcome (Basile 1993).

Idiopathic recurring stupor

In analogy to HE patients, individuals suffering from idiopathic recurring stupor (IRS), an episodic impairment of consciousness, have been seen to improve dramatically with flumazenil administration (Tinuper *et al.* 1994, Shimoe *et al.* 1996). This is supported by a state-dependent increase in BZD-like activity in plasma and CSF (Tinuper *et al.* 1992, Schreiber *et al.* 1994, Montagna *et al.* 1995) and the acceleration of basic EEG activity during the ictal period (Rothstein *et al.* 1992). Until the application of MS techniques to IRS studies, however, pathogenetic implication of BZDs is hypothetical.

Sleep disturbances

Conflicting reports exist on the influence of native BZDs on sleep/wake cycles and the manifestation of sleep disturbances. While the BZR antagonist, flumazenil, has exerted mild stimulating effects in healthy volunteers (Ziegler *et al.* 1986, Higgitt *et al.* 1986, Duka & Dorow 1995), it is far from clear whether naturally occurring BZDs are in any way causative of physiological fluctuations in vigilance. In a recent study in healthy volunteers, variations of natural BZDs in serum did not follow a uniform circadian pattern (Sand *et al.* 1998b). Judging by an anecdotal report, flumazenil is devoid of any clinical effect in narcolepsy (Montagna *et al.* 1995), a condition characterized by excessive daytime sleepiness which does not feature an increase in BZD levels. Still, other manifestations of sleep pathology deserve further investigation to rule out BZD-induced rhythm shifts and influence on sleep architecture.

Anxiety and stress

The participation of BZR agonist ligands in the modulation of anxiety reactions has long been suspected (Nutt *et al.* 1990a, Leonard 1994). Particular attention has been devoted to GABAergic tone in limbic areas of the brain, such as the amygdala, known to play a pivotal role in the mediation of anxiolytic BZD effects (Hodges *et al.* 1987, Shibata *et al.* 1989, Yadin *et al.* 1991). Here, regional changes in immunoreactivity point at a release of BZD-like mole-

cules by the limbic system in proportion to the degree of anxiety experienced by test animals (Da Cunha *et al.* 1992a). The level of anxiety has also been markedly increased in rats as a result of intraamygdala flumazenil injections, supposedly interfering with the regional action of BZD-like molecules (Da Cunha *et al.* 1992b). In humans, panicogenic effects of flumazenil administration (Schöpf *et al.* 1984) were found to be accentuated in patients with panic disorder which has raised the question of an altered homeostasis of natural BZR ligands (Nutt *et al.* 1990a,b).

Anxiogenic properties of flumazenil were found to be stress-inducible in rodents and would appear to confirm earlier findings on increased brain BZR binding in escapable stress settings (Pokk & Zharkovsky 1997, Drugan *et al.* 1994). From these observations, antagonism of putative BZD-type agonist ligands released during performance of stressful tasks has been suggested (Da Cunha *et al.* 1992, Pelissolo 1995). However, pentobarbital toxicity models have yielded contradictory results depending on the paradigm employed (Trullas *et al.* 1988, Ojima *et al.* 1997) and have so far not been supplemented by MS evidence of BZDs' functional contribution to stress-protective mechanisms. If proof hereof can be obtained, the treatment of anxiety states or stress reactions with exogenous BZDs would then correspond to a mere substitution of deficient CNS-active compounds.

Acquisition and memory modulation

The amnesic effects of therapeutic BZDs are well established (Lister 1985, Thiebot 1985) and sometimes viewed as beneficial, e. g., in pre-medication for traumatizing surgical procedures. Whether a modulatory amnesic action of endogenous BZR agonists exists is not clear; however, reports on promnesic effects of BZR inverse agonists and antagonists in drug-naïve rodents have given rise to speculations (Izquierdo & Medina 1991, Forster *et al.* 1995). Other indications include regional changes in brain BZD-like immunoreactivity as an accompaniment of habituation and inhibitory avoidance training in animals (Wolfman *et al.* 1991). A systematical evaluation of memory in relationship to serum BZD concentrations in non-medicated individuals is still lacking.

