Medicinal plants as a source of novel brain GABA A/benzodiazepine receptor ligands

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Ph.D. Thesis

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II. Ai, J., Dekermendjian, K., Wang, X., Nielsen, M. and Witt, M.R. (1997): 6-methylflavone, a benzodiazepine receptor ligand with antagonistic properties on rat brain and human recombinant $GABA_A$Rs *in vitro*. *Drug Dev. Res.* **41**:99-106.

III. Dekermendjian, K., Ai, J., Nielsen, M., Sterner, O., Shan, R. and Witt, M.R. (1996): Characterisation of furanocoumarin phellopterin as a rat brain benzodiazepine receptor partial agonist *in vitro. Neurosci. Lett.* **219**:151-154.

VI. Squires R.F., Ai, J., Witt M.R., Kahnberg, P., Saederup, E., Sterner, O. and Nilsen, M. (1999): Honokiol and magnolol increase the number of [^{3}H]muscimol binding sites three-fold in rat forebrain membranes in vitro using a filtration assay, by allosterically increasing the affinities of low-affinity sites. *Neurochem. Res*. **24**:1595-1604.

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Abbreviations

Ala	Alanine
(+)-TACP	(+)-*trans*-(1S,3S)-3-aminocyclopentane-1-carboxylic acid
βCCE	β-carboline-3-carboxylic acid ethyl ester
βCCM	β-carboline-3-carboxylic acid methyl ester
3-APA	3-aminopropylphosphonic acid
3-APPA	3-aminopropylphosphinic acid
4 –PIOL	5-(4-piperidyl)isoxazol-3-ol
5-HT	5-hydroxytryptamine
AA	Amino acid
AD	Alzheimer's disease
Arg	Arginine
AS	Angelman syndrome
Asn	Asparagine
Asp	Aspartic acid
BBB	Blood-brain barrier
BZD	Benzodiazepine
CACA	*cis*-4-aminocrotonic acid
cDNA	Complementary deoxyribonucleic acid
CHM	Chinese herbal medicine
CNS	Central nervous system
Cys	Cysteine
DA	Dopamine
DHP	α-dihydropicrotoxinin
DMCM	Methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
EC_{50}	Concentration at 50% enhancement
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine

HD	Huntington's disease
HEK	Human embryonic kidney
His	Histidine
HPLC	High performance (pressure) liquid chromatography
IC_{50}	Concentration of compounds causing 50% inhibition of radioligand specific binding to the receptors
Ile	Isoleucine
K_D	Constant of disassociation
Leu	Leucine
Lys	Lysine
Met	Methionine
mRNA	Messenger ribonucleic acid
NA	Noradrenaline
nAch	Nicotinic acetylcholine
NMR	Nuclear magnetic resonance
PD	Parkinson's disease
Phe	Phenylalanine
Pro	Proline
Ser	Serine
SPA	Schizophrenia
TBPS	*t*-butylbicyclophosphorothionate
TCM	Traditional Chinese Medicine
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol
Thr	Threonine
TM	Transmembrane domain
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
ZAPA	Z-3-amidinothiopropenoic acid

Summary

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system. GABA exert its inhibitory action mainly via the γ-Aminobutyric acid$_A$ receptors ($GABA_ARs$), which are ligand-gated ion channels consisting of several classes of subunits (α, β, γ, δ, ε and π). These subunits may assembly into, at least, 10 major $GABA_A$ receptor subtypes in the brain. $GABA_AR$ is known to contain several modulatory binding sites for pharmacologically and clinically important drugs such as benzodiazepines (BZD), barbiturates, neurosteroids and ethanol, with the BZD binding site as the most important one. The anxiolytic, anticonvulsant, muscle relaxant and sedative-hypnotic BZDs are among the most prescribed drugs clinically. Because of the side effects associated with the use of classical BZDs e.g. diazepam and flunitrazepam, two strategies have been generally adopted in development of new generation of anxiolytics. One is to develop BZD receptor partial agonist; the other is to develop BZD receptor subtype selective compounds. The *in vitro* radioligand receptor binding assays performed on various brain tissues and a variety of recombinant $GABA_AR$ subunit combinations expressed in various cell lines has been well established. So was the electrophysiological method such as the patch-clamp technique performed on different recombinant $GABA_AR$ subtypes. These methods make it possible to screen and characterise compounds with partial agonistic or subtype selective properties from various sources. Due to the known allosteric interaction between the various existing binding sites on the $GABA_ARs$, by applying these methodologies mentioned above, it is also possible to discover compounds binding to additional allosteric binding sites on the $GABA_ARs$. The identification of these new sites might be useful as an important tool for the investigation of the interaction of known binding sites, as well as a potential target for new drugs.

The aim of this project is to isolate and characterise active compounds from medicinal plants used in traditional Chinese medicine (TCM) for binding sites on the $GABA_ARs$. The studies will focus on the BZD binding sites.

Totally ca. 50 TCM plants were screened mainly for their binding activity (GABA, BZD and TBPS binding sites) on the $GABA_ARs$ (some of the plants have also been tested against the kainate receptor). Activities were found in extracts of 13 plants. 12

active compounds from 7 plants were purified and chemically elucidated. Because of the lower affinity (Affinity is the property of attraction of a drug for a receptor. It is numerically represented by the equilibrium dissociation constant (K_D) of the ligand-receptor complex) found in the initial experiments, some of the active compounds (acacetin, rutaecarpine) were not investigated further.

The furanocoumarin derivatives phellopterin, byakangelicol and imperatorin were purified from *Angelica dahurica*. Phellopterin, the most potent derivative, was found inhibiting [^{3}H]diazepam and [^{3}H]Ro 15-1788 binding to the BZD binding site of the rat brain $GABA_AR$ *in vitro* with IC_{50} values (Concentration of the compound induces 50% inhibition of the radio-ligand receptor binding) of 400 and 680 nM, respectively. Scatchard plot analysis showed that phellopterin increased the K_D (Constant of dissociation) without changing the B_{max} (Number of maximum binding sites) in [^{3}H]diazepam binding to various rat brain membrane preparations. This indicates that phellopterin induced a competitive inhibition of [^{3}H]diazepam binding. The GABA ratios (The ratio of the IC_{50} values determined for the testing compound in absence and presence of GABA (usually 100 μM) in the [^{3}H]BZD binding assays) of 1.3 for [^{3}H]diazepam binding and 1.7 for [^{3}H]Ro 15-788 binding indicate that phellopterin is a partial agonist at the BZD site. This finding was supported by TBPS shift experiments (TBPS shift (or TBPS ratio) is calculated as the ratio of the specific binding of [^{35}S]TBPS) in the presence and absence of testing compound in a on-rate experiment). The partial agonistic profile of phellopterin makes it an interesting candidate for further development of more potent derivatives.

Honokiol and magnolol were purified from *Magnolia officinalis*. They are previously reported having central depressant effects such as: sedation, ataxia, muscle relaxation as well as having anxiolytic activity in animal experiments, but the mechanism of action remains unknown. Our data shows that both honokiol and magnolol enhance [^{3}H]muscimol binding to rat brain membrane preparations and to various human recombinant $GABA_AR$ subtypes expressed in Sf9/baculovirus system. Saturation binding assays showed that honokiol and magnolol increased the B_{max} for [^{3}H]muscimol binding to $GABA_ARs$ constructed by $\alpha_2\beta_3\gamma_{2S}$ and $\alpha_2\beta_3$ subunit combinations. However, both the B_{max} and the K_D for [^{3}H]muscimol binding to $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_2$ subunit combinations were increased by honokiol and magnolol. The results indicate that

honokiol and magnolol exert different affinities for different $GABA_AR$ subtypes. Honokiol and magnolol (10 μM) potentiated GABA-induced chloride current in whole-cell patch clamp experiments comparable to that of diazepam. Our data suggest that honokiol and magnolol by interacting with the receptor complex could change the neuronal transmission mediated by GABA through $GABA_ARs$. This effect could partially explain their anxiolytic and central depressant effects previously reported. The interaction of honokiol and magnolol with $GABA_ARs$ may be explained by binding to a new unknown binding site. The binding of honokiol and magnolol to these allosteric sites may convert the low affinity GABA binding sites to the high affinity sites, which can be detected by [^{3}H]muscimol binding assays.

The synthetic or semi-synthetic derivatives of natural-occurring compounds take a great part of the total nature-originated drugs. Therefore, based on the available data of nature-occurring compounds to investigate their synthetic or semi-synthetic derivatives for a possible activity would be a natural consideration.

Many flavonoids, such as apigenin, chrysin and amentoflavone, have been purified from plants and shown previously to bind to BZD receptors in the Central nervous system (CNS) *in vitro*. In the present communication screening of 32 commercially available flavonoids, seventeen structurally related flavonoid derivatives were found to inhibit the binding of [^{3}H]diazepam to $GABA_A$/BZD receptors. 6-methylflavone, the most potent one (IC_{50} value of 120 nM) was characterised by binding as well as functional assays. Saturation binding experiments showed that 6-methylflavone is a competitive inhibitor of [^{3}H]Ro 15-1788 binding to rat cortical $GABA_A$/BZD receptors. Data was obtained for the GABA ratios of [^{3}H]diazepam binding to various rat brain membrane preparations in the presence of increasing concentrations of 6-methylflavone. Data was also accumulated for the GABA ratios of [^{3}H]Ro 15-1788 binding to various recombinant human $GABA_A$/BZD receptor subunit combinations expressed in baculovirus/Sf9 insect cell system in the presence of increasing concentrations of 6-methylflavone. Both these types of data were compared with data obtained by assay of the currents assays by the whole-cell patch clamp on Sf-9 insect cells. It was concluded that 6-methylflavone is most likely a antagonist on the BZD binding site. Meanwhile, based on available data, some general structure-activity relationship of flavonoids for their inhibitory effect on the $GABA_A$/BZD receptor has been discussed.

β-carboline-3-carboxylic acid methyl ester (βCCM) and β-carboline-3-carboxylic acid ethyl ester (βCCE) have been isolated from *Pseudostellaria heterophylla* with trace amount. *Pseudostellaria heterophylla* was chosen from a well-known TCM prescription named *Panacea anti-epilepsy.* As seen in the separation process on HPLC, there are at least 10 or more active substances that show inhibition of [^{3}H]flunitrazepam binding with similar potency *in-vitro*. Both βCCM and βCCE are known as potent proconvulsant acting on $GABA_A$/BZD receptor complex. The plant is used in the reverse pharmacological direction as an anti-convulsant treatment in TCM. This raised the intriguing possibility that some other β-carboline derivatives or other compounds having anti-convulsant property may present in this plant. To our knowledge, this is the first report demonstrating a plant origin of these β-carboline-3-carboxylic acid derivatives.

Finally, based on our own experiences in the isolation and characterisation of compounds from medicinal plants, some possible pitfall was also discussed.

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system (CNS). Depending on the brain region, 20 to 50% of all central synapses use GABA as the transmitter (Young and Chu, 1990; Halasy and Somogyi, 1993). GABA mediates inhibitory action throughout the CNS by activating three classes of receptors, $GABA_A$, $GABA_B$ and $GABA_C$ receptors that are classified structurally and pharmacologically (Table 1). A new classification of $GABA_A$Rs has recently been proposed by Barnard et al. (1998). $GABA_A$Rs consist of several classes of subunits (α, β, γ, δ, ε and π) that form a integrated chloride ion channels (for review, see Dunn, et al. 1994; Smith and Olsen, 1995; Davies, et al. 1996). Each subunit possesses extracellular N and C terminal domains and four transmembrane regions. $GABA_A$Rs contain modulatory binding sites for benzodiazepine (BZD), barbiturates, neurosteroids and ethanol, are activated by GABA, muscimol and inhibited by bicuculline and picrotoxin (reviewed by Braestrup and Nielsen, 1983; Macdonald and Olsen, 1994; Rabow et al., 1995; Sieghart, 1995; Johnston, 1996a; Doble and Martin, 1996; Krogsgaard-Larsen et al., 1997; Hevers and Lüddens, 1998). In contrast, $GABA_B$ receptors are members of the seven transmembrane receptor family, which are coupled to either K^+ or Ca^{2+} channels via G-proteins and are regulated by intracellular second messenger systems (reviewed by Bowery, 1989, 1993; Mott and Lewis, 1994; Malcangio and Bowery, 1995; Misgeld et al, 1995). Two splice variants of $GABA_B$ receptor subunits $GABA_B$ R1a and $GABA_B$ R1b were recently cloned that showed sequence similarity to the metabotropic receptors for the excitatory neurotransmitter L-glutamate (Kaupmann et al. 1997; Bettler et al. 1998). $GABA_B$ receptors are selectively activated by GABA and (-)baclofen and are inhibited by phaclofen. Although similar to $GABA_A$Rs directly associated with a Cl^- ion channel, $GABA_C$ receptors differ markedly from $GABA_A$ and $GABA_B$ receptors in their pharmacological properties. They are insensitive to both bicuculline and baclofen as well as to $GABA_A$R modulatory drugs like BZDs, barbiturates or neurosteroids, but stimulated by GABA and certain analogues of GABA, such as *cis*-4-aminocrotonic acid (CACA). $GABA_C$ receptors are though to be composed of the ρ subunits (ρ_{1-3} and two splicing variants of ρ_1) and are predominantly expressed in the vertebrate retina (reviewed by Bormann and Feigenspan, 1995; Johnston, 1996b; Lukasiewicz, 1996;

Enz and Cutting 1998; Feigenspan and Bormann, 1998; Martinez-Torres et al., 1998). Table 1 summarises the main differences among these three receptor systems.

Table 1. A brief summary of different GABA receptors

Receptors	$GABA_A$	$GABA_B$	$GABA_C$
Ion channels	Ionotropic Cl^-	Metabotropic G protein-coupled K^+	Ionotropic Cl^-
Protein subunits	α_{1-6}, β_{1-4}, γ_{1-4}, δ, ε and π	R_{1a}, R_{1b}	ρ_{1-3}
Agonists	GABA Muscimol	GABA (-)baclofen	GABA CACA
Antagonists	Bicuculline Picrotoxin	Phaclofen Saclofen	3-APA 3-APPA
Allosteric modulator	BZDs, barbiturates, neurosteroids etc	No	No
Receptor structure	Hetero-oligomeric pentamers, each subunit with 4 transmembrane domains	7 transmembrane domains couple with G-protein	Homo-oligomeric pentamers, each subunit with 4 transmembrane domains.
Primary locations	Frontal cortex, cerebellar granule cell layer, olfactory bulb etc.	Cerebellar molecular layer, frontal cortex, thalamic nuclei.	Retina

This thesis concerns only the $GABA_A$Rs.

The aim of this project is to isolate and characterise active compounds from medicinal plants used in traditional Chinese medicine (TCM) for the existing binding sites on the $GABA_A$Rs, with a focus on the BZD binding sites. There are several factors contribute to the initiation of the current project: 1). The establishment and routinisation of the radioligand-receptor binding assays for the agonists (e.g. GABA and muscimol) and the modulatory agents (e.g. BZDs and TBPS) of the $GABA_A$R provides a fast simple

screening and characterising system for novel potential ligands from various sources, including medicinal plants. 2). The diversity of the subunit isoforms leads to a variety of different $GABA_AR$ subtypes that show differential expression in various neuronal populations. This diversity could potentially mediate affinities of specific drugs. With the variety of $GABA_AR$ subtypes, the possibility exists to target compounds to a specific $GABA_AR$ subtype and thereby avoid side effects associated with the use of classical BZDs. The establishment of cell lines expressing individual recombinant receptor subtypes (both for binding as well as for electrophysiological studies) provides the opportunity of developing such specific subtype-selective compounds which in turn might be a useful tool for the characterisation of the physiological role of individual $GABA_AR$ subtypes *in vivo*. 3). In order to obtain drugs with new molecular structures and pharmacological profiles, it is necessary to isolate and identify compounds by bioassay-guided fractionation from natural products, especially medicinal plants used in folkloric or traditional medicine, like TCM. TCM and herbs have been widely used in China and outside Chinese communities for thousands years. Throughout the long Chinese history, there has been an accumulation of experience using medicinal plants to treat a variety of diseases. TCM plays a role in the health care of Chinese people through the history, as well as in the modern times. Using modern analytical methods and assay systems, new drugs (single compound drugs, such as artemisinin) have been isolated from Chinese medicinal plants (Xiao, 1983; Qin and Xu, 1998).

It is my desire and belief that the combination of my previous knowledge of TCM (11 years from 1983 ~ 1994) with the knowledge from this laboratory on the $GABA_ARs$ will lead to the identification of some interesting active compounds for the $GABA_A$/BZD receptor system from Chinese medicinal plants.

Within this thesis, the first two sections will focus on the pharmacology and molecular biology of the $GABA_A$/BZD receptors. The third section will discuss natural products as a source of new drugs and pharmacological tools in general, and the general status of TCM and TCM medicinal plants. The fourth section will present the experimental work and the results in identification and characterisation of active compounds from Chinese medicinal plants and semi-synthetic compounds with nature origin to the $GABA_A$ receptors. Based on our own experiences, some possible pitfall will also be discussed.

Section 1. Pharmacology of $GABA_A$Rs

1.1. GABA binding site

$GABA_A$Rs are ligand-gated Cl^- channels. Its neurotransmitter GABA is a small amino acid (AA) derived from glutamate by glutamic acid decarboxylase. Activation of $GABA_A$Rs by GABA results in an increase in neuronal membrane conductance for Cl^- from the prolonged openings of the ion channels, this influx of Cl^- causes a localised hyperpolarisition of the neuronal membrane, which counteracts the effects of depolarising stimuli and results in the inhibition of synaptic transmission (Macdonald and Twyman, 1992). The inhibitory action of GABA can be mimicked by full agonists, such as muscimol (a natural product from the mushroom *Amanita muscaria*), competitively antagonised by competitive antagonists, such as bicuculline (another natural product isolated from the plant *Dicentra cucullaria* and a variety of *Corydalis*, *Dicentra* and *Adlumia* species), and noncompetitively antagonised by picrotoxin (an equimolar mixture of picrotoxinin and picrotin isolated from *Anamirta cocculus* and related poisonous plants). The GABA binding sites on the $GABA_A$R complex in mammalian brain have been characterised in homogenates of various membrane preparations used in specific radioligand-receptor binding assays. There seems to be three different GABA binding sites: 1). the high affinity binding site, 2). the low affinity recognising sites and 3). the very low affinity binding site. These sites have been experimentally characterised. Thus, homogenisation, multiple wash steps, the use of sodium-free buffer and incubation at 0 °C serves to remove endogenous GABA and other potential endogenous ligands and to disable the GABA transport system are necessary steps for characterisation of these sites (Braestrup and Nielsen, 1983). [^{3}H]GABA and [^{3}H]muscimol binding studies in mammalian brain tissue have revealed that both high (K_D values in low nanomolar range) and low affinity (K_D values in high nanomolar range) states of $GABA_A$Rs for GABA exist (Olsen et al., 1981). The low-affinity recognition site can be specifically labelled by the specific GABA antagonist bicuculline, thus this site may be an antagonist-preferring binding sites (Olsen and Snowman, 1983). The fact that micromolar concentrations of GABA are needed to open Cl^- channels in ion flux, electrophysiological experiments and to modulate other binding sites (e.g. BZD sites) on the $GABA_A$R complex, suggest GABA probably exerts its

physiological effects by acting at an additional very low affinity-binding site (K_D values in micromolar range), which can only be opened during synaptic transmission when GABA is massively released into the synaptic cleft (reviewed by Sieghart, 1992; 1995). The apparent separate existence of high, low, very low affinity binding sites for GABA can be explained either in terms of different conformational states of the same receptor, or in term of distinct GABA binding sites on a single $GABA_AR$ (Sieghart, 1995).

Table 2. $GABA_AR$ agonists, partial agonists and antagonists∗

Agonists	
Endogenous	GABA, Taurine and β-alanine
Exogenous	Muscimol, THIP, Isoguvacine, ZAPA, (+)-TACP
Partial Agonist	4 –PIOL, Thio-THIP
Antagonists	
Competitive	Bicuculline, Bicuculline methochloride, SR95531, RU5135, Pitrazepin, Securinine, (+)-Hydrastine, Benzyl penicillin, (+)-Tubocurarine
Noncompetitive	Picrotoxin, δ-guanidinovaleric acid, Cunaniol, Dimefline, Enoxacin, *m*-Benzenesulfonic acid diazonium chloride, Dopamine sulfate, Norfloxacin, Pentylenetetrazole, Furosemide

∗ Data is based on papers (Kerr and Ong, 1992; Johnston, 1996a; Krogsgaard-Larsen et al. 1997).