Epilepsy

It has been proposed that certain forms of epilepsy may be associated with a constitutional deficit in natural BZDs as opposed to an excess of natural BZR ligands encountered in HE and experimental models of HE (De Blas 1993, Bassett *et al.* 1987). Patients with HE show little propensity to seizures (Basile *et al.* 1991) and autoradiographic data confirm that approximately 30 % of BZR are occupied in animal models of HE (Basile et al. 1990), i. e., a percentage sufficient to mediate anticonvulsant effects (Petersen *et al.* 1986). The involvement of naturally occurring BZDs in regulation of basic GABAergic tone and seizure threshold could explain perturbances induced by the BZR antagonist flumazenil (Hoffman & Warren 1993, Davis & Wax 1996), provided that interactions with the serotonergic system are not causative (Da-Rocha *et al.* 1997).

Alcoholism

The well-known cross-tolerance for alcohol and BZDs has helped in establishing BZD treatment of acute alcohol withdrawal states (Miller 1995, Mayo-Smith 1997). As a consequence, the search for endogenous BZDs and their clinical relevance has also been directed towards alcohol-related pathology (Lister 1991, Butterworth 1994, Polc 1995). So far, only one report has addressed the effects of the BZR antagonist flumazenil in early alcohol withdrawal (Nutt et al. 1993): to judge by a mixed emotional response, the results remain inconclusive of an interaction with putative native BZDs.

Observations and outlook

Over the past decade, the natural occurrence of benzodiazepines has been corroborated by innovative biomedical research and has prompted the query for plausible biosynthetic pathways. Chemical structures of natural BZDs match 1,4-benzodiazepines of industrial origin; however, the analogy of precursor molecules is still being debated. The determination of the quinoline alkaloid viridicatin by capillary GC was recently reported (Bringmann et al. 1997) and the search for other structurally related intermediates of natural BZDs is under way. A number of chromatographic techniques, e. g., counter-current (CC), rotation locular CC (RLCCC) and multilayer coil CC (MLCCC) chromatography are being studied to complement existing procedures for reliable tracings of BZDs in biological matrices.

Often referred to as 'endogenous' agents, natural BZDs in the mammalian organism may be entirely derived from nutritive sources: only a fraction of natural products has been analyzed so far and the impact on the food chain remains to be fully elucidated. There is need to confirm the concentration of natural BZDs in the mammillary gland by MS as suggested by preliminary findings on BZD-like substances in human breast milk (Dencker et al. 1992). The authors postulated a functional remnant from the times when sedation of breast-fed infants helped ensure survival.

While it would seem that physiological importance of natural BZDs is not necessarily confined to sleep induction in the new born, evidence of their functional effects is scarce. Insight is to be sought into the etiology of several neuropsychiatric syndromes, e. g., those associated with reduced vigilance or hyperarousal. With the exception of hepatic encephalopathy, where BZR antagonists were found to hold significant therapeutic potential, most candidate clinical conditions have been studied only cursorily. This has been attributed mainly to the analytical impediments associated until recently with reliable quantitation of

BZDs at trace amounts in peripheral blood. With the exception of studies of HE, most measurements were hitherto performed on *post-mortem* material, CSF or human milk samples, thus, rendering large scale investigations difficult. Disease-specific variations in natural BZD levels or BZD stimulus response curves would lend strong support to theories on BZD-related symptomatology, including possible effects on immune status (Zavala 1997).