Structurally different compounds are known to interact with the GABA binding site within the $GABA_AR$ complex as agonist, partial agonist and antagonist (Table 2). Since the specific receptor antagonists are essential tools for the characterisation of the physiological and pharmacological properties of receptors, the discovery of the convulsant alkaloid bicuculline as an antagonist of the inhibitory action of GABA in the CNS provided a vital pharmacological tool to probe GABA mediated inhibition. The $GABA_AR$ agonists may induce rapid desensitisation and the antagonists are potential anxiogenic, proconvulsant or convulsant compounds (Krogsgaard-Larsen et al., 1997). There is considerable interest in $GABA_A$ partial agonists as targets for drug development. Partial agonists of $GABA_ARs$, particularly those of relatively low

efficacy, such as Thio-THIP and Thio-4-PIOL are promising therapeutic agents for Alzheimer's disease (Krogsgaard-Larsen et al., 1994, 1997).

1.2. BZD binding site (BZD receptors)

The most notable phenomenon of $GABA_A$Rs is that they possess a variety of allosteric binding sites (allosteric binding sites are referred to the binding sites that are different from the agonist binding sites within the receptor complex, binding of the corresponding compounds to these sites mediate a different biological response from the agonist) for several clinically important drugs. The activation of these sites often result in an allosteric modulation on the activity of agonists. The most widely investigated allosteric modulatory sites are the BZD sites (Lüddens et al., 1995; Rabow et al., 1995; Sieghart, 1995). BZDs were introduced as clinical therapeutic agents in the early 1960s before GABA was considered as a neurotransmitter. The first clue of the action of BZDs was the proposal that BZDs facilitate GABA-mediated synaptic inhibition (Costa et al., 1975; Haefely et al., 1975; Polc and Haefely, 1976). However, it was the discovery of high-affinity binding sites in vertebrate brain that started the exiting exploration on understanding of the pharmacological actions of BZDs (Squires and Braestrup, 1977; Möhler and Okada, 1977). The binding of BZDs to these sites was stimulated by GABA (Tallman et al., 1978). Because there was a excellent correlation between the clinical potency of BZDs and their affinity for these binding sites, it is now believed that these $GABA_A$R-associated binding sites are the pharmacological receptors by which the BZDs exert their clinically important actions (Haefely et al., 1985). That is why the BZD binding sites were termed BZD receptors (Braestrup and Nielsen, 1983).

1.2.1. BZD receptor subtypes and ligand selectivity

1.2.1.1. Central against peripheral

BZD receptors can be divided into two classes: "central" and "peripheral" receptors, referring to the receptors appeared in CNS and peripheral tissues as kidney, respectively. Flumazenil (Ro 15-1788) only interacts with the "central" receptors, while Ro 5-4868, the 4'-chloro derivative of diazepam, is a selective ligand for the "peripheral" receptors. Only the central BZD receptors are associated with $GABA_A$Rs.

1.2.1.2. BZD receptor subtypes and the selectivity of ligands

Most BZDs bind to the central BZD receptors with similar affinities throughout the brain, but the binding properties of several compounds, most notably CL 218872 (Squires et al., 1979; Sieghart, 1983) and β-carboline-3-carboxylic acid ethyl ester (βCCE) (Nielsen and Braestrup, 1980) demonstrate the heterogeneity of BZD receptors. Two types of BZD receptor were originally proposed based on their pharmacology and distribution. The BZD1 subtype found throughout the brain but predominant in the cerebellum scarcely in the hippocampus (Faull et al., 1987; Faull and Villiger, 1988; Olsen et al, 1990; Lüddens et al., 1995), show high affinity to the triazolopyridazine CL 218872, and βCCE (β-carboline-3-carboxylic acid ethyl ester) (Squires et al., 1979; Braestrup and Nielsen, 1980; Nielsen and Braestrup, 1980; Sieghart, 1983). The BZD2 subtype found principally in hippocampus, spinal cord and cortex show low affinity for these ligands mentioned above and high affinity for flunitrazepam. A third class of BZD receptor was found in cerebellar granule cells, associated with the α_6 $GABA_AR$ subunit, is insensitive to the prototype BZDs e.g. diazepam. It probably functionally involves in mediating the alcohol-induced impairment in motor performance that can be antagonised by the selective ligand Ro 15-4513 (Sieghart et al., 1987; Lüddens et al., 1990). Molecular biology studies have demonstrated that several isoforms of the α subunit of the $GABA_AR$ exist, and the phenotype of the BZD binding site depended on the nature of the α subunit expressed in the receptor (Levitan, et al 1988; Pritchett, et al 1989). This molecular biological basis for the heterogeneity of BZD receptor subtypes will be discussed in detail in Section 2.5.1.

The functional role for the individual BZD receptor subtype was for several years unclear due to the relative low selectivity of available compounds (Doble and Martin, 1996). Thus, compounds with higher selectivity for the different BZD receptor subtypes would be desirable as pharmacological tool for the characterisation of the functional properties of BZD receptor subtypes, as well as a lead for development of new pharmaceuticals avoiding the side effects associated with the classical BZDs.

1.2.2. Intrinsic efficacy of BZD receptor ligands and partial agonism

Intrinsic efficacy is the inherent ability of a given compound to initiate a biological response. It is the property that a ligand induces the conformational change of its

receptor and changes the behaviour of the receptor toward other proteins in the membrane, (Kenakin and Kenakin, 1997). For the BZD receptors, generally, there are three classes of ligands, agonist, antagonist and inverse agonist with different efficacy.

1.2.2.1. Full agonist and partial agonist

Most of the prototypical 1,4-benzodiazepines appear to be agonists at the BZD receptors (For chemical structure, see Section 1.2.3.; Gardner et al., 1993). Electrophysiological experiments in many different neuronal systems have indicated that BZDs such as diazepam, enhance the actions of GABA at the $GABA_AR$ by facilitating its binding to the receptor and by increasing the frequency of Cl^- channel opening (Study and Barker, 1981; Edgar and Schwarz, 1992). However, they do not open Cl^- channels in the absence of GABA (Study and Barker, 1981; Polc, 1988). The principal behavioural effects of classical BZDs in animals are anxiolytic, anticonvulsant, sedative and myorelaxant effect (Doble and Martin, 1996). Clinically, the sedative/myorelaxant effects together with amnestic effect, tolerance and withdrawal effect are generally considered as unwanted side-effects (Haefely et al., 1990; Doble and Martin, 1992). Studies with BZD full agonists have demonstrated that higher doses BZDs and higher occupancy of the BZD receptors are required to produce sedative/myorelaxant effects than, but not for producing of anticonvulsant and anxiolytic effects (Braestrup and Nielsen, 1986; Petersen et al., 1986, Petersen, 1987). Since partial agonists have lower intrinsic efficacy, they do not activate all the receptors they occupied. For partial agonists of sufficiently low efficacy, it may be impossible for them to activate sufficient receptors to induce sedative/myorelaxant effects even at high doses that would be enough to occupy the receptors completely (Haefely, 1994; Doble and Martin, 1996; Figure 1).

Figure 1.

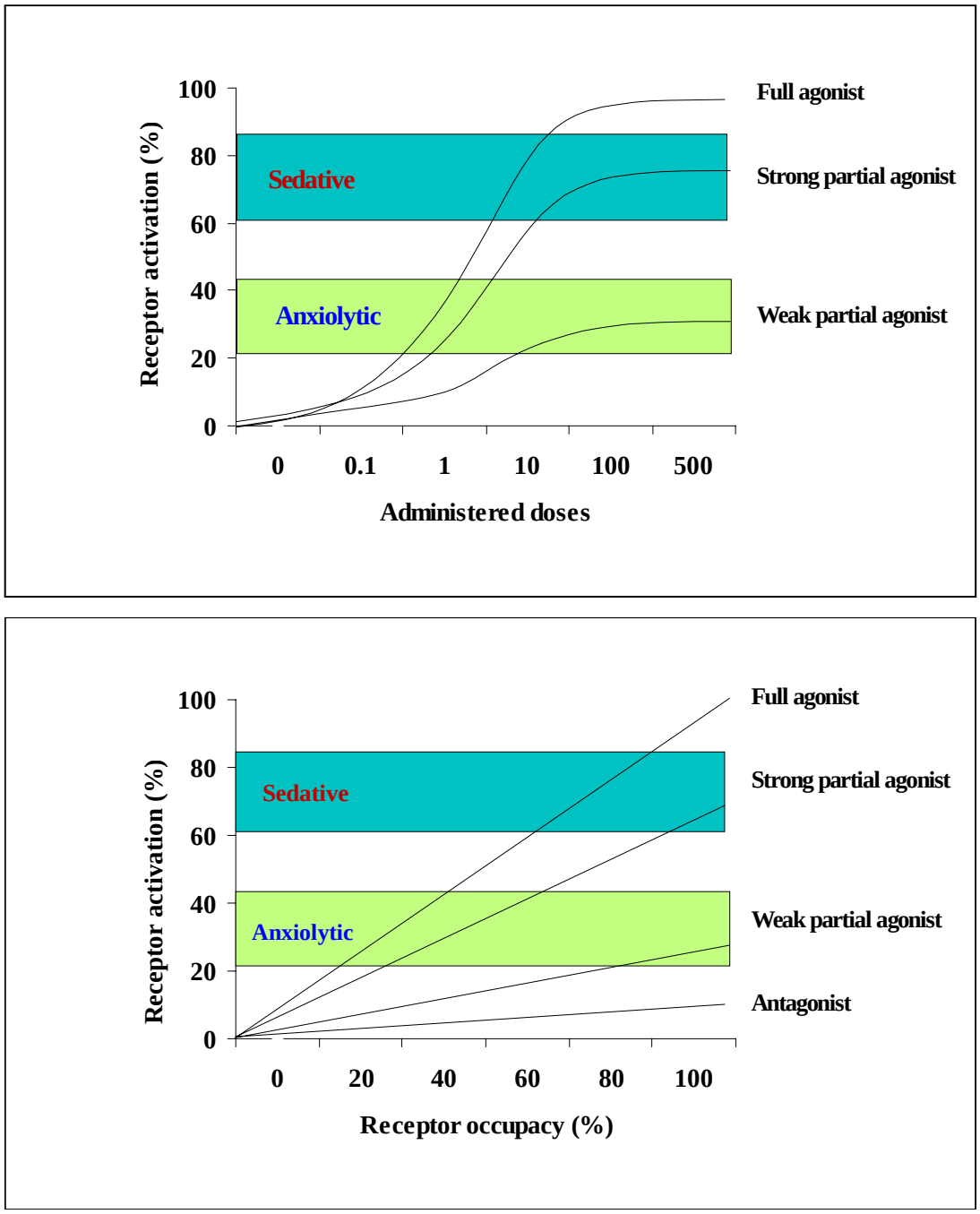


Figure 1. Theoretical receptor activation curves for ligands on BZD receptors. Because of the different intrinsic efficacy, at the some administered dose (Upper diagram) or the same amount of receptor occupied (Lower diagram), BZD receptor partial agonists activate much less portion of the receptor they occupied, thus can only induce certain behavioural effects, e.g. anxiolytic effect (Modified from Doble and Martin, 1996).

1.2.2.2 Antagonists

Ro 15-1788 (flumazenil) was the first one identified as a reference neuronal BZD receptor antagonist (Hunkeler et al., 1981). It has nanomolar affinity (K_D) for the BZD receptors, without BZD-like behavioural properties (anxiolytic, anticonvulsant, sedative and muscle relaxation etc.). It has been shown to potently antagonise the acute central effects of BZD receptor agonists, partial agonists and inverse agonists (Haefely, et al., 1983; Brogden and Goa, 1991). Clinically, flumazenil is principally used for the reversal of BZD-induced sedation/anaesthesia on patients undergoing surgical procedures and in the treatment of overdose with BZDs (Gardner, et al., 1993; Malizia and Nutt, 1995; Upton and Blackburn, 1997; Weinbroum et al., 1997).

1.2.2.3 Inverse agonists and partial inverse agonists

The β-carboline derivatives were discovered in attempts to isolate endogenous ligands for BZD receptors. The βCCE was isolated from ethanol treated human urine at low pH (Nielsen, et al., 1979; Braestrup, et al., 1980). Although subsequently βCCE was shown to be an artefact formed during the isolation procedure by an ethanol esterification of β-carboline-3-carboxylic acid, which possibly derived from tryptophan by a ring closure (Kerr and Ong, 1992). βCCE has been used as a lead structure for the development of psychotropic drugs (e.g. abecarnil) as well as a biological tools (e.g. full inverse agonist methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM)) for the research of $GABA_A$ /BZD receptor complex. The behavioural effects of these compounds are the opposite of those effects produced by classical BZDs, which include anxiogenic, proconvulsant or convulsant effects. This resulted in the concept of “inverse agonist”, in contract to the prototypical BZD agonists. The BZD receptor was the first and for many years the only receptor at which bi-directional (both agonistic and inverse agonistic) efficacy was observed (Braestrup et al., 1983, 1984). In addition to their own intrinsic pharmacological activity, inverse agonists can potently reverse the effects of BZD full agonists (Oakley and Jones, 1980; Tenen and Hirsch, 1980; Cowen, et al., 1981). As partial agonists can antagonise the action of full agonists, partial inverse agonists can also block convulsions evoked by full inverse agonists, such as DMCM (Oakley and Jones, 1982; Corda, et al., 1989). Therapeutic opportunities for inverse agonists were limited due to proconvulsant effect. However, a very weak inverse

agonist that is devoid of proconvulsant activity may be of potential value for enhancing memory function due to their behavioural arousal effect. Thus, these compounds might be useful for the treatment, for example, of Alzheimer's disease (Jensen et al., 1987; Forster et al., 1995; Upton and Blackburn, 1997).

1.2.2.4 Efficacy spectrum of BZD receptor ligands

From the above overview, it is apparent that the pharmacological profile of BZD receptor ligands cover a wide spectrum of intrinsic efficacy, stretching from full agonists (anxiolytic, anticonvulsant and sedative compounds), partial agonists, over antagonists, to partial inverse agonists and inverse agonists (anxiogenic, proconvulsant substances) (Figure 2).

Figure 2.

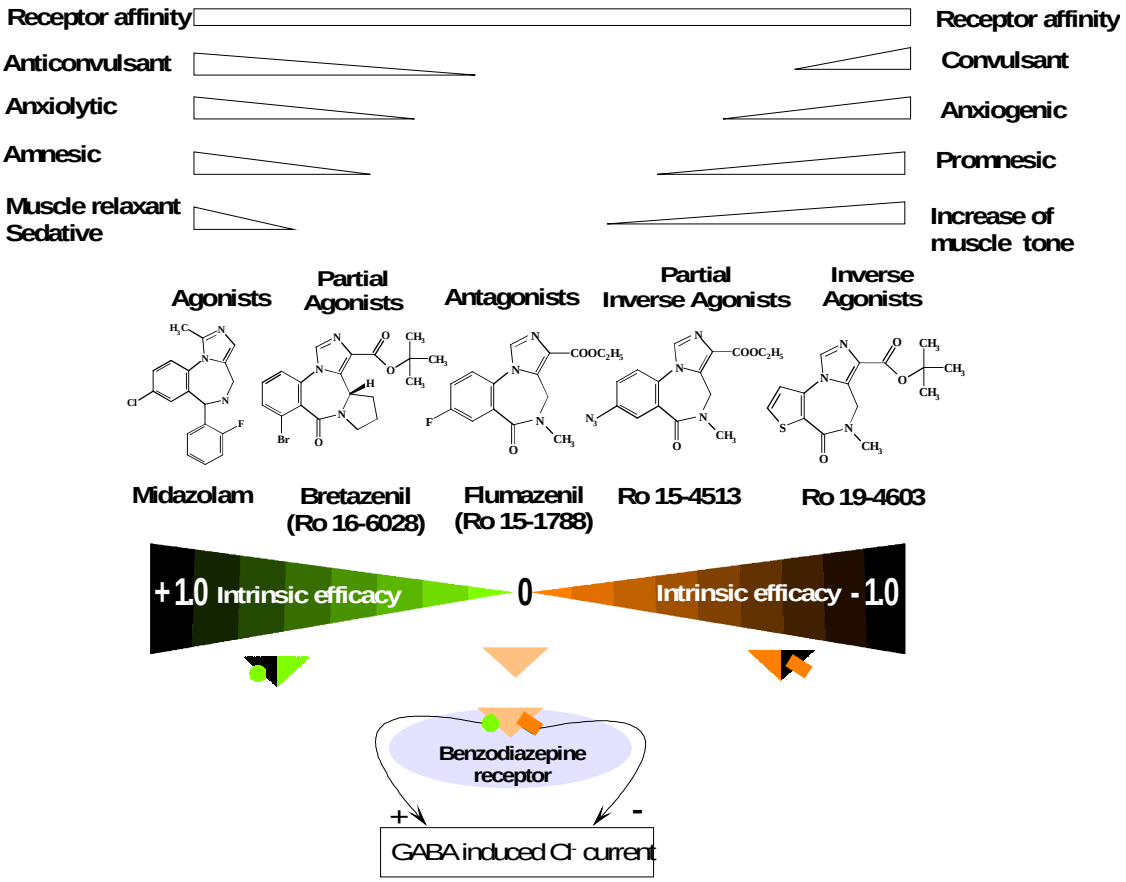


Figure 2. Spectrum of intrinsic efficacy of BZD receptor ligands and their pharmacological properties. The intrinsic efficacy is assigned + 1.0 for full agonists, 0 for competitive antagonists, and – 1.0 for full inverse agonists. The representative

ligands with diverse intrinsic efficacy from BZD class were shown (Modified from Schmiechen et al., 1993; Haefely, 1994).

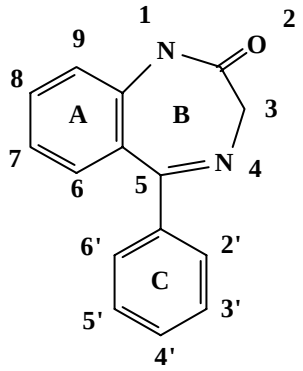
In addition to the BZD and β-carboline series, there are some other structural unrelated groups of compounds acting on the BZD receptors with differential pharmacological activities (for details, see Section 1.2.3.). Some of these compounds are of natural origin (for review, see Ai et al., 1997b).

1.2.3. BZD receptor ligands

The discovery of the high affinity binding sites of BZDs in the brain and the development of radioligand binding assays proved valuable for the later discovery of various ligands with diverse chemical structures, and for the structure-activity relationship studies at the BZD recognition site. This section will just give a brief overview of representative ligands with different intrinsic efficacy from distinct chemical groups. For comprehensive review, see Braestrup and Nielsen, 1983; Haefely et al., 1985; Gardner, 1988; Gardner et al., 1993; Gupta, 1995; Doble and Martin, 1996.

1.2.3.1. BZD analogues

Figure 3.



7-position: NO_2 > CF_3 > Br > CN > Cl > $N(CH_3)_2$ > $SOCH_3$ > SBu > SCH_3 > CH_3 > H > SO_2CH_3 > Ph > F

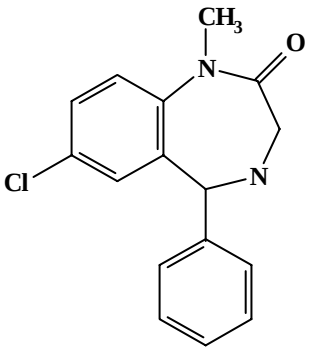
2'-position: Cl > F > Br > NO_2 > CF_3 > H > OCH_3 > CH_3

Figure 3. The chemical structure of 1,4-BZDs, and the rank orders for the individual group contributions to activity against pentetrazol induced seizure at the 7- and 2'-positons (Modified from Gupta, 1995)

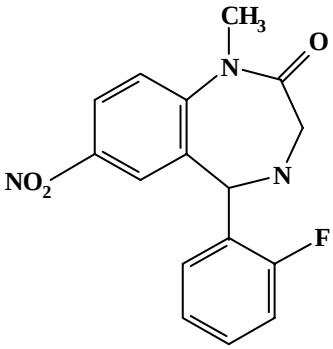
Studies of the structure-activity relationship have suggested that the presence of the seven membered ring (B) is essential for activity and that substitution is advantageous for activity only at positions 1, 3, 7 and 2'. At position 7-, the nitro group is the most effective substitution and fluorine the least; at position 2'- the halogen substitution leads to compounds with increased affinity (Figure 3, for review see Haefely et al., 1985; Gupta, 1995; Doble and Martin, 1996). The carbonyl group at position 2 is important for binding activity; its removal results in a decrease in affinity by two orders of magnitude. Saturation of the 4,5 double bond results in complete loss of *in vitro* activity (Haefely et al., 1985; Doble and Martin, 1996). Most interestingly, the chemical modification of imidazobenzodiazepines (e.g. Ro 15-4513 and Ro 15-1788) have lead to ligands binding selectively to α_5-containing subtype of the $GABA_A$Rs with inverse agonistic activity (K_I value for $\alpha_5\beta_2\gamma_2$ subtype was shown under the chemical structure of these compounds. For all other subtypes (α_1, α_2, α_3 and α_6 combined with $\beta_2\gamma_2$) the K_I values (K_I is the dissociation equilibrium constant for an inhibitor, it can be calculated from IC_{50} from the Cheng-Prusoff correction formula $K_I = IC_{50}/(1+[L]/K_D)$, in which [L] is the concentration of the testing ligand, K_D is the dissociation equilibrium constant of the radioligand, Haylett, 1996) are greater than 300 nM (Zhang, et al., 1995). Based on the basic structure, many derivatives of BZDs are produced. Some representative ones are presented (Figure 4).

Figure 4.

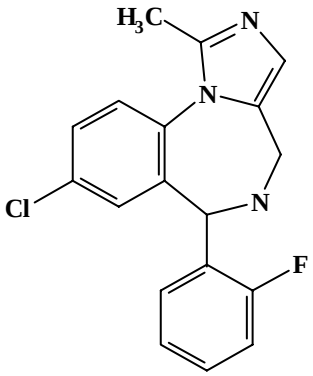
Full agonists



Diazepam

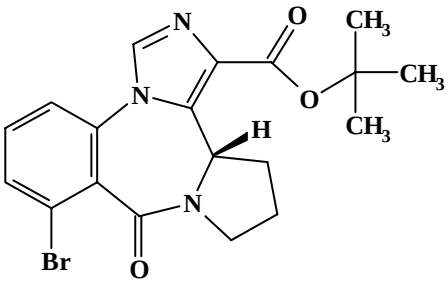


Flunitrazepam

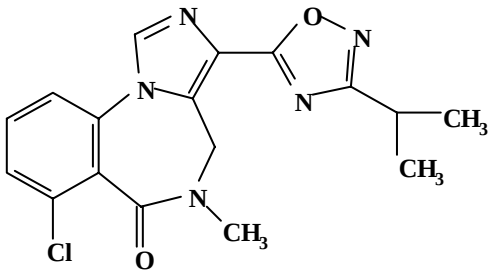


Midazolam

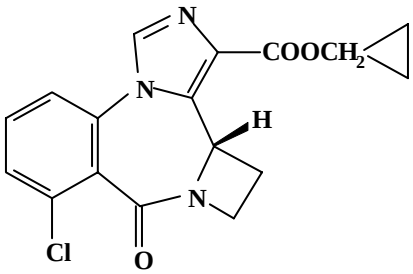
Partial agonists



Ro 16-6028 (bretazenil)

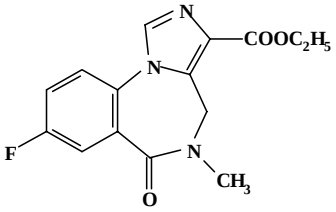


FG 8205



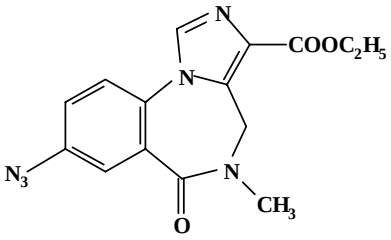
Ro 17-1812

Antagonist

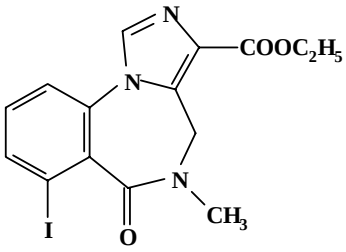


Ro 15-1788 (flumazenil)

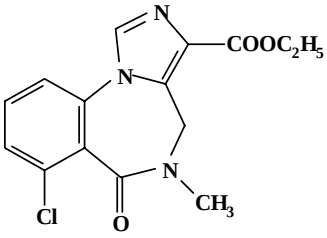
Partial inverse agonists



Ro 15-4513

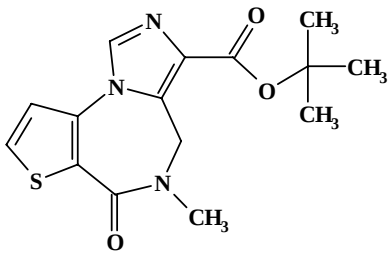


Ro 16-0154



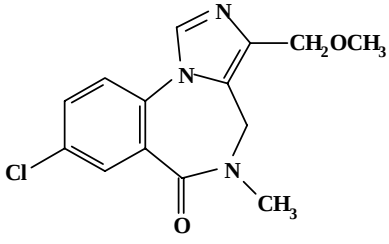
Ro 15-3505

Full inverse agonists

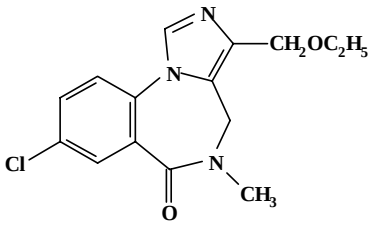


Ro 19-4603

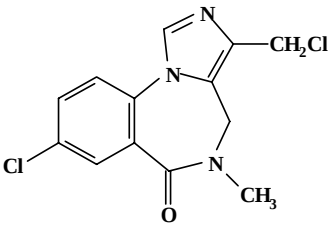
α_5-containing subtype selective compounds



K_I 40 nM



80 nM



30 nM

Figure 4. The representative BZDs with diverse intrinsic efficacy

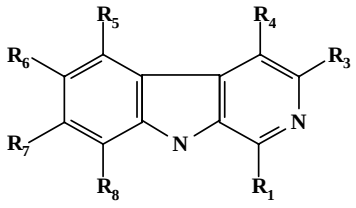
1.2.3.2. β-carbolines

Some general rules were obtained during the structural optimisation of β-carbolines, and a refined lead structure more suitable for optimisation could be described as: 1) A full aromatic plane of β-carboline structure. 2) No substitution in positions 1,8 and 9. 3) A metabolically stable ester group in position 3 (e.g. oxadiazole in ZK 95276). 4) Open to a wide range of substitution in position 5 and 6. 5) Limited freedom of substitution in position 4 and 7 (Schmiechen et al., 1993; Figure 5).

The pharmacological profile of β-carboline derivatives covers a entire activity spectrum, ranging from full agonists (anxiolytic, anticonvulsant, sedative compounds), partial agonists, antagonists, to inverse agonists (anxiogenic, proconvulsant substances) with the inverse agonist as predominant (Gardner et al., 1993; Figure 6).

Figure 5.

A



The basic structure of β-carbolines

B

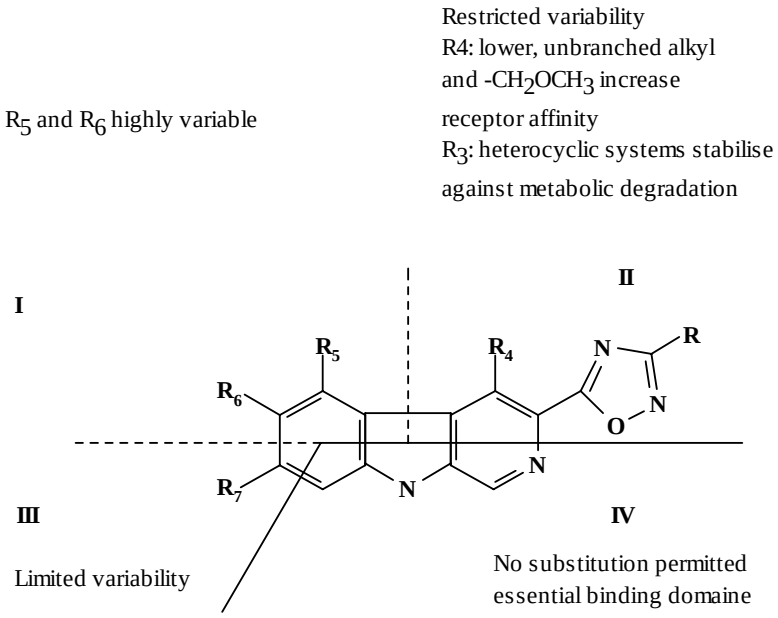
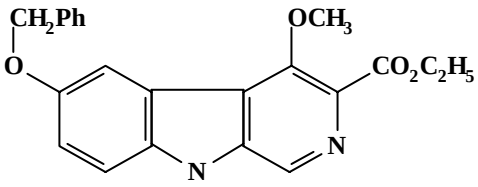


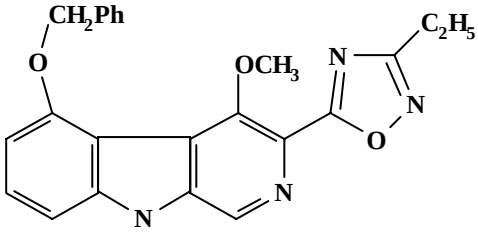
Figure 5. A: The basic structure of β-carbolines. B: The allowed and prohibited substitutions of the β-carboline structure (B, modified from Schmiechen et al., 1993)

Figure 6.

Full agonists

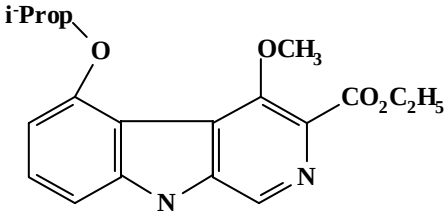


ZK 93423

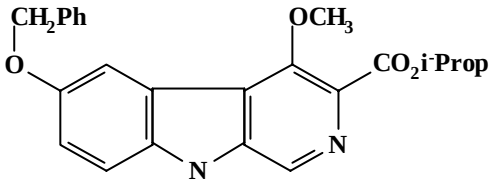


ZK95276

Partial agonists

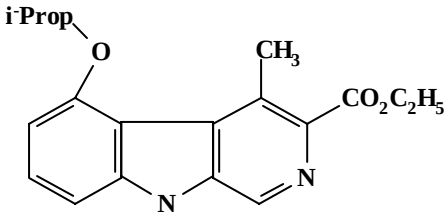


ZK 95962



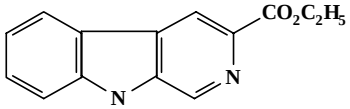
Abecarnil

Antagonist

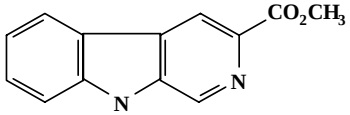


ZK 93426

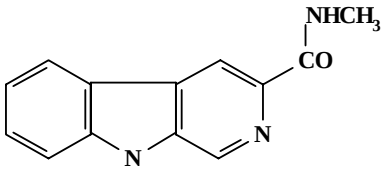
Partial inverse agonists



βCCE

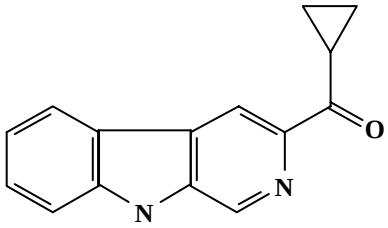


βCCM

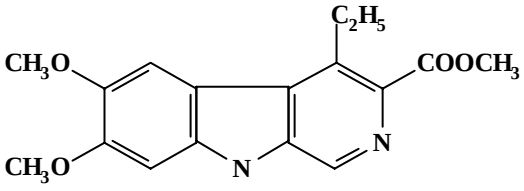


FG 7142

Full inverse agonists



RU 33973



DMCM

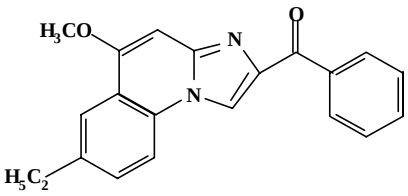
Figure 6. The representative β-carbolines with diverse intrinsic efficacy

1.2.3.3. Imidazoquinolines and imidazopyrimidines

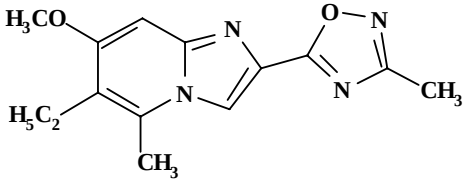
A large number of imidazoquinolines and imidazopyrimidines have been synthesised which show affinity for BZD receptors. Among these compounds, because of its partial agonistic profile, RU 32698 (divaplon) was selected as a candidate for clinical development having the optimum profile for use as a potential non-sedative anxiolytic (Gardner et al., 1993). Derivatives with antagonistic and inverse agonistic activity are also found in these series.

Figure 7.

Full agonists

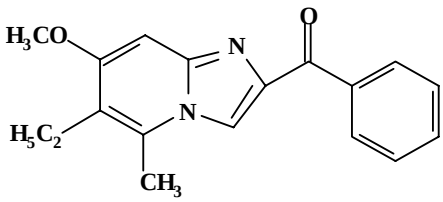


RU 31719

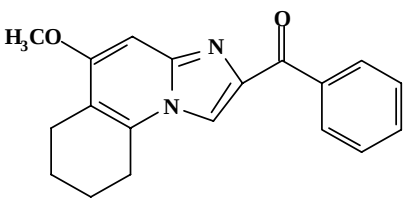


RU 33203

Partial agonists

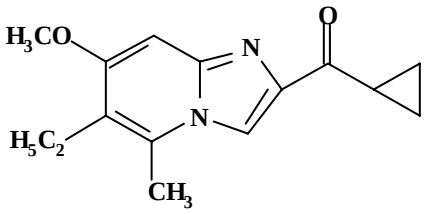


RU 32698



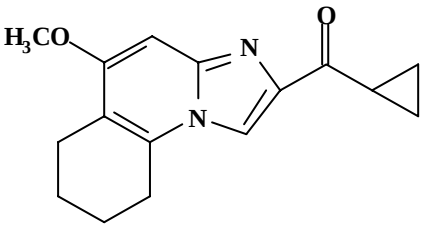
RU 32514

Antagonist

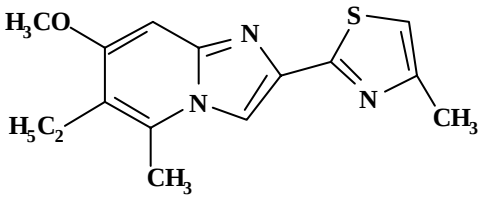


RU 33094

Partial inverse agonists

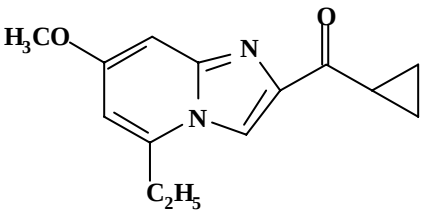


RU 33697

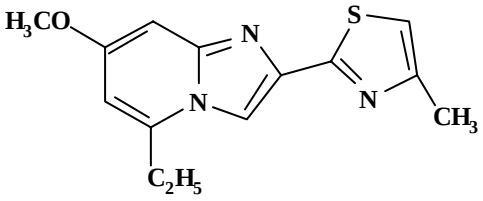


RU 33356

Full inverse agonists



RU 34000



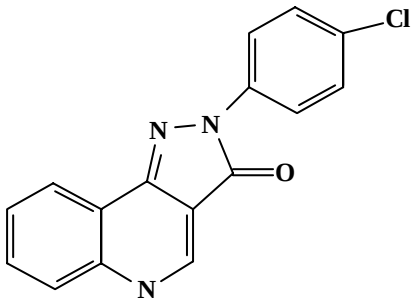
RU 34317

Figure 7. The representative Imidazoquinolines and imidazopyrimidines with diverse intrinsic efficacy

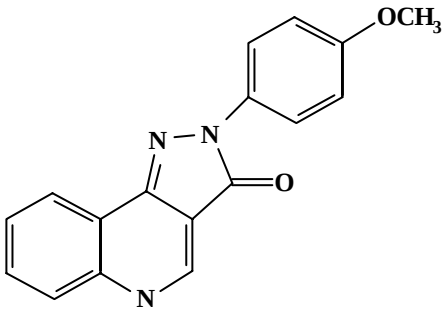
1.2.3.4. Pyrazoloquinolines

Figure 8.

Partial and weak partial agonists



CGS 9896



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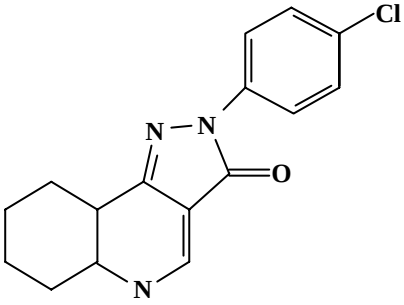

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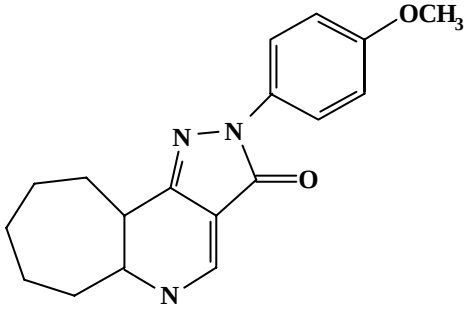
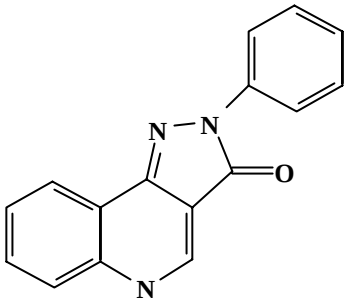


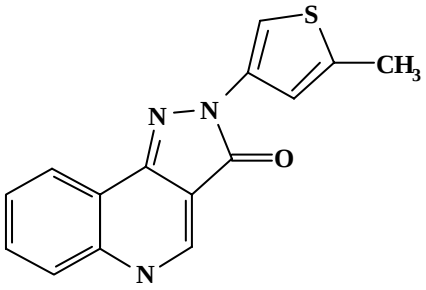
CGS 20625

Antagonist or weak inverse agonist



CGS 8216

Full inverse agonist



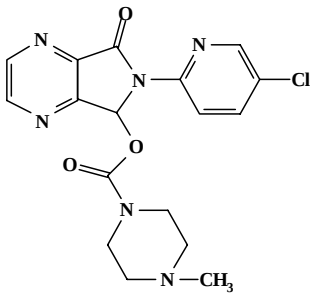
S-135

Figure 8. The representative pyrazoloquinolines and derivatives with diverse intrinsic efficacy

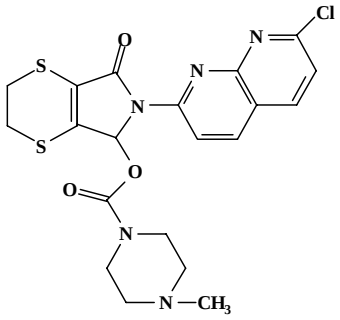
1.2.3.5. Cyclopyrolones and derivatives

Figure 9.

Full agonists

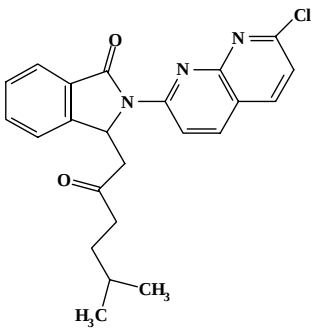


Zopiclone

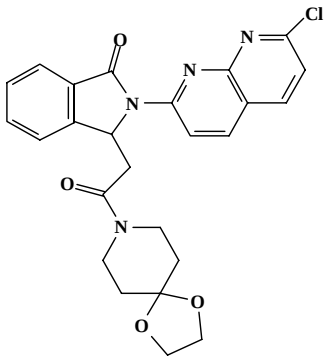


Suriclone

Partial agonists



RP 59037



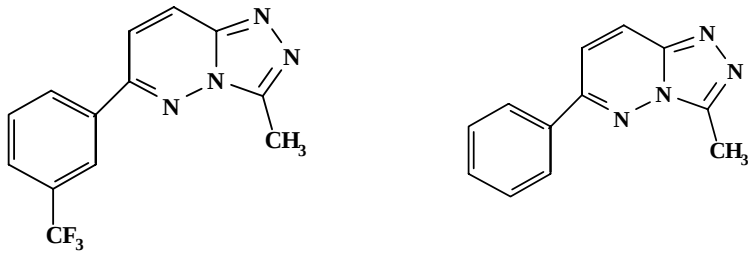
DN 2327

Figure 9. The representative cyclopyrolones and derivatives

1.2.3.6. Triazolopyridazines

CL 218872 was the first BZD receptor ligand to show an affinity for BZD1 receptors and initiated the characterisation of the BZD1 and BZD2 receptor subtypes. It can antagonised the muscle relaxant effects of diazepam in mice, suggested that CL 218872 is a partial agonist. GABA ratio values (defined on page 12) for CL 218872 is smaller than that of zolpidem (full agonist) and greater than flumazenil (antagonist). Similarly, CL 218872 showed low maximum potentiation of GABA-induced chloride currents in isolated frog sensory neurones (Haefely et al., 1985; Gardner et al., 1993; Doble and Martin, 1996). SR 95195 is a derivative of CL 218872, shows inverse agonists properties with low affinity to BZD receptors (IC_{50} 4 ~ 8 μM).

Figure 10.