Among the clinical entities deserving future attention is generalized anxiety disorder (GAD), a common anxiety disorder characterized by hyperarousal, increased muscular tension, excessive worry, anxiety, irritability and sleep disturbances. Pharmacotherapy of GAD relies in part on BZDs and while short-term regimens are usually recommended, it is known that some patients function only when they take BZDs regularly (Hoehn-Saric 1998). Most patients continue to derive benefit from the agents over time without the need for an increase in dosage (Hollister 1977, Uhlenhuth et al. 1988) which may serve as a clue to specific alterations in GABAergic tone in a model of physiological anxiolysis.

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References

Avallone R, Zeneroli ML, Venturini I, Corsi L, Schreier P, Kleinschnitz M, Ferrarese C, Farina F, Baraldi C, Pecora N, Frigo M, Baraldi M (1998) Endogenous benzodiazepine-like compounds and diazepam binding inhibitor in serum of patients with liver cirrhosis with and without overt encephalopathy. Gut 42: 861–867

Bansky G, Meier PJ, Ziegler WH, Walser H, Schmid M, Huber M (1985) Reversal of hepatic coma by benzodiazepine antagonist (Ro 15–1788). Lancet 1: 1324–1325

Bansky G, Meier PJ, Riederer E, Walser H, Ziegler WH, Schmid M (1989) Effects of the benzodiazepine antagonist flumazenil in hepatic encephalopathy in humans. Gastroenterology 97: 744 –750

Basile AS, Gammal SH (1988) Evidence for the involvement of the benzodiazepine receptor complex in hepatic encephalopathy. Implications for treatment with benzodiazepine receptor antagonists. Clin Neuropharmacol 11: 401–422

Basile AS, Ostrowski NL, Gammal SH, Jones EA, Skolnick P (1990) The GABAA receptor complex in hepatic encephalopathy: Autoradiographic evidence for the presence of an endogenous benzodiazepine receptor ligand. Neuropsychopharmacology 3: 61–71

Basile AS, Jones EA, Skolnick P (1991) The pathogenesis and treatment of hepatic encephalopathy: Evidence for the involvement of benzodiazepine receptor ligands. Pharmacol Rev 43: 27–71

Basile AS (1993) The role of benzodiazepine receptor ligands in the pathogenesis of encephalopathy. In: Izquierdo I, Medina JH (eds.) Naturally occurring benzodiazepines. Structure, distribution and function. Ellis Horwood London 89–114

Basile AS, Harrison PM, Hughes RD, Gu ZQ, Pannell L, McKinney A, Jones EA, Williams R (1994) Relationship between plasma benzodiazepine receptor ligand concentrations and severity of hepatic encephalopathy. Hepatology 19:112–21

Bassett ML, Mullen KD, Skolnick P, Jones EA (1987) Amelioration of hepatic encephalopathy by pharmacologic antagonism of the GABAsubA-benzodiazepine receptor complex in a rabbit model of fulminant hepatic failure. Gastroenterology 93: 1069–1077

Beck O, Lin Z, Brodin K, Borg S, Hjemdahl P (1997) The online screening technique for urinary benzodiazepines: comparison with EMIT, FPIA and GC-MS. J Anal Toxicol 21: 554–557

Beckmann H, Haas S (1984) Benzodiazepine therapy: an evaluation. Nervenarzt 55: 111–121

Braestrup C, Squires RF (1978) Brain specific benzodiazepine receptors. Br J Psychiatry 133: 249–260

Bringmann G (1992) A first biosynthetic proposal for the *in vivo* formation of naturally occurring diazepam-like benzodiazepines. J Neural Transm 88: 77–82

Bringmann G, Mader T (1995) *In vivo* formation of diazepam-like 1,4-benzodiazepines by *Penicillium verrucosum* var. *verrucosum* after administration of 2-aminobenzophenones and glycine. J Neural Transm 101: 169–181

Bringmann G, Mader T, Feineis D (1997) Determination of viridicatin in Penicillium cyclopium by capillary gas chromatography. Anal Biochem 253:18–25

Butterworth RF (1994) Cerebral dysfunction in chronic alcoholism: role of alcoholic liver disease. Alcohol Suppl 2, pp 259–265