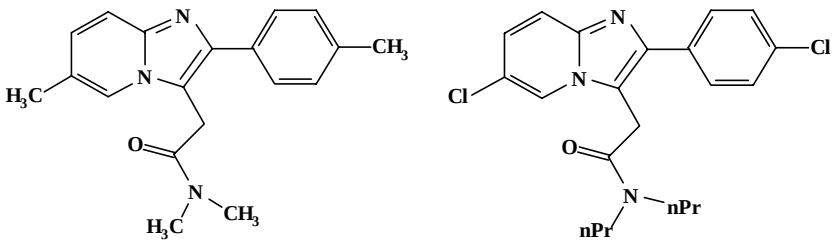
CL 218872 SR 95195

Figure 10. The representative triazolopyridazines

1.2.3.7. Imidazopyridines

The imidazopyridines, zolpidem and alpidem, exhibit high affinity for the BZD receptors. Zolpidem shows selectivity for the BZD1 receptor subtype, and shows strong agonist efficacy as measured *in vitro* (e.g. GABA ratio of 3). Alpidem, a close analogue of zolpidem, also shows selectivity to the BZD1 receptor subtype in the brain but also has high affinity for peripheral BZD receptors. Behavioural profiles indicate that alpidem may be a partial agonist.

Figure 11.



Zolpidem Alpidem

Figure 11. The representative imidazopyridines

1.3. Picrotoxin and TBPS binding site

Picrotoxin is an equimolar mixture of picrotoxinin and picrotin isolated from *Anamirta cocculus* and related poisonous plants of the moonseed family (Johnston, 1996a). The cage convulsants *t*-butylbicyclophosphorothionate (TBPS) shares, with picrotoxin, the same unique binding site on $GABA_A$Rs, where they act to inhibit the amplitude of neuronal GABA-activated Cl^- currents (Bowery et al., 1976; Squires et al., 1983). Picrotoxin acts noncompetitively to decrease GABA currents (Newland and Cull-Candy, 1992), and is thought to either act at the channel itself or at the site near the Cl^- channel (Barker et al., 1983; Akaike and Oomura, 1984; Akaike et al., 1985; Bormann, 1988). These binding sites were originally identified by [^{3}H]α-dihydro-picrotoxinin (DHP) or [^{35}S]TBPS (Olsen, 1982; Squires et al., 1983). Convulsant compounds that bind to the TBPS binding sites reduce the Cl^- conductance directly by hindering the entry of Cl^- across the ion channel (Sieghart, 1992).

Picrotoxin and cage convulsants do not or only partially and allosterically inhibit GABA receptor binding and do not displace BZD from their high-affinity binding sites, but allosterically modulated BZD receptor binding (Olsen, 1982; Karobath et al., 1981; Skerritt and Johnston, 1983; Johnston, 1996a).

GABA, at micromolar concentrations (e.g. 1μM GABA), allosterically inhibited [^{35}S]-TBPS binding, which could be reversed by $GABA_A$R blockers (Maksay and Simonyi, 1986; Squires and Saederup, 1987; Squires and Saederup, 1993; Squires and Saederup, 1998). In addition to GABA, most other substances known to interact with $GABA_A$Rs allosterically modulate these convulsant binding sites labelled by [^{35}S]TBPS. Agents that mimic or facilitate the effects of GABA on the opening of Cl^- channels, such as barbiturates, etazolate, etomidate and steroids, potently inhibit [^{35}S]TBPS binding by reducing its binding affinity (as seen by the increase of the IC_{50} value) (Gee, 1988, Im and Blakeman, 1991). The binding affinity of [^{35}S]TBPS is enhanced by drugs that reduce GABA-ergic transmission (Squires et al., 1983; Supavilai and Karobath, 1984; Gee et al., 1986; Concas et al., 1988). BZD receptor agonists enhance and inverse BZD receptor agonists reduce [^{35}S]TBPS binding affinity at non-equilibrium conditions, whereas BZD receptor antagonists are without effect in this binding assay. These effects are called “TBPS shift”, which is a useful method to predict the efficacy of BZD receptor ligands. Thus, pure BZD agonists generally give 40% enhancement, whereas

inverse agonists give 40% inhibition of [^{35}S]TBPS binding (Supavilai and Karobath, 1983; Braestrup et al., 1984; Braestrup and Nielsen, 1986). BZDs agonists can inhibit the binding of [^{35}S]TBPS only in the presence of micromolar quantities of GABA (Gee, 1988), and stimulate [^{35}S]TBPS binding when GABA was antagonised by bicuculline (Im and Blakeman, 1991). The latter finding coincides with the observation that BZDs affect the membrane permeability of Cl^- ions only in the presence of GABA.

1.4. Barbiturate binding site

Electrophysiological studies indicate that the sedative hypnotic barbiturates, such as pentobarbital and secobarbital, enhance the effects of GABA by increasing the average duration of channel opening (Study and Barker, 1981; Macdonald et al., 1989; Macdonald and Twyman, 1992; Macdonald and Olsen, 1994). In addition, at high anaesthetic concentrations (> 50 μM), barbiturates directly increase the time of channel remains in the opening state, even in the absence of GABA (Bormann, 1988). These distinct effects of barbiturates indicate the existence of at least two sites of interaction of barbiturates with the $GABA_AR$ (Sieghart, 1995). Recombinant $GABA_AR$ studies indicate different domains within the β-subunits are required for forming a Cl^- channel that can be activated by GABA and pentobarbitone, although, unlike BZDs, the presence of a γ_2 subunit is not necessary (Schofield et al., 1987; Amin and Weiss, 1993; Horne et al., 1993).

In the binding studies, there are no barbiturates can be radiolabeled with high specific radioactivity. The affinity for the radiolabeled barbiturates is relatively low. No true antagonists for barbiturate binding site are available (Ito et al., 1996). However, the interaction of barbiturates with other binding sites on $GABA_ARs$ showed that barbiturates enhanced the binding affinity of [^{3}H]GABA, [^{3}H]muscimol and [^{3}H]flunitrazepam, and inhibit [^{35}S]TBPS binding, in a manner that correlated with their rank order of potency as anaesthetics and hypnotics (Olsen, 1982; Squires et al., 1983). The investigations of the interaction of barbiturates and BZDs showed that barbiturates appear to stimulate the binding of [^{3}H]BZDs by an effect on their K_D (Leeb-Lundberg et al., 1980). Binding of the inverse agonists (e.g. β-carbolines) is, however, reduced by barbiturates, and this resulted in the proposal that the extent of this “barbiturate shift”

may be a measure of efficacy for ligands acting at the BZD site (Wong et al., 1984; Honore et al., 1984).

1.5. Neurosteroids binding site

Electrophysiological studies showed that alphaxolone selectively enhanced the inhibitory effects of GABA via $GABA_A$Rs, which provided a mechanism of the anaesthetic action of this compound (Harrison and Simmonds, 1984). A number of structurally related endogenous steroids, such as progesterone metabolites 5α-pregnan-3α-ol-20-one and 5β-pregnan-3α-ol-20-one and deoxycorticosterone metabolite 5α-pregnan-3α, 21-diol-20-one have been shown to mimic this effect and were even more potent than alphaxolone (Callachan, et al., 1987; Peters et al., 1988; Lambert et al., 1995; Olsen and Sapp 1995). At low concentrations (30 to 300 nM) these compounds enhance GABA-stimulated chloride conductance (Majewska, 1992; Kokate et al., 1994). At high concentrations (> 1μM), these steroids, like barbiturates, produce a direct opening of the $GABA_A$R associated Cl^- channel in the absence of GABA that could be inhibited by the $GABA_A$R antagonist bicuculline (Callachan et al., 1987; Majewska, 1992). The activation of $GABA_A$Rs by steroids increased both the frequency (BZD-like effect) and duration (barbiturate-like effect) of Cl^- channel opening (Peters et al., 1988; Twyman and Macdonald 1992).

In the binding studies, neurosteroids enhanced the binding of the [^{3}H]muscimol ($GABA_A$R agonist) both by an increase in binding affinity and an increase in the number of binding sites (Harrison et al., 1987; Lopez-Colome et al., 1990). Neurosteroids can also enhance the affinity of the BZD receptor agonist [^{3}H]flunitrazepam in a picrotoxin-sensitive way and allosterically inhibit binding of [^{35}S]TBPS to $GABA_A$R (Gee, 1988; Schumacher and McEwen, 1989; Majewska, 1992). Although many of the effects of neurosteroids are similar to those of barbiturates on $GABA_A$Rs, the data from pharmacological analyses utilising steroid-barbiturate and steroid-BZD drug combinations in electrophysiological and radioligand binding experiments indicated that they appear to interact with different sites on $GABA_A$Rs (Gee, 1988; Lambert et al., 1995, 1996). The behavioural effects of neurosteroids acting on the $GABA_A$Rs include anxiolytic, anticonvulsant, sedative-hypnotic and anaesthetic activities both in animals and in humans (Majewska, 1992; Gee et al., 1995; Lambert et

al., 1995). The doses of neurosteroids required for their anticonvulsant action and the sedative effect are not identical but close to each other. Thus, the development of steroid with a more subtle influence on the $GABA_AR$ (e.g. pregnanediols) may prove to be a useful strategy to overcome the sedative properties of the steroids (Lambert et al. 1995).

1.6. Ethanol binding site

Ethanol exhibits a large variety of different effects on the nervous system. It not only influences membrane fluidity, neuronal electric activity and synaptic transmission, but also exhibit specific actions on both voltage-gated (Ca^{2+} and K^+ channels) and transmitter-gated ion channels (e.g. glutamate receptor, $GABA_AR$, 5-hydroxytryptamine (5-HT) receptor, nicotinic acetylcholine (nAch) receptor and glycine receptor ion channels) (Deitrich et al., 1989; Morrow, 1995; Crews et al., 1996; Miczek et al., 1997). Many pharmacological effects of ethanol are similar to those of BZDs and barbiturates, including anticonvulsant, anxiolytic, ataxic and sedative-hypnotic effects, suggest the facts that $GABA_AR$ may be the target mainly responsible for many of the actions of ethanol (Crews et al., 1996). At pharmacologically relevant concentrations, ethanol has been shown to stimulate or potentiate $GABA_AR$-mediated $^{36}Cl^-$ uptake in subcellular brain preparations from rat cerebral cortex and cultured embryonic neurons, (Suzdak et al 1986, 1987; Mehta and Ticku, 1988). Electrophysiological studies have confirmed that the enhancing effect of ethanol on the $GABA_AR$-mediated Cl^- conductance depends on the molecular composition of $GABA_ARs$ ($GABA_AR$ subtypes) appeared in specific brain regions or cell populations (Givens and Breese, 1990a, b; Aguayo, 1990; Reynolds and Prasad, 1991; Criswell et al., 1993; 1995). The enhancement of $GABA_AR$ responses induced by ethanol at relatively low concentrations (20 mM), but not at higher anaesthetic concentrations (50 ~ 400 mM), is dependent on the presence of the alternatively spliced variant of the γ_2 subunit (γ_{2L}). This subunit contains an extra 8 amino acids (as compared to the short form γ_{2S}) in the region between the third and fourth transmembrane domain (TM3 and TM4) (Wafford et al., 1991; Wafford and Whiting, 1992; Mihic et al., 1994a). Cerebellar granule cells contain recognition sites for the BZD inverse agonist Ro 15-4513, which can antagonise the effects of ethanol on some behavioural effects of ethanol in animals (such as the exploration and anxiety) as the effect of ethanol on GABA-mediated responses (Lister and Nutt, 1988; Ticku and

Kulkarni, 1988; Lüddens et al., 1990). Receptors containing the α_6 subunits, which confer the sensitivity to the BZD inverse agonist Ro 15-4513, may be responsible for the action of ethanol in the cerebellar granule cells (Lüddens et al., 1990). In thalamus, Ro 15-4513 binds with high affinity to the $GABA_A$Rs consisting of α_4 subunits, suggesting the α_4 containing $GABA_A$Rs could be another target for ethanol (Wisden et al., 1991). Chronic ethanol treatment differentially alters the expression of $GABA_A$R subunit mRNAs in the cerebral cortex and cerebellum. The levels of $GABA_A$R α_1 subunit mRNAs are reduced whereas α_4 subunit mRNAs are increased by approximately equal amounts in cerebral cortex. In the cerebellum, decreases in $GABA_A$R α_1 subunit mRNAs and increase in α_6 subunit mRNA levels are found. These changes in mRNA levels suggest alterations in the expression of the corresponding proteins that could account for the alterations in receptor function and binding that have been observed (Morrow et al., 1990, 1992; Mhatre and Ticku, 1993; Ticku and Mhatre, 1994; Devaud et al., 1995).

1.7. $GABA_A$Rs and neurological disease

1.7.1. Epilepsy

The deficit in GABA-ergic inhibitory synaptic transmission might contribute to the neuronal epileptic activity and the spread of focal seizure activity (Gale, 1992; Olsen et al., 1992). Compounds that potentiate the synthesis, synaptic release, postsynaptic action of GABA show anticonvulsant activity. Actually, many of the antiepileptic drugs exert their action by enhancing the brain GABA activity (e.g. BZDs, barbiturates, vigabatrin etc.) (Emilien and Maloteaux, 1998). The modulatory mechanism of BZDs and barbiturates on the $GABA_A$/BZD receptors has been discussed (see Section 1.2. and 1.4.). Vigabatrin, a synthetic derivative of GABA, increases brain GABA levels by irreversibly inhibiting GABA-transaminase (the primary presynaptic degradative enzyme of GABA), thus enhances GABA-ergic transmission decreases seizure activity (Macdonald and Kelly, 1995; Emilien and Maloteaux, 1998).

The alteration of $GABA_A$Rs has been observed in animal models as well as in human with epilepsy. In kindling-induced seizure in rats, a regionally specific alteration of $GABA_A$/BZD receptor binding has been observed (Titulaer et al., 1994; 1995a, b, c).

Detected by [^{3}H]muscimol (Titulaer et al., 1994), [^{3}H]flunitrazepam (Titulaer et al., 1995a), [^{35}S]TBPS binding (Titulaer et al., 1995b) and muscimol-stimulated ^{36}Cl-uptake experiments (Titulaer et al., 1995c), a significant decrease of receptor binding was found in hippocampal CA1 area, while a profound increase was observed in fascia dentata area in the kindled rat hippocampus, which means an increased synaptic inhibition in the dentate and a loss of GABA-ergic inhibition in the CA1. The alteration of subunit mRNA of the $GABA_AR$ was also regional specific. For example, an increase of α_5 subunit mRNA was found in the dentate gyrus, while significant reduced levels of α_2 and α_5 subunit mRNA were seen in the hippocampal CA1, CA2 and CA3 regions in rat pilocarpine-induced epilepsy model (Rice et al., 1996). In the rat model of absence seizures induced by γ-hydroxybutyric acid, a marked up-regulation in α_1 mRNA and a corresponding down-regulation in α_4 mRNA in thalamic relay nuclei, but not in hippocampus, was observed 2-4 hours after the onset of seizure (Banerjee et al., 1998). In the kainic acid-induced seizure model, the impairment of GABA-mediated neurotransmission was found mainly due to the lasting loss of $GABA_AR$ containing cells, and this loss of $GABA_ARs$ in the hippocampus may partially be compensated by increased expression of some $GABA_AR$ subunits (e.g. α_1, α_2, α_4, α_5, β_1, β_2, γ_2 and δ) within the molecular layer of the dentate gyrus and in pyramidal cells (Tsunashima et al., 1997; Schwarzer et al., 1997). In human epilepsy, the changes of GABA-ergic inhibition are controversial, the functional GABA-ergic inhibition can be reduced, normal or slightly enhanced (Mody, 1998).

1.7.2. Angelman Syndrome

So far, the gene of β_3 subunit seems to the only one among genes encoding the $GABA_AR$ subunits, associated with a defined genetic disease i.e. the Angelman syndrome (AS). AS is characterised by severe mental retardation, epilepsy, hyperactivity, sleep disturbances, motor incoordination and craniofacial abnormalities (Williams et al., 1995). Approximately 70% of AS cases result from a *de novo* deletion of 4 Mb of DNA in maternal chromosome 15q11-q13 (Knoll et al., 1989), which co-localises the genes coding α_5, β_3 and γ_3 subunits of the $GABA_ARs$. A mutant mouse line which lack all three genes (α_5, β_3 and γ_3) has a lethal cleft palate defect. Those mice, who do survive show a variable neurological phenotype (e.g. tremor, jerky gait), while

the mice lacking the α_5 and/or γ_3 appear phenotypically normal. This indicates that deletion of the β_3 gene alone is responsible for the defects (Nakatsu et al., 1993; Culiat et al., 1994). The β_3 gene knock-out mice do have a epilepsy phenotype and show marked similarities (e.g. elctroencephalographic abnormalities, seizures, learning and memory deficits, poor motor skills on a repetitive task, hyperactivity and disturbed rest-activity cycle) to the clinical feature of AS, indicating that impaired expression of the β3 gene in humans probably contributes to the overall phenotype of AS (DeLorey et al., 1998).

1.7.3. Huntington's disease

Huntington's disease (HD) is an inherited neurodegenerative disorder characterised by progressive involuntary choreiform movements, psychopathological changes, and dementia**.** The pathogenesis is unknown, therefore there is currently no known cure for this disease. One neuropathological characteristic of HD is a profound loss of striatal projection neurons, leading to atrophy of the caudate nucleus and putamen (Quinn and Schrag, 1998; Walling et al., 1998). In HD chorea, a decrease in GABA and BZD receptor binding was found in caudate nucleus and putamen, an increase of binding was seen in lateral and medial pallidum (Möhler and Okada, 1978; Penney and Young, 1982). For both GABA and BZD binding sites, the decreased binding in striatum, and the increased binding in globus pallidus, superficial frontal cortex and midfrontal cortex was found due to the changes in the number of receptors rather than in their affinity (Whitehouse et al., 1985; Trifiletti et al., 1987). [^{35}S]TBPS binding to the postmortem brain of HD patient was unchanged, but the enhancement of [^{35}S]TBPS binding by alpidem, flunitrazepam and clozepam was reduced (Lloyd et al., 1991). The loss of $GABA_A$/BZD receptors was also shown by positron emission tomographic, postmortem autoradiographic and immunohistochemical studies (Holthoff et al., 1993; Faull et al., 1993). On animal model of HD (quinolinic acid lesions in striatum), a marked loss of GABA immunoreactivity and a increase in the density of $GABA_AR$ in the substantia nigra pars reticulata was observed (Nicholson et al., 1995). A reduced anxiety was seen in HD transgenic mice as tested in the elevated plus-maze tests (File et al., 1998).

1.7.4. Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disorder, in which neurons of various neuronal systems degenerate, a deficiency of dopamine in the nigrostriatal system is one of the most notable characteristics (Birtwistle and Baldwin, 1998; Gelb et al., 1999). In PD, GABA receptor binding, as well as glutamic acid decarboxylase was decreased in the substantia nigra and caudate nucleus relative to normal controls (Rinne et al., 1984; Nishino et al., 1988). $GABA_AR$ densities were significantly decreased in both the cortical and subcortical brain regions, which possibly reflects the degeneration of neurons on which the receptor is localised (Nishino et al., 1988). Specific binding of [^{3}H]flunitrazepam to the BZD receptors is reduced (in the lateral segment of the globus pallidus, Griffiths et al., 1990), unchanged (in caudate and putamen, Griffiths et al., 1990) or increased (in caudate nucleus, Maloteaux et al., 1988) in PD. GABA can still enhance the [^{3}H]flunitrazepam binding, indicated that the functional link between GABA and BZD receptor remained intact in PD (Maloteaux et al., 1988). In autoradiographic study, a loss of BZD receptors in the mid and caudal portions of the putamen was noted (Griffiths et al., 1994). Besides the neuronal BZD, the Platelet peripheral BZD receptors are also found decreased in PD, which implicated the abnormalities in mitochondrial function (Bonuccelli et al., 1991). Iomazenil is a high affinity partial inverse agonist of the central BZD receptors. Assay of cellular up-take of ^{123}I labelled ([^{123}I]iomazenil) iomazenil at the moment evaluated as a useful diagnostic tool for PD (Kawabata et al., 1996; 1997). The correlation between motor disability and decrease in [^{123}I]iomazenil uptake in the cerebral cortex suggesting that BZD receptors in the cerebral cortex of patients with PD may be impaired in proportion to the severity of the disease, thus [^{123}I]iomazenil will be useful for evaluating the pathophysiological condition in PD (Kawabata et al., 1996; 1997).