Cirimele V, Kintz P, Mangin P (1997) Testing human hair for flunitrazepam and 7-amino-flunitrazepam by GC/MS-NCI. Forensic Sci Int 84: 189–200

Conn HO (1987) Cirrhosis. In: Schiff L, Schiff ER (eds.) Diseases of the liver. Lipincott Philadelphia, pp 725–864

Da Cunha C, Levi de Stein M, Wolfman C, Koya R, Izquierdo I, Medina JH (1992a) Effect of various training procedures on performance in an elevated plus maze: possible relation with brain regional levels of benzodiazepine-like molecules. Pharmacol Biochem Behav 43: 677–681

Da Cunha C, Wolfman C, Levi de Stein M, Ruschel AC, Izquierdo I, Medina JH (1992b) Anxiogenic effects of the intraamygdala injection of flumazenil, a benzodiazepine receptor antagonist. Funct Neurol 7, pp 401–405

Da Cunha C, Wolfman C, Izquierdo I, Medina JH (1993) Anxiety and brain benzodiazepine-like molecules. In: Izquierdo I, Medina JH (eds.) Naturally occurring benzodiazepines. Structure, distribution and function. Ellis Horwood London, pp 81–88

Da-Rocha MA Jr, Puech AJ, Thiebot MH (1997) Influence of anxiolytic drugs on the effects of specific serotonin reuptake inhibitors in the forced swimming test in mice. J Psychopharmacol 11: 211–8

Davis CO, Wax PM (1996) Flumazenil associated seizure in an 11-months-old child. J Emerg Med 14: 331–333

De Blas AL (1993) Benzodiazepines and benzodiazepine-like molecules are present in brain. In: Izquierdo I, Medina JH (eds) Naturally Occurring Benzodiazepines. Structure, Distribution and Function. Ellis Horwood London, pp 1–27

Dencker SJ, Johansson G, Milsom I (1992) Quantification of naturally occurring benzodiazepine-like substances in human breast milk. Psychopharmacology 107: 69–72

Drugan RC, Basile AS, Ha JH, Ferland RJ (1994) The protective effects of stress control may be mediated by increased brain levels of benzodiazepine receptor agonists. Brain Res 661: 127–136

Duka T, Dorow R (1995) Human experimental psychopharmacology of benzodiazepine receptor inverse agonists and antagonists. In: Benzodiazepine Receptor Inverse Agonists. Sarter M, Nutt DJ, Lister RG (eds) Wiley-Liss Publ, New York, pp 243–270

Duthel JM, Constant H, Vallon JJ, Rochet T, Miachon S (1992) Quantitation by gas chromatography with selected-ion monitoring mass spectrometry of "natural" diazepam, N-desmethyldiazepam and oxazepam in normal human serum. J Chromatogr 579: 85–91

Ferenci P, Grimm G, Meryn S, Gangl A (1990) Successful long-term treatment of portal-systemic encephalopathy by the benzodiazepine antagonist flumazenil. Gastroenterology 96: 240–243

Fernandez-Teruel A, Escorihuela RM, Tobena A, Driscoll P (1991) Stress and putative endogenous ligands for benzodiazepine receptors: the importance of characteristics of the aversive situation and of differential emotionality in experimental animals. Experimentia 47: 1051–1056

Forster MJ, Prather PL, Patel SR, Lal H (1995) The benzodiazepine receptor inverse agonist RO 15–3505 reverses recent memory deficits in aged mice. Pharmacol Biochem Behav 51: 557–560

Fraser AD, Howell P (1998) Oxaprozin cross-reactivity in three com-

mercial immunoassays for benzodiazepines in urine. J Anal Toxicol 22: 50–54
Grassi G, Giraudi G, Moschella A, Giovannoli C, Baggiani C (1998) Production of antibodies against benzodiazepines for competitive enzyme immunoassay detection in plant extracts. Ital J Food Sci 1: 5–15
Grimm G, Ferenci P, Katzenschlager R, Madl C, Schneeweiss B, Laggner AN, Lenz K, Gangl A (1988) Improvement of hepatic encephalopathy treated with flumazenil. Lancet 2: 1392–1394
Higgitt A, Lader M, Fonagy P (1986) The effects of the benzodiazepine antagonist Ro 15–1788 on psychophysiological performance and subjective measures in normal subjects. Psychopharmacology 89: 395–403
Hodges H, Green S, Glenn B (1987) Evidence that the amygdala is involved in benzodiazepine and serotonergic effects on punished responding but not on discrimination. Psychopharmacol 92: 491–504
Hoehn-Saric R (1998) Psychic and somatic anxiety: worries, somatic symptoms and physiological changes. Acta Psychiatr Scand Suppl 393: 32–38
Hoffman EJ, Warren EW (1993) Flumazenil: a benzodiazepine antagonist. Clin Pharm 12: 641–656
Hollister LE (1977) Valium: a discussion of current issues. Psychosomatics 18: 44–58
Izquierdo I, Medina J (1991) GABAA receptor modulation of memory: the role of endogenous benzodiazepines. Trends Pharmacol Sci 12: 260–265
Jinno K, Han Y, Nakamura M (1996) Analysis of anxiolytic drugs by capillary electrophoresis with bare and coated capillaries. J Capillary Electrophor 3: 139–145
Kapczinski F, Curran HV, Przemioslo R, Williams R, Fluck E, Fernandes C, File SE (1996) Cognitive impairments of alcoholic cirrhotic patients: correlation with endogenous benzodiazepine receptor ligands and increased affinity of platelet receptors. J Neurol Neurosurg Psychiatry 60: 676–680
Kavvadias D, Abou-Mandour AA, Czygan FC, Beckmann H, Sand P, Riederer P, Schreier P (2000) Identification of benzodiazepines in *Artemisia dracunculus* and *Solanum tuberosum* rationalizing their endogenous formation in plant tissue Biochem Biophys Res Commun 269: 290–295
Kleinschnitz M, Herderich M, Schreier P (1996) Determination of 1,4-benzodiazepines by high-performance liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr B Appl 676: 61–67
Klotz U (1990) "Natural" benzodiazepines in man. Lancet 335: 922
Klotz U (1991) Occurrence of "natural" benzodiazepines. Life Sci 48: 209–215
Lambert WE, Meyer E, Xue-Ping Y, De Leenheer AP (1995) Screening, identification and quantitation of benzodiazepines in postmortem samples by HPLC with photodiode array detection. J Anal Toxicol 19: 35–40
Leonard BE (1994) Sleep disorders and anxiety: biochemical antecedents and pharmacological consequences. J Psychosom Res 38 Suppl 1: 68–87
Lippa AS, Greenblatt EN, Russell WP (1978) The use of animal models for delineating the mechanisms of anxiolytic agents. In: Hanin I, Usdin E (eds) Animal Models in Psychiatry and Neurology. Oxford, pp 279–292
Lister RG (1985) The amnesic action of benzodiazepines in man. Neurosci Biobehav Rev 9: 87–93
Lister RG, Linnoila M (1991) Alcohol, the chloride ionophore and endogenous ligands for benzodiazepine receptors. Neuropharmacology 30: 1435–1440
Mayo-Smith MF (1997) Pharmacological management of alcohol withdrawal. A meta-analysis and evidence-based practice guideline. American Society of Addiction Medicine Working Group on Pharmacological Management of Alcohol Withdrawal. JAMA 278: 144–151
Medina JH, Peña C, Piva M, Paladini AC, De Robertis E (1988) Presence of benzodiazepine-like molecules in mammalian brain and milk. Biochem Biophys Res Commun 165: 547–553
Medina JH, Peña C, de Stein ML, Wolfman C, Paladini AC (1989) Benzodiazepine-like molecules, as well as other ligands for the brain benzodiazepine receptors are relatively common constituents of plants. Biochem Biophys Res Comm 165: 547–553