1.7.5. Alzheimer's disease

Alzheimer's disease (AD) is a degenerative mental disease characterised by progressive brain deterioration and dementia. It is the most common cause of dementia in the elder people, affecting approximately 5 to 10% of those over 65 years of age and possibly 50% of those over 85 years old persons. It is characterised pathologically by senile, neurofibrillary, and neuropil threads from the examination of the brain tissue after the

patient's death, with limited information about its aetiology and pathogenesis (Small, 1995; Sloane, 1998; Small, 1998). In addition to the possible involvement of genetic, immune, oxidative and inflammatory mechanisms, the cholinergic and other neurotransmitter changes are probably also involved in the process of AD (Small, 1998). For the $GABA_A$/BZD receptors, a general loss of the receptors in different brain area is evidenced. Thus, receptor density of both $GABA_A$ and $GABA_B$ receptors in layers II, III and V of frontal cortex (Chu et al., 1987a), and hippocampus (Chu et al., 1987b) in AD patients are significantly different with the age matched controls, while the binding affinity for GABA to these two receptors is remained. Similarly, the number of BZD receptors in frontal and temporal cortex and hippocampus was also found significantly reduced in AD (Shimohama et al., 1988; Jansen et al., 1990). The specific [^{35}S]TBPS binding was significantly reduced in frontal cortex membranes from AD patients as compared to normal control, but alpidem, flunitrazepam and clonazepam can still enhance the [^{35}S]TBPS binding in AD implicating the intact linkage between the BZD sites and the Cl^- channels (Lloyd et al., 1991). Immunohistochemical studies showed that the $GABA_AR$ subunits ($\beta_{2/3}$) in the hippocampus are well preserved even until the terminal stages of AD (Mizukami et al., 1997), while a marked decrease in neuropil immunolabeling (of α_1 subunit) within the CA2, CA1 subregions and prosubiculum are found in severe AD (Mizukami et al., 1998a). Concerning the mRNAs levels, β_2 mRNA is well-preserved, while β_3 mRNA was decreased in hippocampus in the later stage of AD (Mizukami et al., 1998b). The cholinergic deficits plays a key role in AD dementing process, an increased cholinergic transmission resulting from available pharmacotherapy is the only approach shown to be efficacious for the treatment of AD (Small, 1998). The mnemonic function (memory enhancing function) of cholinergic neurons may be enhanced by treatments that reduce GABA-ergic inhibition (Smith et al., 1994). Two partial inverse agonists, FG 7142 (see Figure 6 for chemical structure, Smith et al., 1994) and S-8510 (2-(3-isoxazolyl)-3,6,7,9-terahydroimidazo[4,5-d]pyrano+++[4,3-b] pyridine monophosphate monodydrate) (Kawasaki et al., 1996) have been shown to enhance the mnemonic function with little risk for inducing anxiety or convulsion in rats, and therefore can be used for development of therapeutic drug for AD.

1.7.6. Schizophrenia

Apart from hypotheses of genetic, neuroimmunovirological, retroviral infection, birth and pregnancy complication related aetiology, the dopamine hypotheses, proposing that the schizophrenia (SPA) results from the excessive activity at dopamine synapses in the brain, and that neuroleptic drugs may control the symptoms of SPA by antagonising dopamine at synaptic receptors, is one of the most important hypotheses for understanding the mechanism of SPA (Carpenter and Buchanan, 1995; Willner, 1997). Contrary to the theory that dopamine receptor system is thought directly involved in the pathogenesis of schizophrenia (SPA), the involvement of GABAA/BZD receptors may have a rather indirect impact. Nevertheless, some general dysfunctions of $GABA_A$/BZD receptors have been discovered. A significant increase of *in vitro* GABA and/or BZD receptor binding activity has been observed on neurons in, e.g. the prefrontal cortex (Hanada et al., 1987; Benes et al., 1996a), hippocampus (Benes et al., 1996b) and other brain regions from the post-mortem tissue of SPA (Kiuchi et al., 1989). This hyperactivity of $GABA_A$Rs may be involved in the pathogenesis and some symptoms of SPA. Both *in vitro* (Pandey et al., 1997) and *in vivo* studies (Ball et al., 1998) showed, a reduced $GABA_A$/BZD receptor binding in SPA patients on medication with neuroleptics but not the drug free ones. However, the $GABA_A$R subunit genes (α_1, α_2, α_4, α_5, α_6, β_1 and β_3), and mRNAs (α_1, α_2, α_5, β_1, β_2 and γ_2) in the prefrontal cortex of SPA were not significantly changed (Byerley et al., 1995; Akbarian et al., 1995). Contrary to that, a reduction in relative abundance of γ_{2S} mRNAs and the associated relative increase in γ_{2L} mRNAs of the $GABA_A$R in prefrontal cortex of SPA were found (Huntsman et al., 1998). Both GABA site and BZD site agonists may produce SPA-like symptoms, while the BZD site partial inverse agonists or partial agonist (e.g. bretazenil) being able to reduce SPA symptoms (Squires and Saederup, 1991; Delini-Stula and Berdah-Tordjman, 1996).

Section 2. Molecular biology of $GABA_A$Rs

2.1. Multiplicity of $GABA_A$R subunits revealed by molecular cloning

The first two cDNA sequences were identified in 1987, referred to as α_1 and β_1, encoding synthetic polypeptides containing 456 and 474 amino acids, respectively (Schofield et al., 1987). By screening brain cDNA libraries with cDNA probes and degenerate oligonucleotide probes constructed from sequences conserved between these subunit of cDNAs, a family of $GABA_A$R subunit cDNAs was subsequently identified (for review, see Olsen and Tobin, 1990; Vicini, 1991; Wisden and Seeburg, 1992; Macdonald and Olsen, 1994; Whiting et al., 1995; Dunn et al, 1994; Doble and Martin 1996). To date, a total of 6α, 4β, 4γ, 1δ and 1ε subunits of the $GABA_A$R have been cloned and sequenced from mammalian brain (Table 3). Some of them (α_1, β_2, β_3, γ_1 and γ_2) were also found in chicken, including some additional subtype of the β subunits (β_4) and γ subunits (γ_4). Another new class subunit of $GABA_A$R (π subunit) has so far only been cloned from the reproductive tissues of human and rat (Hedblom and Kirkness, 1997). The amino acid sequence identity within a particular subunit class is about 70 to 80% while between classes it is around 30 to 40% (Figure 12). The homology of amino acid sequences (20 ~ 30%) of $GABA_A$R subunits with the nAch receptor, the glycine receptor and the 5-HT_3 receptor subunits suggest that these receptors are members of a superfamily of ligand-gated ion channels (Schofield et al., 1987; Whiting et al., 1995; Barnard et al., 1998; Hevers and Lüddens, 1998; Vafa and Schofield, 1998).

The electron-microscopic image analysis performed on $GABA_A$Rs purified from porcine brain (Nayeem et al., 1994), as well as molecular study performed on recombinant $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_2$ $GABA_A$Rs expressed in Sf9 cells (Knight et al., 1998) suggest that the $GABA_A$R channels were formed from five subunits.

Figure 12.

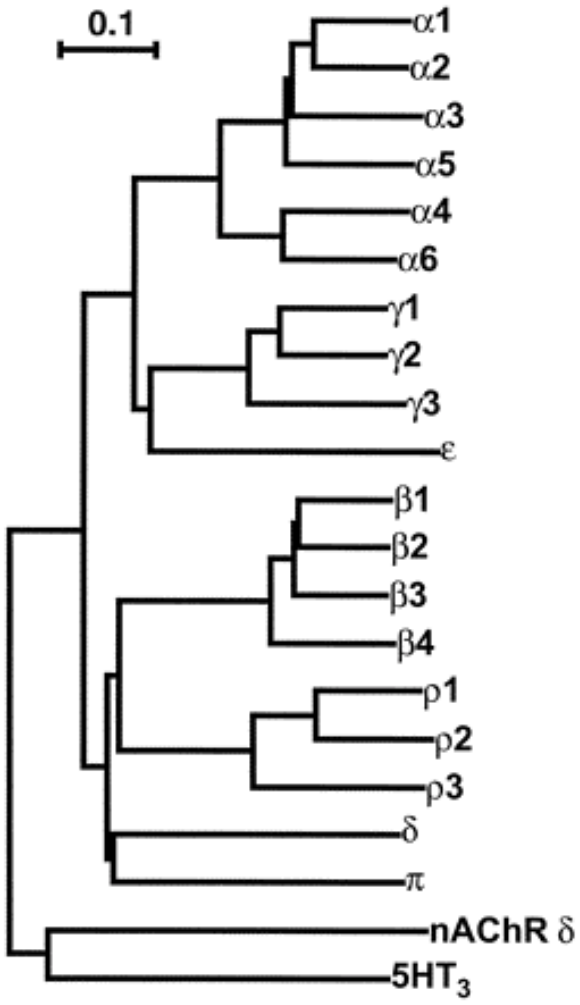


Figure 12. Dendrogram showing the relevance of amino acid sequences between $GABA_AR$ subunits. All sequences are from the rat, except of β_4 (chicken) and ε (human). The total lengths of the horizontal branch connecting any two sequences represents the divergence in their amino acid sequence. The scale bar corresponding to 10% sequence divergence (Adopted from Barnard et al., 1998).

Table 3. Cloned $GABA_AR$ subunits

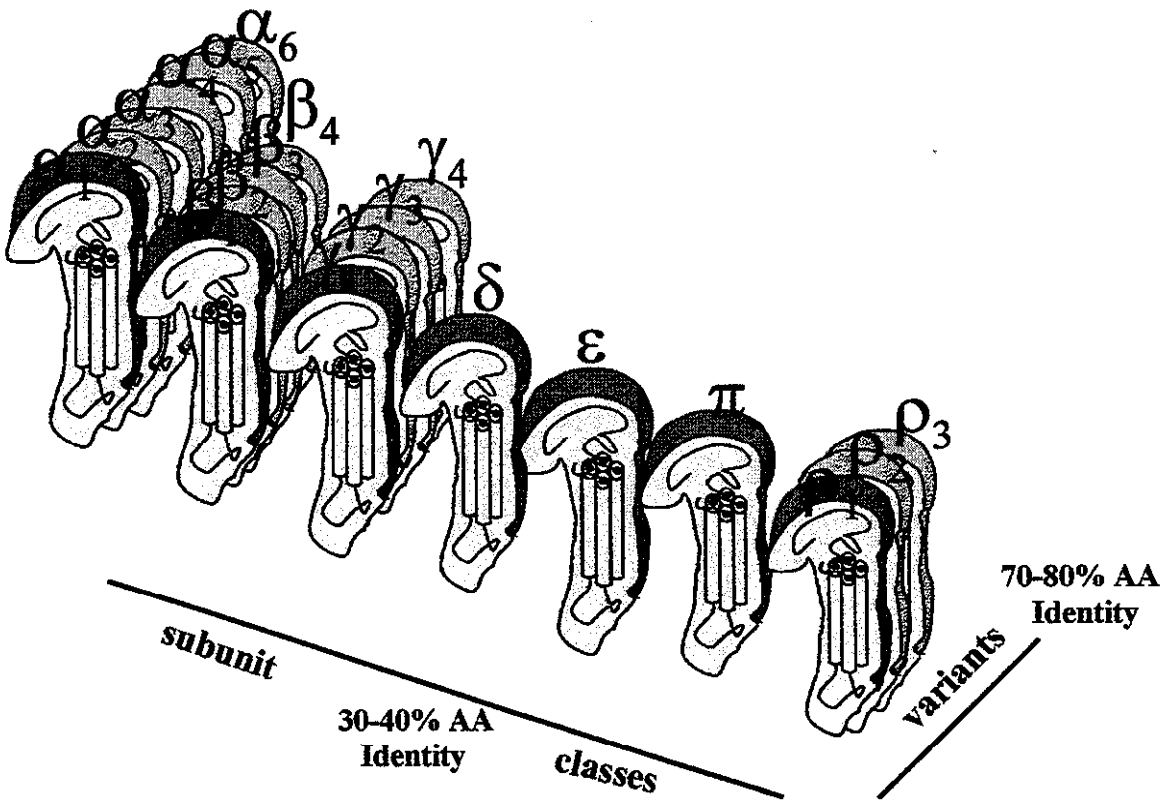
Subunit	Human	Rat	Bovine	Chicken
α_1	Schofield et al., 1989	Lolait et al., 1989	Schofield et al., 1987	Bateson et al., 1991a
α_2	Hadingham et al., 1993a	Seeburg et al., 1990	Levitan et al., 1988	
α_3	Hadingham et al., 1993a	Malherbe et al., 1990	Levitan et al., 1988	
α_4	Yang et al., 1995	Seeburg et al., 1990	Ymer et al., 1989a	
α_5	Hadingham et al., 1993a	Pritchett et al., 1990		
α_6	Hadingham et al., 1996	Lüddens et al., 1990	Lüddens et al., 1990	
β_1	Schofield et al., 1989	Lolait et al., 1989	Schofield et al., 1987	
β_2	Hadingham et al., 1993b	Ymer et al., 1989b	Ymer et al. 1989b	Harvey et al., 1994
β_3	Wagstaff et al., 1991	Ymer et al., 1989b	Ymer et al., 1989b	Bateson et al., 1990
β_4				Bateson et al., 1991b
γ_1	Ymer et al., 1990	Ymer et al., 1990	Ymer et al., 1990	Glencorse et al., 1993
γ_2	Pritchett et al., 1989	Shivers et al., 1989	Whiting et al., 1990	Glencorse et al., 1990
γ_3	Hadingham et al., 1995	Knoflach et al., 1991		
γ_4				Harvey et al., 1993
δ	AF016917	Shivers et al., 1989		
ε	Davies et al., 1997			
π	Hedblom and Kirkness, 1997	Hedblom and Kirkness, 1997		

2.2. Molecular structure of $GABA_AR$ subunits

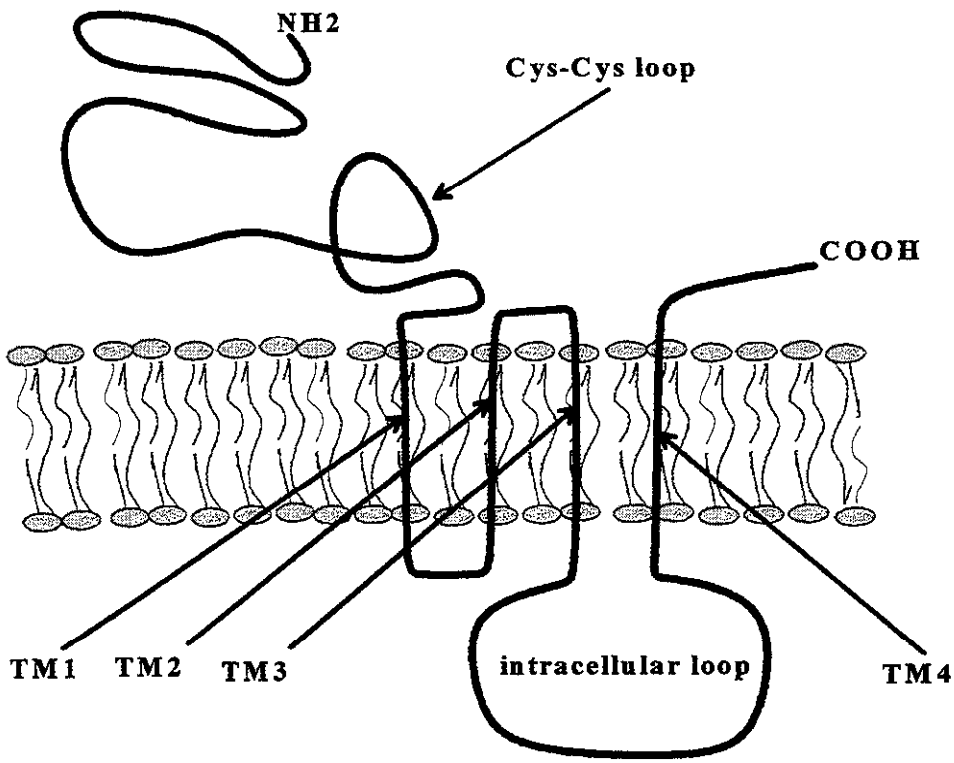
All subunits of ligand-gated ion channels including the $GABA_ARs$ have a similar molecular structure. A single subunit contains: (1) a large extracellular hydrophilic NH_2-terminus with several potential glycosylation sites and a cysteine bridge formed by two conserved cysteines; (2) four transmembrane domains (TM1-TM4); (3) a variable (in length and composition) intracellular loop between TM3 and TM4, which contains possible phosphorylation sites for protein kinase A and C and tyrosine protein kinase; (4) a short extracellular C-terminus. The second transmembrane domain (TM2) is thought to be part of the chloride channel core (Figure 13, Olsen and Tobin, 1990; Tyndale et al., 1995; Hevers and Lüddens, 1998).

Figure 13.

A.


α6
α5
α4
α3
α2
α1
β4
β3
β2
β1
γ4
γ3
γ2
γ1
δ
ε
π
ρ3
ρ2
ρ1
subunit
30-40% AA
Identity
classes
variants
70-80% AA
Identity

B.


NH2
Cys-Cys loop
COOH
TM1
TM2
TM3
intracellular loop
TM4

C.

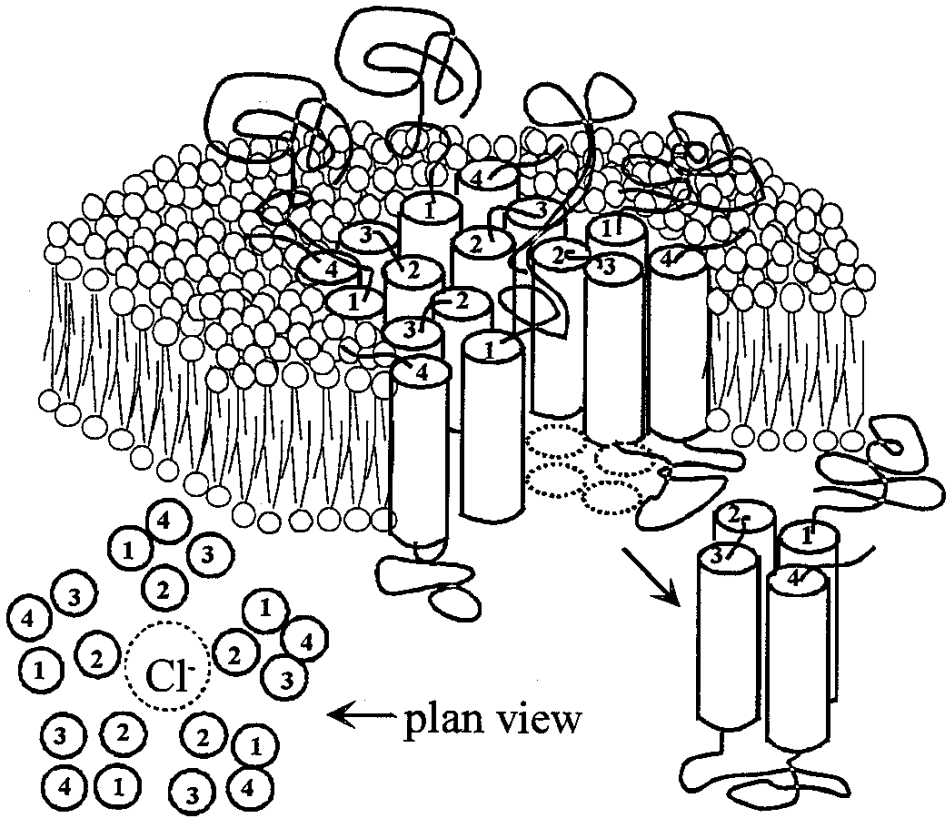


Figure 13. Schematic representation of $GABA_AR$ subunits, molecular structure and assembly. A: Multiplicity of $GABA_AR$ subunit isoforms. B: Molecular structure of $GABA_AR$ subunit. C: A model of the structure of the $GABA_AR$ chloride channel complex. (A, modified from Hevers and Lüddens, 1998; C, modified from Olsen and Tobin, 1990; Tyndale et al., 1995).

2.3 Alternative splicing forms of $GABA_AR$ subunit

The understanding of the heterogeneity of the $GABA_AR$ subunit isoforms is extended by the identification of alternate splice variants, in which additional sequences can be included or omitted from the mature mRNA species. Within the $GABA_AR$ gene family, alternative splicing have been identified in four subunits, namely α_6, β_2, β_4, γ_2 (Whiting et al., 1995). The splice variant of the α_6 subunit has an omission of 10 amino acids from the N-terminal domain and constitutes 20% of the total α_6 population, but it seems to form non-functional receptors (Korpi et al., 1994). All other three (β_2, β_4 and γ_2)

variants occur within the putative large cytoplasmic domain that lies between TM3 and TM4. The two β_2 subunit variants differing by the presence (β_{2L}) and absence (β_{2S}) of 17 amino acids have been identified in both chicken and man (Harvey et al., 1994; McKinley et al., 1995). Similarly, the two γ_2 variants arising by inclusion (γ_{2L}) or omission (γ_{2S}) of an additional exon, giving an additional 8 amino acids have been found in bovine, human, mouse and chicken brain (Whiting et al., 1990; Kofuji et al., 1991; Glencorse et al, 1992). The γ_{2L} subunit have been suggested to be necessary for the ethanol potentiation of $GABA_A$Rs (see Section 1.6.). The splice variants of β_4 subunit ($\beta_{4'}$) with a extra four amino acids has been demonstrated only in chicken (Bateson et al., 1991b).

2.4 Chromosome assignment of $GABA_A$R subunit genes

The genes coding $GABA_A$R subunits have been assigned to individual chromosomes (Table 4).

Table 4. Chromosome assignment of $GABA_A$R subunit genes

$GABA_A$R Subunit gene	Chromosome Assignment	$GABA_A$R Subunit gene	Chromosome Assignment
Human		Mouse	
α_1, α_6, β_2, γ_2	5q32-q33	α_5, β_3, γ_3	7
α_2, α_4, β_1, γ_1	4p13-q11	α_2, β_1	5
α_5, β_3, γ_3	15q11-q13	ρ_1, ρ_2	4
α_3, β_4, ε	Xq28	α_1	11
ρ_1, ρ_2	6q14-q21	α_3	X
δ	1p		

Data are collected from different papers (Tyndale et al., 1995; Rabow et al., 1995; Whiting et al., 1995; McKernan and Whiting 1996; Doble and Martin, 1996; Levin et al., 1996; Wilke et al., 1997).

2.5 Pharmacological role of $GABA_AR$ subunits

Expression of various combinations of recombinant subunit subtypes in oocytes or cultured mammalian cell lines has suggested that each of the subunit classes serve particular roles in defining the functionality of the assembled receptors. The role of $GABA_AR$ subunits has been most well characterised in pharmacological studies of BZDs. The binding profiles of BZD receptor ligands to the recombinant $GABA_AR$ subtypes expressed in various cell lines are similar to that found in native $GABA_AR$ subtypes (Table 5, Möhler et al., 1997).

2.5.1 α subunit class

The α subunit class is the largest, comprising six members, and it is believed that they specify the heterogeneity of the BZD binding site (Lüddens et al., 1995; Dunn et al., 1994). The described BZD1 receptor (see Section 1.2.1.2.) has been shown to consist primarily of receptors containing an α_1 subunit (Pritchett et al., 1989). The subunit combination $\alpha_1\beta_2\gamma_2$ is the major $GABA_AR$ subtype in the brain, which amounts to at least 60% of the BZD-sensitive $GABA_ARs$, as demonstrated by immunoprecipitation and immunohistochemical localisation (see Section 2.6.) of the three subunits in the same neurons (Wisden et al., 1992; Ruano et al., 1994; Fritschy and Möhler, 1995; Möhler et al., 1997). Expressed in transfected cells or *Xenopus* oocytes, receptors of $\alpha_1\beta\gamma_2$ combination showed high affinity for compounds such as CL 218872 and zolpidem both in biochemical and functional studies (Pritchett et al., 1989; Wafford et al., 1992; Hadingham et al., 1993a). Receptors containing the α_2 and α_3 subunits, represent about 25% of the BZD-sensitive $GABA_ARs$ and are most abundant in regions where the α_1 subunit is absent or expressed at low levels (Marksitzer et al., 1993; Benke et al., 1994; Fritschy and Möhler, 1995; Möhler et al., 1997). The subunits combinations $\alpha_2\beta\gamma_2$ and $\alpha_3\beta\gamma_2$ have lower affinity for CL 218872 and zolpidem, are corresponding to the described BZD2 receptors (see Section 1.2.1.2., Hadingham et al., 1993 a, b). The α_5 subunit containing recombinants (e.g. $\alpha_5\beta\gamma_2$) also exhibited a BZD2 phenotype but with a very low affinity for zolpidem, and having some 10-fold selectivity for Ro 15-4513 over all other receptor subtypes (Pritchett and Seeburg, 1990; Hadingham et al., 1993 a). α_4 and α_6 subunit containing receptors have high affinity for the compound Ro 15-4513 and bretazenil and very low affinity for classical BZDs such as diazepam and

flunitrazepam (Lüddens et al., 1990; Wisden et al., 1991). These receptors localised primarily in cerebellar granule cells (α_6 containing receptors) and thalamus (α_4 receptors) are termed “diazepam-insensitive” receptors (Malminiemi and Korpi, 1989; Lüddens et al., 1990, 1995; Wisden et al., 1992; Laurie et al., 1992). Using site-directed mutagenesis, the mutant of Glu225 to Gly on the α_3 subunit (glycine is the equivalent residue of α_3 with Glu225 in the α_1 subunit) confers a BZD1 pharmacological properties (Pritchett and Seeburg, 1991). The α_1, α_2, α_3 and α_5 subunits have a histidine at position 101, whereas the α_4 and α_6 subunits have an arginine, the mutant by substitution of arginine for histidine in α_6 subunits confers high-affinity binding of diazepam (Wieland et al., 1992). This histidine in the α_1, α_2, α_3 and α_5 appears to be a key residue for the action of clinically used BZD ligands (see Section 2.8.2.1.; Wieland et al., 1992; Kleingoor et al., 1993).

Table 5. Binding profiles of BZD site ligands to the native $GABA_AR$ subtypes

K_I (μM), [^{3}H]flumazenil binding Receptor population immunoprecipitated by subunit-specific antisera								
	$\alpha_1\beta_2\gamma_2$	$\alpha_2\beta_3\gamma_2$	$\alpha_3\beta_3\gamma_2$	$\alpha_4\beta_x\gamma_2$	α_5	γ_1	γ_3	δ
Flumazenil	0.6*	1.2*	1.1*	130	0.6*	+	1.1*	0.5*
Flunitrazepam	7	7	8	+	3	38*	854	2
Diazepam	20	25	20	+	20	ND	ND	5
Ro 15-4513	ND	ND	ND	16*	1	+	6.9	0.4
βCCM	2	6	7	ND	3	1550	11	0.2
Zolpidem	12	100	83	+	30	+	+	43
CL 218872	195	960	670	+	280	400	227	400

K_I was determined for receptor populations immunoprecipitated from whole rat brain with the respective α, β, γ or δ subunit specific antiserum. +: K_I > 10,000 μM; *: values represent K_D from Scatchard plot. ND: not determined (from Möhler et al., 1997).

2.5.2 β subunit class

The particular β subunit isoform appears to have little influence on the characteristics of the BZD recognition site (Pritchett et al., 1989; Wisden and Seeburg, 1992; Hadingham

et al., 1993b). However, it was demonstrated that the β_1, β_2 or β_3 subunits differentially influenced the [^{35}S]TBPS binding of recombinant receptors and its modulation by BZDs (Lüddens et al., 1994). The action of the anticonvulsant compound loreclezole was demonstrated depended on the type of the β subunit present in recombinant receptors. A more than 300-fold higher affinity for receptors containing β_2 or β_3 subunits over those containing β_1 subunits was observed (Wingrove et al., 1994). A single amino acid, β_2 Asn289 (β_3 Asn290), located at the carboxyl-terminal end of the putative channel-lining domain TM2, confers the sensitivity to the modulatory effects of loreclezole (Wingrove et al., 1994).

2.5.3 γ subunit class

The most important γ subunit is γ_2 that was found necessary to form a fully functional $GABA_AR$ with a high-affinity BZD binding site (Pritchett et al., 1989). Among the two splicing forms (see Section 2.3., γ_{2S} and γ_{2L}), most of the studies so far performed have used the short form of the γ_2 subunit (γ_{2S}) for the construction of recombinant receptors (Sieghart, 1995). γ subunits (γ_{1-3}) when expressed together with α and β subunits, confer BZD recognition properties on the expressed receptors, but receptors containing γ_1 and γ_3 exhibit pharmacological differences from those containing a γ_2 subunit with regard to both BZD affinity and efficacy (Pritchett et al., 1989; Ymer et al., 1990; Knoflach et al., 1991; Puia et al., 1991; Wafford et al., 1993; Herb et al., 1992 Lüddens et al., 1994). Combining with an α and a β subunit, receptors containing the γ_1 subunit have a marked decrease in affinity for the antagonist flumazenil and the inverse agonist DMCM as compared to the receptors containing γ_2 subunit (Ymer et al., 1990; Wafford et al., 1993). In contrast, $\alpha_1\beta_2\gamma_3$ shows a marked decrease in BZD agonist affinity (but not the affinity to BZD antagonists or inverse agonists) as compared to that of the $\alpha_1\beta_2\gamma_2$ (Wafford et al., 1993). Recombinant receptor combinations containing a γ_2 subunit confer insensitivity to Zn^{2+} (Draguhn et al., 1990; Smart et al., 1991). Both the α and γ subunits are necessary for a receptor to exhibit sensitivity to BZDs, and it have been proposed that the BZD binding domain is formed with contributions from both subunits (see Section 2.8.; Stephenson et al., 1990; McKernan et al., 1995; Wingrove et al., 1997).

2.5.4 δ subunit class

This subunit (455 AA) form homo-oligomers in human embryonic kidney 293 (HEK293) cells that display GABA-gated channels with small currents that are sensitive to picrotoxin, bicuculline and pentobarbital (Shivers et al., 1989). δ subunit was found co-localised with α_1, α_4 and β_2 mRNAs in large number of regions (principally thalamic nuclei), with α_1, α_6 and $\beta_{2/3}$ in the cerebellum granule cells, to possibly form $\alpha_1\alpha_4\beta\delta$ and $\alpha_1\alpha_6\beta\delta$ containing receptors which have high affinity to muscimol but lack BZD binding sites (Wisden et al., 1992). The subunit combinations $\alpha_1\beta_{2/3}\gamma_2\delta$ and $\alpha_2\alpha_5\delta$ may also exist in some brain regions (Fritschy and Möhler, 1995). Co-expressed with α_1 and β_1, the formed subunit combination $\alpha_1\beta_1\delta$ has a slowed rate of desensitisation and is potently blocked by zinc and is insensitive to diazepam (Saxena and Macdonald, 1994). *In vivo*, the receptors containing δ subunit as detected by immunoprecipitation, comprise a novel population of $GABA_A$Rs which do not bind BZDs but have a 5-fold higher affinity for muscimol as compared with the receptors containing γ_2 subunit (Quirk et al., 1995). Transfected in HEK293 cells, the presence of the δ subunit inhibits $GABA_A$R modulation but not the direct activation by neurosteroids (3α, 21-dihydroxy-5α-pregnan-20-1 and Pregnenolone sulphate) (Zhu et al., 1996a). The δ subunit mRNA expression in cerebellum was up-regulated in pentobarbital-tolerant mice and was down-regulated in pentobarbital-withdrawn mice (Lin and Wang, 1996), inhibited by the inactivation of α_6 subunit gene in cerebellar granule cells (Jones et al., 1997), and selectively modulated by KCl-induced depolarisation, a condition that mimics the effects of neuronal activity through a pathway involving calcium entry and activation of a Ca^{2+}/calmodulin-dependent protein kinases in cultured rat cerebellar granule neurons (Gault and Siegel, 1997).

2.5.5 ε subunit class

This polypeptide is 506 amino acids in length and exhibits its greatest amino acid sequence identity with the γ_3 subunit within the $GABA_A$R (Whiting et al., 1997). It can assemble with α and β subunits and confer an insensitivity to the potentiating effects of intravenous anaesthetic agents (Davies et al., 1997). The $\alpha_1\beta_1\varepsilon$ $GABA_A$Rs, like $\alpha_1\beta_1\gamma_{2S}$ receptors, are modulated by pentobarbital and the steroid 5α-pregnan-3α-ol-20-1 but,

unlike $\alpha_1\beta_1\gamma_{2S}$ receptors, are insensitive to flunitrazepam. Additionally, $\alpha_1\beta_1\varepsilon$ receptors exhibit rapid desensitisation kinetics, as compared with $\alpha_1\beta_1\gamma_{2S}$ (Whiting et al., 1997). In situ hybridisation and immunocytochemistry reveal a pattern of expression of ε subunit in the brain restricted primarily to the hypothalamus (Whiting et al., 1997). Clustered together with the α_3 and β_4, the gene coding human $GABA_AR$ subunit ε has been assigned to chromosome band Xq28, located in the candidate regions of two different neurological diseases: early onset Parkinsonism and X-linked mental retardation (Wilke et al., 1997).

2.5.6 π subunit class

This subunit (440 AA) displays 30-40% amino acid identity with other subunit classes (e.g. α, β, γ) within the $GABA_AR$ subunit family and represents a distinct subunit class. It has been identified in human and rat tissues (Hedblom and Kirkness, 1997). Transcripts of the π subunit were detected in several human tissues and were particularly abundant in the uterus. Transient expressing π subunit cDNA alone, HEK293 cells did not express binding sites for the $GABA_AR$ ligands, [^{3}H]muscimol or [^{35}S]TBPS (Hedblom and Kirkness, 1997). These cells also failed to elicit chloride currents in response to 100 μM GABA or 100 μM glycine. When cells were cotransfected with cDNA encoding the π subunit, and either an α_1 or a β_1 subunit, there was also a failure to detect expression of any ligand binding activities. The transfection of cells with a combination of α, β and π subunits resulted in the expression of both [^{3}H]muscimol and [^{35}S]TBPS binding sites, but not the binding sites for [^{3}H]Ro 15-1788 or [^{3}H]flunitrazepam. The cells cotransfected with β_3/π showed a significant reduced sensitivity (factor 9) to the endogenous steroid, pregnolone, as compared to the β_3 homomeric receptors (Hedblom and Kirkness, 1997).

2.6 $GABA_AR$ subtypes in the brain

The multiplicity of $GABA_AR$ subunit isoforms suggests that a large number of receptor subtypes may exist. Without restriction on subunit assembly, a theoretical maximum of 15^5 (759375) different receptor subtypes would be possible for the 15 subunit isoforms found in human brain. However, the number of theoretical possibilities of $GABA_AR$

subtypes in brain is considerably reduced (ca. 10,000) concerning the fact that at least one α subunit, one β subunit and one γ subunit are required to form a fully functional receptor (Pritchett et al., 1989). The evidence currently available suggests that there are perhaps only 10 to 15 (Doble and Martin, 1996) or less (McKernan and Whiting, 1996) major subtypes occurring *in vivo* (Table 6).

Two approaches mainly contributed to this conclusion. The first is tracing sites of gene expression using *in situ* hybridisation. Because of overlapping co-localisation, certain subunit combinations are considered as likely to represent native receptor isoforms (e.g. Wisden et al., 1992). The second is mapping the site of protein expression using subunit-specific antibodies (immunocytochemistry, e.g. Fritschy and Möhler, 1995).

Table 6. Distribution of the major $GABA_AR$ subtype in the rat brain∗

$GABA_AR$ subtype	Relative abundance in rat brain (%)	Location and putative function
$\alpha_1\beta_2\gamma_2$	43	Present in most brain areas. Localised to interneurons in hippocampus and cortex and cerebral Purkinje cells
$\alpha_2\beta_{2/3}\gamma_2$	18	Present on spinal cord motoneurones and hippocampal pyramidal cells
$\alpha_3\beta_n\gamma_{2/3}$	17	Present on cholinergic and monoaminergic neurones where they regulate Ach and monoamine turnover
$\alpha_2\beta_n\gamma_1$	8	Present on Bergmann glia, nuclei of the limbic systems and in pancreas
$\alpha_5\beta_3\gamma_{2/3}$	4	Predominantly present on hippocampal pyramidal cells
$\alpha_6\beta\gamma_2$	2	Present on cerebellar granule cells
$\alpha_6\beta\delta$	2	Present on cerebellar granule cells
$\alpha_4\beta\delta$	3	Present in thalamus and hippocampal dentate gyrus
Other minor subtypes	3	Present throughout brain

∗ Other minor subtypes include $\alpha_1\alpha_6\beta\gamma_2$, $\alpha_1\alpha_3\beta\gamma_2$, $\alpha_2\alpha_3\beta\gamma_2$ and $\alpha_5\beta\gamma_2\delta$ subtypes and are represented together as a small population (Adopted from McKernan and Whiting, 1996).

According to these two approaches, the $\alpha_1\beta_2\gamma_2$ subunit combination represent the most abundant $GABA_AR$ subtype in the brain (Laurie et al., 1992; Wisden et al., 1992; Benke et al., 1991, 1994; Fritschy et al., 1992; Fritschy and Möhler, 1995; Möhler et al., 1997). It amounts almost 50% of all $GABA_AR$ population in the brain (McKernan and Whiting, 1996). It is not only the main component in GABA-ergic signal transduction, but also mediates the basic pharmacological spectrum of the classical BZD receptor ligands (see Section 2.5.1., Möhler et al., 1997). Concerning identified neurons, $\alpha_1\beta_2\gamma_2$ receptors are expressed in numerous populations of GABA-ergic neurons, particularly the GABA-ergic neurons in the cerebellum, brainstem reticular formation, pallidum, substantia nigra and basal forebrain, as well as interneurons in cerebral cortex and hippocampus (Möhler et al., 1997).

The second possibly most abundant subtypes of native $GABA_ARs$ are $\alpha_2\beta_3\gamma_2$ and $\alpha_3\beta_3\gamma_2$, which account to approximately 35% of the total $GABA_AR$ population (McKernan and Whiting, 1996). The $\alpha_2\beta_3\gamma_2$ subtype are most abundant in regions as striatum, hippocampus (e.g. hippocampal pyramidal cells) and olfactory bulb, while $\alpha_3\beta_3\gamma_2$ subtype are abundant in lateral septum, reticular nucleus of the thalamus and several brainstem nuclei (Fritschy and Möhler, 1995; Möhler et al., 1997).

The $\alpha_5\beta_{2/3}\gamma_2$ subtype represents one of the numerous minor $GABA_AR$ subtypes in the brain (Möhler et al., 1997). They are relatively highly expressed in the hippocampus, in which they constitute approximately 20% of the $GABA_AR$ population (McKernan et al., 1991). In addition, $\alpha_5\beta_{2/3}\gamma_2$ receptors are also found in olfactory bulb, hypothalamus and trigeminal sensory nucleus (Fritschy and Möhler, 1995). Based on mRNA and protein colocalisation further subunit combinations have been suggested such as $\alpha_6\beta\gamma_2$, $\alpha_6\beta\delta$ in the cerebellar granule cells, $\alpha_4\beta\delta$ in thalamus and hippocampal dentate gyrus, and $\alpha_1\alpha_4\beta\delta$, $\alpha_1\alpha_6\beta\delta$, $\alpha_1\alpha_6\beta\gamma_2$ or $\alpha_1\alpha_3\beta_{2/3}\gamma_2$ etc. (Wisden et al., 1992; De Blas, 1996; Fritschy et al., 1992; Laurie et al., 1992; Fritschy and Möhler, 1995; Jechlinger et al., 1998).

2.7. Functional expression of recombinant $GABA_A$Rs

There are several expression systems for the investigation of recombinant $GABA_A$Rs. One rapid and simple model is made by directly injection of mRNA encoding for $GABA_A$R subunits into *Xenopus* oocytes to elicit the biosynthesis of these receptors that can be investigated by electrophysiological techniques (Levitan et al., 1988; Sigel et al., 1990). Other expression systems employing the transfection of the cDNAs coding different $GABA_A$R subunits into mammalian cells (such as HEK293 cells, Chinese hamster ovary cells and mouse fibroblasts etc.) and insect cells (e.g. Sf9 cells) that can be investigated by electrophysiological techniques as well as binding studies (Verdoorn et al., 1990; Knoflach et al., 1992; Porter et al., 1992; Horne et al., 1993; Pregenzer et al., 1993; Hartnett et al., 1996; Knight et al., 1998).

The expression efficiency of recombinant receptors in Sf9 insect cell system seems better than other systems (Sieghart, 1995). A recent study suggested that the Sf9 expression system reproduces the pharmacological properties of native receptors expressed in the brain (functional $GABA_A$Rs with respect to both GABA and BZD binding pharmacology) and allows reproducible construction of receptors of known composition by an ordered assembly process (Hartnett et al., 1996). The high level of expression and the ease of co-expression of selected subunit combinations appears to make the Sf9/baculovirus an ideal system for the study of receptor assembly, subunit stoichiometry of the receptor complexes, as well as in drug screening studies (Hartnett et al., 1996; Ai et al., 1997a, c; Knight et al., 1998).

Using the expression systems mentioned above, receptors consisting of a single, two, three or more subunits of $GABA_A$Rs have been investigated.

Except of the recently described ε and π subunits, α, β, γ and δ subunits can assemble into functional homo-oligomeric channels. However, compared to most dual or triple subunit combinations, the homo-oligomeric channels generally have a lower efficiency of expression and give rise to smaller currents activated by GABA (Sigel et al., 1990; Verdoorn et al., 1990; Davies et al., 1997). Since these homo-oligomeric Cl^- channels can be activated by rather high concentrations of GABA, stimulated by barbiturates or steroids but not BZDs, inhibited by bicuculline and picrotoxinin, the binding sites for GABA, picrotoxinin, barbiturate and steroid may be present on each of these subunits, or formed on assembly of these subunits (Pritchett et al., 1989; Shivers et al., 1989;

Verdoorn et al., 1990; Puia et al., 1990; Atkinson et al., 1992; Joyce et al., 1993; Sieghart, 1995; Krishek et al., 1996).