Medina JH, Danelon JL, Wasowski C, de Stein ML, Paladini AC (1991) Production of benzodiazepine-like compounds in bovine rumen. Biochem Biophys Res Commun 181: 1048–1055
Medina JH, Paladini AC (1993a) Occurrence of benzodiazepine, benzodiazepine-like molecules and other ligands for the benzodiazepine receptor in nature. In: Izquierdo I, Medina JH (eds) Naturally Occurring Benzodiazepines. Structure, Distribution and Function. Ellis Horwood London, pp 28–43
Medina JH, de Stein ML, Wolfman C, Wasowki C, De Blas A, Paladini AC (1993b) In vivo formation of benzodiazepine-like molecules in mammalian brain. Biochem Biophys Res Commun 195: 1111–1118
Miller NS (1995) Pharmacotherapy in alcoholism. J Addict Dis 14: 23–46
Montagna P, Sforza E, Tinuper P, Provini F, Plazzi G, Cortelli P, Schoch P, Rothstein JD, Lugaresi E (1995) Plasma endogenous benzodiazepine-like activity in sleep disorders with excessive daytime sleepiness. Neurology 45: 1783
Mullen KD, Mendelson WB, Martin JV, Roessle M, Maynard TF, Jones EA (1988) Could an endogenous benzodiazepine ligand contribute to hepatic encephalopathy? Lancet 1: 457–459
Mullen KD, Szauter KM, Kaminsky-Russ K (1990) "Endogenous" benzodiazepine activity in body fluids of patients with hepatic encephalopathy. Lancet 336: 81–83
Nutt DJ, Glue P, Lawson C (1990a) The neurochemistry of anxiety: an update. Prog Neuropsychopharmacol Biol Psychiatry 14: 737–752
Nutt DJ, Glue P, Lawson C, Wilson S (1990b) Flumazenil provocation of panic attacks. Arch Gen Psycho 47: 917–925
Nutt D, Glue P, Wilson S, Groves S, Coupland N, Bailey J (1993) Flumazenil in alcohol withdrawal. Alcohol Suppl 2: 337–341
Ojima K, Matsumoto K, Watanabe H (1997) Flumazenil reverses the decrease in the hypnotic activity of pentobarbital by social isolation stress: are endogenous benzodiazepine receptor ligands involved? Brain Res 745: 126–133
Overwil BH, Meyer HR (1973) Verfahren zur Herstellung von Benzodiazepin-Derivaten. DOS 2 252 378
Pelissolo A (1995) The benzodiazepine receptor: the enigma of the endogenous ligand. Encephale 21: 133–140
Peña C, Medina JH, Piva M, Diaz LE, Danilowicz C, Paladini AC (1991) Naturally occurring benzodiazepines in human milk. Biochem Biophys Res Commun 175: 1042–1050
Petersen EN, Jensen LH, Drejer J, Honore T (1986) New perspectives in benzodiazepine receptor pharmacology. Pharmacopsychiatry 19: 4–6
Piva MA, Medina JH, de Blas AL, Peña C (1991) Formation of benzodiazepine-like molecules in rat brain. Biochem Biophys Res Commun 180: 972–981
Pokk P, Zharkovsky A (1997) The effects of flumazenil, Ro 15–4513 and beta CCM on the behaviour of control and stressed mice in the plus-maze test. J Physiol Pharmacol 48: 253–261
Polc P (1995) Involvement of endogenous benzodiazepine receptor ligands in brain disorders: therapeutic potential for benzodiazepine antagonists? Med Hypotheses 44: 439–446
Rothstein JD, Garland W, Puia G, Guidotti A, Costa E (1991) The role of endogenous benzodiazepines in physiology and pathology. In: Transmitter Amino Acid Receptors: Structures, Transductions and Models for Drug Development. Barnard EA, Costa E (eds) Thieme Publ, New York, pp 325–340
Rothstein JD, Guidotti A, Tinuper P, Cortelli P, Avoni P, Plazzi G, Lugaresi E, Schoch P, Montagna P (1992) Endogenous benzodiazepine receptor ligands in idiopathic recurring stupor. Lancet 340: 1002–1004
Rothstein JD, Guidotti A (1993) Endozepines: non-benzodiazepine endogenous allosteric modulators of GABAA receptors. In: Izquierdo I, Medina JH (eds) Naturally Occurring Benzodiazepines. Structure, distribution and function. Ellis Horwood London, pp 115–130
Sangameswaran L, Fales HM, Friedrich P, De Blas AL (1986) Pu-