As compared to the homo-oligomeric channels, the co-expression of two different subunits (e.g. $\alpha_1\beta_2$) of $GABA_A$Rs can form Cl^- channels with higher efficiency for GABA activation (Sigel et al., 1990; Knoflach et al., 1992). There are apparently some differences in the expression efficiency of dual subunit combinations. For example, combinations as $\alpha_1\beta_1$ or $\alpha_1\beta_2$ are found consistently expressed in various expressing systems (Verdoorn et al., 1990; Draguhn et al., 1990; Angelotti et al., 1993). Other subunit combinations appears to be expressed less efficiently, e.g. the $\alpha_5\beta_3$ (Burgard et al., 1996) or the $\alpha_6\beta_3$ isoform (Saxena and Macdonald, 1996). The $\beta_2\gamma_2$ isoform is reported to show only small Cl^- currents (Sigel et al., 1990; Verdoorn et al., 1990; Draguhn et al., 1990), whereas the $\beta_1\gamma_{2S}$ or $\beta_3\gamma_{2L}$ isoforms failed to assemble into functional channels (Sigel et al., 1990; Angelotti and Macdonald, 1993; Burgard et al., 1996). The binary combinations of ε subunit (e.g. αε or βε) are non-functional (Davies et al., 1997). Data summarised by Sieghart indicated that the recombinant $GABA_A$Rs consisting α and β subunits are activated by GABA and inhibited by bicuculline and picrotoxin, and further modulated by barbiturates, steroids, propofol, chlormethiazole, inhalation anaesthetics and Zn^{2+}, but not modulated by BZDs, La^{3+} or Ro 5-4864 (Sieghart, 1995). In contrast to receptors consisting of αβ, the BZD binding sites (but not Zn^{2+}) were found present on recombinant receptors containing αγ or βγ subunit combinations (Puia et al., 1989; Im et al., 1993; Slany et al., 1994, 1995), which is consistent with the results from molecular biology that the binding domain for BZD ligands are located at the α/γ subunit interface (see Section 2.8.2.).

A cellular explanation for the different expression of certain isoforms is reported that some murine subunits and subunit combinations, including homo-oligomeric α_1, β_2 and binary $\alpha_1\gamma_{2L}$, $\beta_2\gamma_{2L}$, are retained in the endoplasmatic reticulum of oocytes and HEK293 cells, and only $\alpha_1\beta_2$, $\alpha_1\beta_2\gamma_{2L}$ could produce functional surface expression (Connolly et al., 1996). These results suggest that receptor assembly occur by defined pathways, which may serve to limit the diversity of $GABA_A$Rs that exist on the surface of neurons (Connolly et al., 1996).

Recombinant receptors containing ternary combination as αβγ, αβδ or αβε can consistently form functional channels and most closely resemble $GABA_A$Rs found in the brain (McKernan and Whiting, 1996; Hevers and Lüddens, 1998). The influences of different subunit isoforms on the pharmacology of the different receptor combinations have been discussed in Section 2.5.

2.8 Mutational analysis of ligand binding domains in the $GABA_A$Rs

The molecular cloning of the subunits of the $GABA_A$Rs provides the opportunity to evaluate the function of the individual amino acids within the subunit sequence, which are responsible for the binding and interaction with corresponding ligands. Mutational analysis studies have revealed some of the functional domains for the recognition of GABA, BZDs within the $GABA_A$Rs (Figure 14, Smith and Olsen, 1995; Davies et al., 1996; Doble and Martin, 1996; Sigel and Buhr, 1997; Hevers and Lüddens, 1998; Vafa and Schofield 1998).

2.8.1 GABA binding sites

Mutation studies indicate the GABA binding sites are determined by both the α and the β subunits, and the GABA binding sites may be located at the α-β interface of the $GABA_A$Rs (Figure 14., Olsen and Tobin 1990; Macdonald and Olsen, 1994; Mihic et al., 1995). A Point mutation in the rat α_1 subunit at phenylalanine (Phe)64 (phenylalanine to leucine) in the putative N-terminal extracellular domain produced a marked decrease in agonist and antagonist affinities when co-expressed with β_2 and γ_2 subunits (Sigel et al., 1992). In our laboratory, the amino acid Ile at position 121 on the human α_1 subunit (homologous to rat α1Ile120) was found to affect the agonist sensitivity (Westh-Hansen et al., 1997). When coexpressed with $\beta_2\gamma_2$ in the baculovirus/Sf9 in sect cell system, the mutation of α_1Ile121Val produced receptors with ca. 30-fold lower affinity to the agonists GABA and muscimol in the antagonist [^{3}H]SR 95531 binding. Electrophysiological measurements of GABA induced whole-cell Cl^- currents showed a 10-fold decrease in $GABA_A$R sensitivity produced by this mutation (Westh-Hansen et al., 1997).

Mutational analysis of the β subunit has provided some evidence for the involvement of tyrosine and threonine residues in GABA recognition. Using rat clones of α_1, β_2 and γ_2

subunits expressed in *Xenopus* oocytes, Amin and Weiss identified two domains in the β subunit which appear to be crucial for the activation of the receptor by GABA (Amin and Weiss, 1993). The two domains (Tyr157-Thr160 and Thr202-Tyr205), situated between the disulphide loop and TM1. Mutation of Tyr157 or Tyr205 to Phe, or Thr160 or Thr202 to Ser in the β_2 subunit significantly reduced binding affinity (20- to 50-fold) for both agonists and antagonists at the GABA binding site. Mutations in the corresponding residues in α and γ subunits or other residues in these two domains had little or no effect. The mutated receptors could still be activated by pentobarbital (Amin and Weiss, 1993).

The structural and sequence similarities shared by the members of the ligand-gated ion channels family of receptors suggest that functional domains of the receptor may also be located in homologous portions of different receptor subunits (Vafa and Schofield, 1998). Within the nAch receptor, four discontinuous sequence loops have been shown to constitute the ligand-binding domains (Changeux, 1995; Hucho et al., 1996). Since these four extracellular domains appear to be conserved across all of the ligand-gated ion channel receptors as homologous domains, the four-loop (Loop A, B, C and D) model of ligand binding domains has been proposed to apply for all members of the ligand-gated ion channel receptor superfamily (Changeux, 1995; Hucho et al., 1996; Vafa and Schofield, 1998). For example, the $GABA_AR$ β subunit residues Tyr157 and Thr160 are homologous to the loop B domain residues of the nAch receptor (Trp149, Tyr151) and the glycine receptor (Phe159, Tyr161), Whereas the residues Thr202 and Tyr205 are homologous to the loop C ligand-binding domain to residues of nAch receptor (Tyr190, Cys192 and Cys193) and the glycine receptor Lys200, Tyr202 and Thr204), respectively (Vafa and Schofield, 1998).

2.8.2BZD binding sites

Pharmacological analyses of $GABA_AR$ subtypes have suggested that both the α and γ subunits, but not the β subunit, contribute to the BZD binding site. Like the agonist binding site, the BZD site are believed to be located at the α/γ subunit interface (Figure 14., Smith and Olsen, 1995; Sigel and Buhr, 1997). Several important amino acid residues on the different α subunit as well as the γ_2 subunit involved in the binding of BZD ligands have been identified.

2.8.2.1 The important residues on the α subunits

The histidine residue in rat α_1 subunit (His101) was shown to confer diazepam-sensitive BZD binding (Wieland et al, 1992). While mutation of this residue to Arg (a homologous residue in α_4 and α_6 subunit) produced a subunit which was insensitive to BZD agonists diazepam, zolpidem and CL 218872 when co-expressed with β_2 and γ_2 subunits in HEK293 cells. There was also a 200-fold decrease in the affinity of the receptor for the BZD antagonist, Ro 15-1788, while the binding affinity for [^{3}H]Ro 15-4513 was retained. The receptors containing the reverse mutation of α_6 subunit (Arg101 to His101) gained sensitivity to BZD agonists (Wieland et al., 1992). Replacement of the Tyr159 of the α_1 subunit by Ser resulted in the loss of [^{3}H]Ro 15-1788 binding (Amin et al., 1997). Co-expressed with β_2 and γ_2 in HEK293 cells, receptors containing the mutant of α_1 (Tyr209 to Gln) showed approximately 40-fold decrease in affinity for [^{3}H]Ro 15-1788 and diazepam, while the α_1 (Tyr209 to Phe) mutant containing receptors showed a small to moderate decrease in affinity for [^{3}H]Ro 15-1788, diazepam, DMCM and CL 218872 (Buhr et al., 1997b). The α_1Tyr209Gln mutation confers loss of any detectable binding affinity for [^{3}H]Ro 15-1788 and [^{3}H]flunitrazepam, while the affinity to agonist [^{3}H]muscimol remain unchanged (Buhr et al., 1997b). Another two mutations on the α_1 subunit (Tyr161Ala and Thr206Ala) increase the sensitivity to both diazepam, CL 218872 and zolpidem (Buhr et al., 1996; 1997b).

In the rat α_3 subunit, if Glu225 is mutated to a Gly (the homologous residue to α_1), there is a more than tenfold increase in binding affinities for zolpidem and Cl218872 (Pritchett and Seeburg, 1991).

When four residues in the rat α_6 subunit (Arg100, Pro161, Glu199 and Ile121) were substituted with the corresponding residues present in the α_1 subunit (His101, Thr162, Gly200 and Val212), the mutant receptors gained more sensitivity to BZD agonist (Wieland and Lüddens, 1994).

Figure 14.

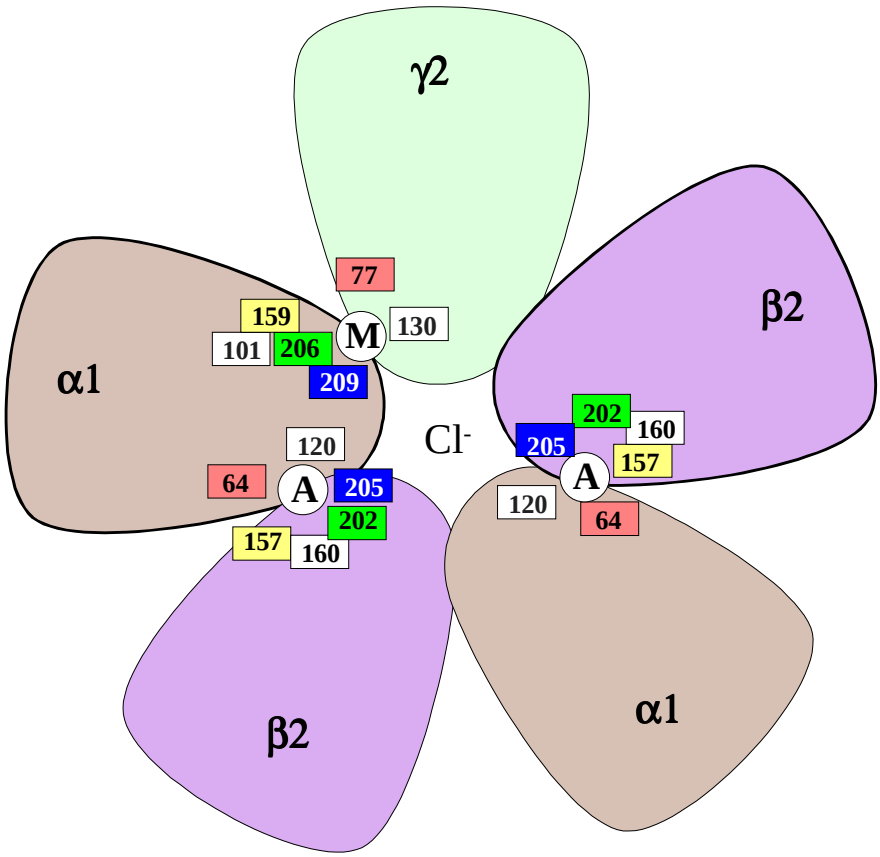


Figure 14. The binding domains for GABA and the BZD ligands identified on different subunits of the $GABA_A$/BZD receptors. The numbers indicate amino acid residues of the corresponding mature rat subunit isoform. The identical or directly homologous amino acid residues of GABA and BZD binding sites are shown in the same colour. A, agonist (GABA) binding site; M, modulatory site for BZD ligands. (Modified from Sigel and Buhr, 1997.

2.8.2.2. The important residues on the γ_2 subunit

Expression of recombinant receptors indicates that a γ subunit is absolutely required for the formation of a BZD binding site (Pritchett et al., 1989; Sigel et al., 1990). Upon deletion of γ_2 from mice the [^{3}H]Ro 15-1788 binding site nearly disappears, confirming the essential role of γ subunits (Günther et al., 1995).Two amino acid residues on the γ_2 subunit (Phe77 and Met130) showed pronounced influences on the receptor binding pharmacology (Buhr et al., 1996; Buhr and Sigel, 1997; Buhr et al., 1997a; Wingrove et

al., 1997). Depending on the amino acid side-chain present at position 77 of the γ_2 subunit, BZD site ligands specificity and affinity were differentially affected. The Phe77Tyr substitution increased the affinity of the receptors to zolpidem and CL 218872 had little effect on Ro 15-1788 binding, but reduced the affinity of the receptors to diazepam with a factor of 230 (Buhr et al., 1997a),. In contrast, the Phe77Ile mutation resulted in little change in the diazepam affinity, whereas there was a strongly reduced affinity for zolpidem and DMCM with a factor of 300 and 900, respectively (Buhr et al., 1997a). This effect of Phe77Ile mutation is in agree with the results of Wingrove et al. (Wingrove et al., 1997).

γ_2Met130 was required for high affinity binding of flunitrazepam, clonazepam and triazolam but not Ro 15-1788, Cl 21 8872 or βCCM (β-carboline-3-carboxylic acid methyl ester) and did not affect BZD efficacy (Wingrove et al., 1997). Substitution of Met130 to Leu of γ_2 subunit resulted in a 51-fold reduction in zolpidem affinity whereas the affinity to [^{3}H]Ro 15-1788 remained unchanged (Buhr and Sigel, 1997). The affinity for diazepam was only decreased by about 2-fold, and for CL 218872 increased 9-fold (Buhr and Sigel, 1997).

Another amino acid in the γ_2 subunit that has also been shown to directly affect the efficacy of BZD compounds is Thr142, which when mutated to serine increased the efficacy of BZD ligands, changing Ro 15-1788 and Ro 15-4513 to agonists, while BZD affinity remained (Mihic et al., 1994b).

2.8.3. Homology of the domains between the BZD site and the agonist site

Although no homologous region on other subunit isoforms involved in GABA interaction has been described for residue α_1H101, it is equivalent to the α subunit residue Tyr93 of the nicotinic acetylcholine receptor, which was shown to be involved in the binding of agonists and the competitive antagonists (Galzi and Changeux, 1994). In addition, in the model proposed by Smith and Olsen, two α subunit regions, beginning at Thr60 and Thr95 are proposed likely involved in the GABA and BZD binding sites, respectively. The common sequence ThrXAspXThrThr (X is variable amino acid) and the conservation of both regions in all α subunits, suggest that part of the BZD binding site on the $GABA_AR$ α subunit may be a modified form of an agonist site (Smith and Olsen, 1995). The following residues, which are important for BZD

binding, are identical or homologous to the residues important for the GABA binding, γPhe77 to αPhe64, αTyr159 to βTyr157, αThr206 to βThr202 and αPhe209 to βPhe205. αGly200, αVal211 and γMet130 of the BZD binding site are located in homologous regions to βTyr205 and αIle120 of the GABA binding site, respectively. Based on the available data, a hypothetical model of the binding pocket for agonist and modulatory BZDs is proposed (Figure 14; Sigel and Buhr, 1997).

2.8.Subunits stoichiometry and arrangement of $GABA_A$Rs

The subunit stoichiometries for recombinant $\alpha_3\beta_2\gamma_2$ receptors have been proposed as 2α, 1β, and 2γ; 2α, 2β and 1γ or 1α, 2β and 2γ, of which the 2:1:2 (α:β:γ) composition may be favoured (Backus et al., 1993). Other studies, in which either only one β subunit is present (Benke et al., 1994) or both the γ_2 and γ_3 subunits can be co-assembled in native $GABA_A$Rs (Benke et al., 1996), seem to support this finding. Recently, two different studies reach the same conclusion that the recombinant $GABA_A$R is a pentamer composed of two α, two β and one γ subunit (Chang et al., 1996; Tretter et al., 1997). The first study probed the stoichiometry of the $GABA_A$R by site-directed mutagenesis of a conserved leucine (to serine) in the putative second membrane-spanning domain of the rat α_1 (αLeu263Ser), β_2 (βLeu259Ser) and γ_2 (γLeu274Ser) subunit isoform (Chang et al., 1996). Co-expression of wild type and mutant subunits of each class (e.g. α and αLeu263Ser), along with their wild type counterparts (e.g. β and γ), in *Xenopus* oocytes resulted in mixed populations of receptors with different GABA sensitivities. The apparent number of incorporated subunits for each class (α, β and γ) could then be determined from the number of fractions comprising the GABA dose-response relationship curves. For example, when a mutated α (α_m) co-expressed with wild type α, β and γ in the same oocyte, the resulting dose-response relationship had three fractions, and two of the EC_{50}s (the concentration of GABA yielding a current half of the maximum) deduced from these three fractions are corresponding to the EC_{50}s deduced from composition of αβγ and α_mβγ, respectively. The third EC_{50} (deduced from the intermediate fraction) indicated that the GABA receptor must contain more than one α subunits (α_mαβγ combination) (Chang et al., 1996). In the other study, the ratio of subunits in recombinant $\alpha_1\beta_3\gamma_2$ receptors was determined in Western blots from

the relative signal intensities of antibodies directed against the N terminus or the cytoplasmic loop of different subunits. The relative reactivity of these antibodies had been determined with $GABA_AR$ subunit chimeras composed of the N-terminal domain of one and the remaining part of the other subunit (Tretter et al., 1997). In the same study, the composition of $\alpha_1\beta_3$ receptors expressed on the surface of HEK293 cells was found to form pentamer with a stoichiometry of two α and three β subunits, whereas combinations of $\alpha_1\gamma_2$ or $\beta_3\gamma_2$ subunits predominantly form heterodimers (Tretter et al., 1997).

One possible explanation for the discrepancy between these studies could be the stoichiometry of the $GABA_AR$ pentamers might not be unique. It might vary depending on receptor subtypes, as suggested from the investigation of native receptor (Li and De Blas, 1997).

There are six possible subunit arrangement in pentameric receptors consisting of two α, two β and one γ subunit (see Figure 15.). Among them the first two (Figure 15a and 15b) are believed to be energetically favoured, and consistent with the observation that α and γ or β and γ subunits predominantly form dimers (Sieghart et al., 1995; Tretter et al., 1997).

Figure 15.

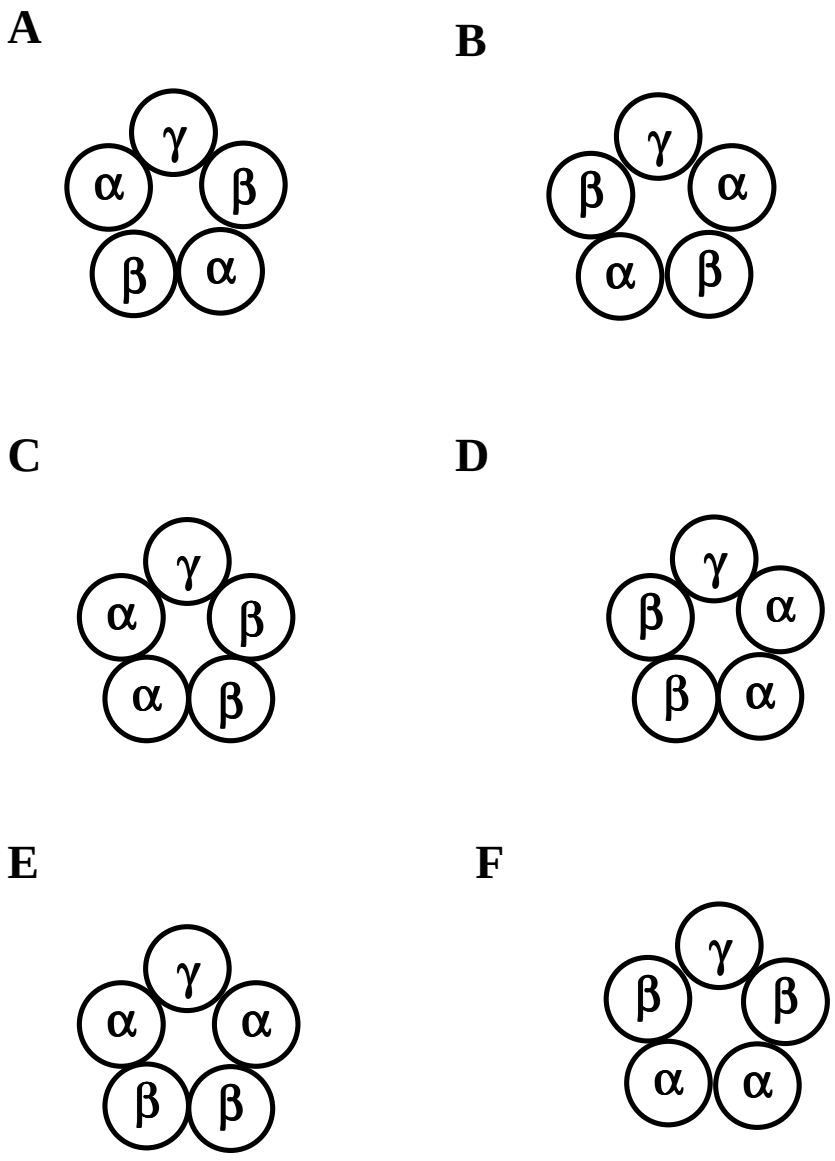


Figure 15. Possible subunit arrangements of receptors composed of two α subunits, two β subunits and one γ subunit

Section 3. Natural products as a source of new drugs and pharmacological tools

Throughout the history, natural products have been an important source for both new drugs and pharmacological tools. This section will first give a general introduction in this respect, following by an analysis of current status of TCM and the development of new drugs from TCM medicinal plants.