rification of a benzodiazepine from bovine brain and detection of benzodiazepine-like immunoreactivity in human brain. Proc Natl Acad Sci 83: 9236–9240

Sand P, Gsell W, Riederer P, Beckmann H, Czygan FC, Abou-Mandour A, Kleinschnitz M, Schreier P (1998a) Hinweise auf die Präsenz natürlicher Liganden des Benzodiazepinrezeptors in sterilem und nicht sterilem Pflanzengewebe. Arch Pharm Med Chem 331 Suppl 1: 45

Sand P, Kleinschnitz M, Vogel P, Beckmann H, Schreier P, Strik W, Riederer P (1998b) Circadian patterns of serum endogenous benzodiazepines. In: Touitou Y (ed) Biological Clocks, pp 567–571

Schöpf J, Laurian PK, Gaillard JM (1984) Intrinsic activity of the benzodiazepine antagonist Ro 15–1788 in man: an electrophysiological investigation. Stuttgart, Georg Thieme Verlag, Pharmacopsychiatry 17: 79–83

Schreiber V (1994) New findings on endogenous drug receptor ligands. Cas Lek Cesk 133: 137–139

Schütz H (1989) Benzodiazepines II. A Handbook. Basic Data, Analytical Methods, Pharmacokinetics and Comprehensive Literature. Springer Berlin New York

Schütz H (1990) Screening and detection of benzodiazepines – pitfalls and clearance. GIT Fachz Lab 4: 441–454

Scollo-Lavizzari G, Steinmann E (1985) Reversal of hepatic coma by benzodiazepine antagonist (Ro 15–1788). Lancet 1: 1324

Shafer DF, Jones EA (1982) Hepatic encephalopathy and the gamma-aminobutyric acid neurotransmitter system. Lancet 1: 18–20

Shibata S, Yamashita K, Yamamoto E, Ozaki T, Ueki S (1989) Effects of benzodiazepine and GABA antagonists on anticonflict effects of antianxiety drugs injected into the rat amygdala in a water-lick suppression test. Psychopharmacol 98: 38–44

Shimoe Y, Fukutake T, Nakai R, Hirayama K (1996) Recurring consciousness disturbance with elevation of endogenous benzodiazepine-like activity. Rinsho Shinkeigaku 36: 495–498

Sioufi A, Dubois JP (1990) Chromatography of benzodiazepines. J Chromatography 531: 459–480

Thiebot MH, Soubrie P, Sanger D (1988) Anxiogenic properties of beta-CCE and FG 7142: A review of promises and pitfalls. Psychopharmacology 94: 452–463

Tinuper P, Montagna P, Cortelli P, Avoni P, Lugaresi A, Schoch P, Bonetti EP, Galassi R, Sforza E, Lugaresi E (1992) Idiopathic recurring stupor. A case with possible involvement of the GABAergic system. Ann Neurol 31: 503–506

Tinuper P, Montagna P, Plazzi G, Avoni P, Cerullo A, Cortelli P, Sforza E, Bonetti EP, Schoch P, Rothstein JD et al. (1994) Idiopathic recurring stupor. Neurology 44: 621–625

Trullas R, Havoundjian H, Skolnick P (1987) Stress-induced change in t-[3H]butylbicyclophosphorothionate binding to gamma-aminobutyric acid-gated chloride channel are mimicked by in vitro occupation of benzodiazepine receptors. J Neurochem 49: 968–974

Trullas R, McIntyre T, Skolnick P (1988) The benzodiazepine/GABA receptor chloride ionophore complex as a biowarning system. In: Biowarning systems in the brain. Takagi H, Oomura Y, Ito M, Otsuka M (eds) University of Tokyo Press, Tokyo, pp 227–241

Uhlenhuth EH, De Wit H, Balter MB, Johanson CE, Mellinger GD (1988) Risks and benefits of long-term benzodiazepine use. J Clin Psychopharmacol 8: 161–167

Unseld E, Krishna DR, Fischer C, Klotz U (1989) Detection of desmethyldiazepam and diazepam in brain of different species and plants. Biochem Pharmacol 38: 2473–2478

Unseld E, Fischer C, Rothemund E, Klotz U (1990) Occurrence of "natural" diazepam in human brain. Biochem Pharmacol 39: 210–212

Warner A (1989) Interference of common household chemicals in immunoassay methods for drugs of abuse. Clin Chem 35: 648–651

Wildmann J, Möhler H, Vetter W et al. (1987) Diazepam and N-desmethyldiazepam are found in rat brain and adrenal and may be of plant origin. J Neural Transm 70: 383–398

Wildmann J (1988a) Increase of natural benzodiazepines in wheat and potato during germination. Biochem Biophys Res Commun 157: 1436–1443

Wildmann J, Ranalder U (1988b) Presence of lorazepam in the blood plasma of drug free rats. Life Sci 43: 1257–1260

Wildmann J, Vetter W, Ranalder UB, Schmidt K, Maurer R, Möhler H (1988c) Occurrence of pharmacologically active benzodiazepines in trace amounts in wheat and potato. Biochem Pharmacol 37: 3549–3559

Wolfman C, Da Cunha C, Jerusalinsky D, Levi de Stein M, Viola H, Izquierdo I, Medina JH (1991) Habituation and inhibitory avoidance training alter brain regional levels of benzodiazepine-like molecules and are affected by intracerebral flumazenil microinjection. Brain Res 548: 74–80

Yadin E, Thomas E, Strickland CE, Grishkat HL (1991) Anxiolytic effects of benzodiazepines in amygdala-lesioned rats. Psychopharmacol 103: 473–479

Yurdaydin C (1993) Benzodiazepine receptor ligands are elevated in an animal model of hepatic encephalopathy: relationship between brain concentration and severity of encephalopathy. J Pharmacol Exp Ther 265: 565–571

Yurdaydin C, Walsh TJ, Engler HD, Ha JH, Li Y, Jones EA, Basile AS (1995) Gut bacteria provide precursors of benzodiazepine receptor ligands in a rat model of hepatic encephalopathy. Brain Res 679: 42–48

Zavala F (1997) Benzodiazepines, anxiety and immunity. Pharmacol Ther 75: 199–216

Zeneroli ML, Venturini I, Avallone R, Farina F, Corsi L, Ardizzone G et al. (1996) Hepatic encephalopathy in liver transplant recipients precipitated by benzodiazepines present in transfused blood. Transplantation 62: 764–777

Zeneroli ML, Venturini I, Corsi L, Avallone R, Farina F, Ardizzone G, Centanaro M, Arrigo A, Schreier P, Kleinschnitz M, Baraldi M (1997) Benzodiazepine-like compounds in the plasma of patients with fulminant hepatic failure. Scand J Gastroenterol 33: 310–313

Ziegler G, Ludwig L, Fritz G (1986) Effects of the specific benzodiazepine antagonist Ro 15–1788 on sleep. Pharmacopsychiatry 19: 200–201