3.1. Natural products as a source of new drugs

Many clinically useful drugs that currently play a major role in the treatment of various human diseases are developed from natural sources such as by microbial fermentations or plant extracts (for review, see Bruhn, 1989; Phillipson, 1994; Cott, 1995; Houghton, 1995; Clark, 1996; Pettit, 1996; Nisbet and Moore, 1997; De Smet, 1997; Shu, 1998). Atropine, reserpine, Quinine, penicillin G, morphine, digoxin, cyclosporin etc., are the most well known natural products-originated drugs as milestones in the history of modern medication. It has been estimated that about half of the best selling pharmaceuticals were found either natural products or their derivatives, and approximately 60% of the antitumor and antiinfective agents that are commercially available or in late stages of clinical trials today are of natural product origin (Table 7; Cragg et al., 1997). Natural products have not only contributed to the development of new modern drugs as mentioned above, but also still play a very important role in most of the developing countries. Thus, it is estimated that 80% of the non-industrialised world still relies on plants as the major source of medicines in their primary health care (Farnsworth and Soejarto, 1985; Wijesekera, 1991).

Concerning natural products as source of new drugs, besides the microbial products, plants are one of the main resources for new drugs or drug candidates. It has been reported that ca. 120 compounds derived from 90 plant species may be considered as important drugs currently in use in one or more countries, 77% of which are derived from plants used in traditional medicine (Table 8; Farnsworth et al., 1985). However, there are only a small proportion of the estimated 400,000 – 500,000 plant species around the globe have been phytochemically investigated, and the number for biological or pharmacological screening is ever lower (Hostettmann et al., 1997). It is urgent to

investigate the pharmacology of plant compounds as soon as possible due to the widespread loss of habitats, especially the tropical forests, which contain the greatest bio-diversity in the world. It is estimated that more than 60,000 species of flowering plant will become extinct by the year 2050 (Hamann 1991).

Table 7. Analysis of origin of 520 new drugs approved by the FDA in USA or comparable entities in other countries in the period of 1983-1994

Clinical Indication	Origin of drugs				
	B	N	ND	S	S*
Antiallergic			1	5	
Antibacterial		6	44	14	
Anticancer and adjuvants	6	4	11	12	4
Antidiabetic		2		2	
Antihypertensive				27	25
Antiinflammatory	1		12	27	
Antithrombotic	2		3	5	
Antiulcer		1	9	11	
Antiviral				3	7
Bronchodilator			2		4
Hypocholesterolemic		2	1	1	
Immunostimulant	2	3	1	1	
Immunosuppressant	1	2	1		1
Nootropic		1	1	6	
Platelet aggregation inhibitor			3	1	
Progestogen			4		
Others	16	9	34	174	5
Total (520)	28	30	127	289	46
Percentage (%)	5	6	24	56	9

B: Biologics, including vaccines, monoclonals etc. N: Natural product from an unmodified source. ND: Natural product derivatives. S: Synthetic. S*: Synthetic, modelled on a natural product parent. Modified from Cragg et al., 1997.

Table 8. Representative drugs with plant origin (Farnsworth et al., 1985)

Drugs	**Clinical uses**	***Source***
Atropine	Anticholinergic	*Atropa belladonna*
Caffeine	CNS stimulant	*Camellia sinensis*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Codeine	Analgesic, antiussive	*Papaver somniferum*
Colchicine	Antigout	*Colchicum autumnale*
Digitoxin	Cardiotonic	*Digitalis purpurea*
Digoxin	Cardiotonic	*Digitalis lanata*
Ephedrine	Sympathomimetic	*Ephedra sinica*
Gossypol	Male contraceptive	*Gossypium herbaceum*
Hyoscyamine	Anticholinergic	*Hyoscyamus niger*
Kawain	Tranquilliser	*Piper methysticum*
Levodopa	Antiparkinsonian	*Mucuna deeringiana*
Morphine	Analgesic	*Papaver somniferum*
Noscapine	Antiussive	*Papaver somniferum*
Ouabain	Cardiotonic	*Strophanthus gratus*
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi*
Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
Quinine	Antimalarial	*Cinchona ledgeriana*
Reserpine	Antihypertensive	*Rauvolfia serpentina*
Scopolamine (hyoscine)	Sedative	*Datura metel*
Tubocurarine	Muscle relaxant	*Chondrodendron tomentosum*
Vinblastine	Anticancer	*Catharanthus roseus*
Vincristine	Anticancer	*Catharanthus roseus*
Yohimbine	Aphrodisiac	*Pausinystalia Yohimbe*

The most notable progress in identification of new drugs from plants has been made in the area of chemotherapy of cancer (Wall and Wani, 1995; Pettit, 1996; Cragg et al., 1997; Kinghorn et al, 1998; Pezzuto et al., 1998). For example, the two alkaloids vincristine and vinblastine, isolated from *catharanthus roseus*, are important therapeutic compounds for the treatment of acute childhood leukemia (vincristine), Hodgkin's

disease and metastatic testicular tumours (vinblastine) (Nelson, 1982; Baker et al., 1995). Their semisynthetic derivative vinorelbine exerts antitumor effect by tubulin binding leading to inhibition of microtubule assembly, thus are used for the treatment of advanced breast cancer (Sorensen, 1995). Two important additions to the cancer chemotherapeutic list are etoposide and teniposide, the semisynthetic derivatives of podophyllotoxin, which was originally isolated from plant *Podophyllum peltatum* (may-apple) (Buss and Waigh, 1995). Etoposide is active against many tumour types and one of the most active single agents for small cell lung cancer, while teniposide is used for the treatment of refractory acute lymphoblastic leukaemia in children (Buss and Waigh, 1995). The most exiting advance in cancer chemotherapy would be the discovery of paclitaxel (taxol). In the large scale-screening programme of American National Cancer Institute initiated some 40 years ago, thousands of extracts of randomly selected plants were tested for cytotoxic activity. Paclitaxel was identified as the active component of the crude extract from the stem bark of *Taxus brevifolia* (Pacific yew), and showed activity against many tumours (Wall and Wani, 1995). It was approved for the treatment of ovarian cancer and breast cancer in 1992 and 1993, respectively. The ability of taxol to polymerise tubulin into stable microtubules in the absence of any cofactors and to induce the formation of stable microtubule bundles in cells characterised taxol as unique antitumour drug with microtubules as the new target of action (Horwitz, 1992). Docetaxel (taxotere), a semisynthetic derivative of 10-deacetylbaccatin III, which is isolated from the needles of *Taxus baccata* (European yew), is closely related to taxol with improved water solubility and similar action against various tumours. It is the most active single agent against metastatic breast cancer (Crown, 1998).

3.2. Natural products as a source of pharmacological tools

While natural products have served and still will serve as a important source of new drugs, another important role of natural products in medical research is that they can be used as pharmacological tools for the identification and investigation of the physiological functions of receptors and enzymes, which serve or possibly will serve as drug targets. For example, nicotine, physostigmine and curare have made a great contribution to the research of the nicotinic receptor. The same is true for muscarine, pilocarpine to the muscarinic receptor. In the field of intracellular signal transduction,

for example, has been developed, in large part, through the study of cyclopsorin, FK506 and rapamycin, (Cardenas, et al., 1998). The phorbol esters from the *Euphorbiaceae* or *Thymelaeaceae* families have played a very important role in the investigation of the protein kinase C and have contributed to the identification of a further receptor RX-kinase (RX is the C-20 homovanillate of 9,13,14-orthophenylacetyl-resiniferonol). These enzymes are of importance in the understanding of disease conditions such as inflammatory response, cancer, cell proliferation, viral expression etc. (Evans, 1991). For the $GABA_A$Rs, the research process might be different without the contribution of muscimol, bicuculline and picrotoxin, all of which are natural products. Some representatives of compounds from natural sources as important pharmacological tool are shown in Table 9.

Table 9. Representative compounds with natural origin as important pharmacological tool for neurotransmitter receptors, neuropeptid receptors and enzymes

Receptors	**Compounds**	**Origin**
Nicotinic Ach receptor		
Acetylcholinesterase inhibitor	Physostigmine	*Physostigma venenosum*
Agonist	Nicotine	*Nicotiana tabacum* etc.
	Arecoline	*Areca catechu*
Antagonist	d-tubocurarine	*Chondrodendron tomentosum*
Muscarinic Ach receptor		
Agonist	Muscarine	*Amanita muscaria* (fungus)
	Pilocarpine	*Pilocarpus jaborandi*
	Arecoline	*Areca catechu*
Antagonist	Atropine	*Atropa belladonna*
	Scopolamine	*Datura metel*
	Anisodine	*Anisodus tanguticus*
	Anisodamine	*Anisodus tanguticus*

Table 9. continued

$GABA_AR$		
Agonist	Muscimol	*Amanita muscaria*
Competitive antagonist	Bicuculline	*Corydalis humosa* *Dicentra cucullaria*
Non-competitive antagonist	Picrotoxin	*Anamirta cocculus*
Glycine receptor		
Antagonist	Strychnine	*Strychnos nux-vomica*
NA receptor		
Selective α-2 receptor antagonist	Yohimbine	*Pausinystalia Yohimbe*
NA, DA, 5-HT receptor		
Vesicular transport and storage inhibitor	Reserpine	*Rauvolfia serpentina*
Opioid receptor		
μ subtype agonist	Morphine	*Papaver somniferum*
Adenylate cyclase		
Activator	Forskolin (colforsin)	Coleus forskohlii
Protein kinase C		
Activator	Phorbol esters	Euphorbiaceae or thymelaeaceae families

3.3. Medicinal plants in TCM

3.3.1. The general status of TCM

TCM is an integral part of the Chinese culture. Over the past several thousand years, it has made great contributions to the health care of Chinese nation through out the history, and still plays a important role in the modern China. Its remarkable efficacy, accumulated experiences and available historical literature make it an important source for new drug development research in China. TCM is one of the dual medical systems

running in China along with the Western medicine. An overview of the current status of TCM in China is presented in Table 10.

Table 10. A general status of TCM in China∗

Administration	State Administration of TCM (equivalent to State Administration of Medicine and Pharmacy for the Western medicine)
Hospitals	2457 TCM hospitals, 39 hospitals of integrated Western and Traditional Chinese Medicine, 129 hospitals of national minorities medicine, 75% counties have TCM hospital
	222,000 beds, 249,000 TCM doctors.
Education	30 TCM universities with a enrolment of 37,000 students
	51 TCM colleges with 29,000 students
	64,000 people educated from TCM night school, correspondence schools etc.
Research	77 independent national research institutions
	100 institutions affiliated to TCM universities, colleges and provincial hospitals
	10,000 academic professionals
Pharmaceuticals	500,000 employees
	600 productive bases, 13,000 farms and 340,000 specialised households for crude drugs production, with a planting area of 5,220,000 mu (1 mu = 667 m^2)
	800 TCM pharmaceutical companies
	40 forms, 5000 varieties with 200,000 ton yearly yield
	3.87 billion yuan output value (1 yuan = 0.8 Danish krone)

∗ Data is based on a review of TCM history appeared on the official Web site of China Academy of TCM (http://china-window.com/zhongy/zydl/e_zyls.html). Figures are referring to that in 1993.

3.3.2. TCM medicinal plants as a source of new drugs

The Materia Medica of TCM includes substances from plants, animals and minerals. A nationwide survey of Chinese herbal medicine (CHM) covering 80% of China's land area conducted over a period of five years from 1983 revealed that there are now 12,807 medicinal agents in the Chinese Materia Medica: 11,146 of plant origin, 1,581 of animal origin, and 80 from mineral sources (Zhang et al., 1995). According to the ways they are used, CHM can be classified into three main categories: 1). TCM herbal medicine. they are used by TCM doctors. Their usage are directed by the systematic and self-contained TCM theories. They are the main component of the CHM and comprise ca. 50% of that commonly used totally. 2). National minorities drug. They are used by doctors of various national minorities of which Tibetan, Mongolian are the most important ones. They are all closely connected with TCM, but have their own characteristics. 3). Folk herbal drugs. They are scattered throughout China with a large number, and are characterised by application on the basis of trial and error without theoretical direction. Some of the truly effective herbal drugs and prescriptions are being kept secret, open only to the owners and to be taught only to their descendants or favourite apprentices.

Concerning the new drug development process, there are mainly two different ways to develop new drugs from TCM substances. The first way, basically according to the standard of that in the Western countries, used single compound, identified from TCM plants or its derivatives with profound activity (e.g. Artemisinin against malaria). The going-through procedures include: 1). Conclusively approving of the compound structure; 2). Production technology of the compound. 3). Animal toxicity experiment (acute and long term); 4). Pharmaceutical experiment on animal model. 5). Animal pharmacodynamic experiment; 6). Stability experiment of the preparation. 7). Clinical trials. 8). Government census. The drugs developed in this way are classified as first class drugs. The second way, which is unique in China, is to develop drugs from TCM prescriptions. These prescriptions could come from an ancient known literature, an experienced TCM expert or a secret formula from an ethnical group etc., but the prerequisite is a profound clinical effectiveness. Going through a similar but fewer procedures, the developed drugs are classified as second or third class drugs. Although the exact knowledge of the active components of these drugs are unknown (there could

be hundred and thousands of compounds in it), the quality control were achieved by setting up a standard for the major known active compounds (which has been shown responsible for the relevant pharmacology by use of modern medicine methods) appeared in the herbs composing the prescription. The standard is set up by using modern analytical methods as HPLC, mass spectroscopy, atomic absorption spectrophotometry, ultraviolet spectrophotometry, infrared spectrophotometry and NMR etc. In a similar way, a huge amount of on-the-counter products (similar to the *Ginkgo biloba* products in the Western) from CHM were produced for the purpose of general health care like tonic, cosmetic, weight-reducing etc.

Extensive research has been done on the basic pharmacology of Chinese medicinal plants and their products. These researches including 1). Botanical classification and differentiation of medicinal plants; 2). Microscopic, chromatographic and chemical identification standards for medicinal plants; 3). Identification and characterisation of active constituents; 4). Pharmacological investigation of the active constituents in appropriate *in vitro* and *in vivo* experimental models (A book about all animal models used in TCM has been compiled by me and my Chinese colleagues. Chen et al, 1993); 5) Pharmacokinetic, pharmacodynamic and toxicological studies. 6). Investigation of the processing of TCM crude drugs. The results are predominantly published in Chinese (e.g. *The handbook of active components of Chinese herbal medicine*, compiled by Jiang and Xiao, 1986 included all the compounds isolated from CHM by then. *The handbook of chemical components and pharmacology of most commonly used Chinese herbal medicine*, compiled by Huang, 1993 with 1900 pages reviewed 429 plants in detail on different names in the literature, botanical identity, commercial available forms and quality standards, brief description of traditional use, general, microscopic and physiochemical identification, known chemical components and pharmacology). Over the years, many drugs or promising drug candidates have been identified from CHM, some representative ones are listed in Table 11.

Table 11. Representative examples of Chinese plants-originated drugs or drug candidates⁎

Drugs	**Origin**	**Indications or functions**
5,7,3',4'-tetrahydroxyflavone-3-nol C4-C6 dimer	*Fagopyrum cymosum*	Pulmonary abscess
α-dichroine β-dichrooine	*Dichroa febrifuga*	Malaria
Agrimorphol	*Agrimonia pilosa*	Taeniasis
Anisodamine	*Anisodus tanguticus*	Fulminant epidemic meningitis, hemorrhagic enteritis
Anisodine	*Anisodus tanguticus*	Migraine, organophosphorus poisoning, acute paralysis caused by vascular accident
Artemisinin Artemether⁎⁎ Arteether⁎⁎ Artesunate⁎⁎ Dihydroartemisimin ⁎⁎	*Artemisia annua*	Malaria
Camptothecin Ironotecan⁎⁎	*Camptotheca acuminata*	Lung, ovarian and cervical cancers
Curcumol Curdione	*Curcuma aromatica*	Early stage of cervix cancer
Daizin Genistin Daidzein	*Pueraria lobata* (Chinese Kudzu)	Alcoholic Hypertensive disease, angina pectoris, migraine and sudden deafness (daidzein)

Table 11. continued

Gastrodin	*Gastrodia elata*	Insomnia, anxiety and vertigo
Schisandrin A, B, C Schisandrol A, B Schisantherin A, B, C	*Schisandra chinensis* *Schisandra sphenanthera*	Hepatitis
Gossypol	*Gossypium herbaceum* (Cotton seed)	Male contraceptive
Homoharringtonine	*Cephalotaxus harringtonia* *Cephalotaxus fortunei*	Leukaemia
Houttynin	*Houttuynia cordata*	Antimicrobial, for infections
Huperzine A	*Huperzia serrata*	Alzheimer's disease
Indirubin	*Indigofera tinctora* *Baphicacanthus cusia* *Isatis tinctoria* *Couroupita guianensis*	Chronic myecytic leukemia
Methyl hydroquinone	*Pyrola rotundifolia*	For infections in respiratory, digestive and urinary system
Nevadensin	*Lysionotus pauciflorus*	Expectorant, antiussive, antituberculosis
Piperine Antilepsirine**	*Piper nigrum*	Epilepsy
Rubescensin (oridonin) Ponicidin	*Rabdosia rubescens*	Esophageal and liver cancer
Terpenoids	*Brucea javanica*	Malaria
Triptolide	*Tripterygium wilfordii*	Rheumatoid arthritis
Tutin	*Coriaria sinica* *Loranthus parasiticus*	Schizophrenia
Yingzhaosu A Arteflene**	*Artabotrys uncinatus*	Malaria
Yuanhuacine A and B	*Daphne genkwa*	Induces premature abortion

* Data are collected from reviews (Xiao, 1983; Jiang and Xiao, 1986 (Chinese); Tang and Eisenbrand, 1992; Wang et al., 1995; Clark, 1996; De Smet, 1997; Zhu et al., 1996b; Qin and Xu, 1998; Shu, 1998; Huang, 1999).

** Synthetic derivatives

Section 4: Medicinal plants as a source of novel brain $GABA_A$/BZD receptor ligands- The experimental work

As discussed above, natural products, especially medicinal plants are important source of new drugs and pharmacological tools. The apparent side-effects associated with the use of classical BZDs, the promising profiles of BZD receptor partial agonists, the lack of high selective ligands for the BZD receptor subtypes, as well as the possible existence of other binding sites as potential drug targets on the $GABA_A$/BZD receptors motivate chemists, biochemists and pharmacologists all over the world to seek and develop compounds with advantageous or new pharmacological profiles and/or new chemical structures.

One line of development is to isolate $GABA_A$/BZD receptor ligands from natural sources by bioassay-guided fractionation. Special effort has been to isolate these compounds from medicinal plants used in traditional or folkloric medicine as TCM. The purpose of current work is to combine the knowledge advantage of TCM medicinal plants with the strategies for developing $GABA_A$/BZD receptor ligands, to isolate and characterise $GABA_A$/BZD receptor active compounds from TCM plants as well as from semi-synthesised naturally-occurring compounds by using methods as HPLC, radio-ligand binding assays and electrophysiology etc.. Based on our own experiences, some general strategies and methodologies of identifying compounds from medicinal plants will be illustrated. The results of our screening work and some possible pitfall will be discussed.

4.1. General methodology in identifying $GABA_A$/BZD receptor ligands from TCM plants

The discovery process for biologically active natural products involves a number of particular steps included: 1). The selective identification of a biological target (e.g. $GABA_A$Rs in the brain). 2). The selection, collection and identification of the organisms to be studied (e.g. plants, marine organisms, fungi, etc.). 3). The extraction and biological evaluation of the extracts. 4). Set priority for further studies. 5). Bio-activity-directed fractionation of the extracts and the structure elucidation of the biologically active principles. 6). Biological evaluation and mechanistic studies of the isolates. 7).

Synthetic modification and molecular modelling studies with the anticipation of identifying the pharmacophoric units. 8). A strategic decision for further development (see Figure 16, Cordell et al., 1998).

Using the $GABA_A$/BZD receptor-binding assay as primary screening method, new structurally diverse ligands were isolated and identified from medicinal plants (see **Appendix I** for overview).

Figure 16.

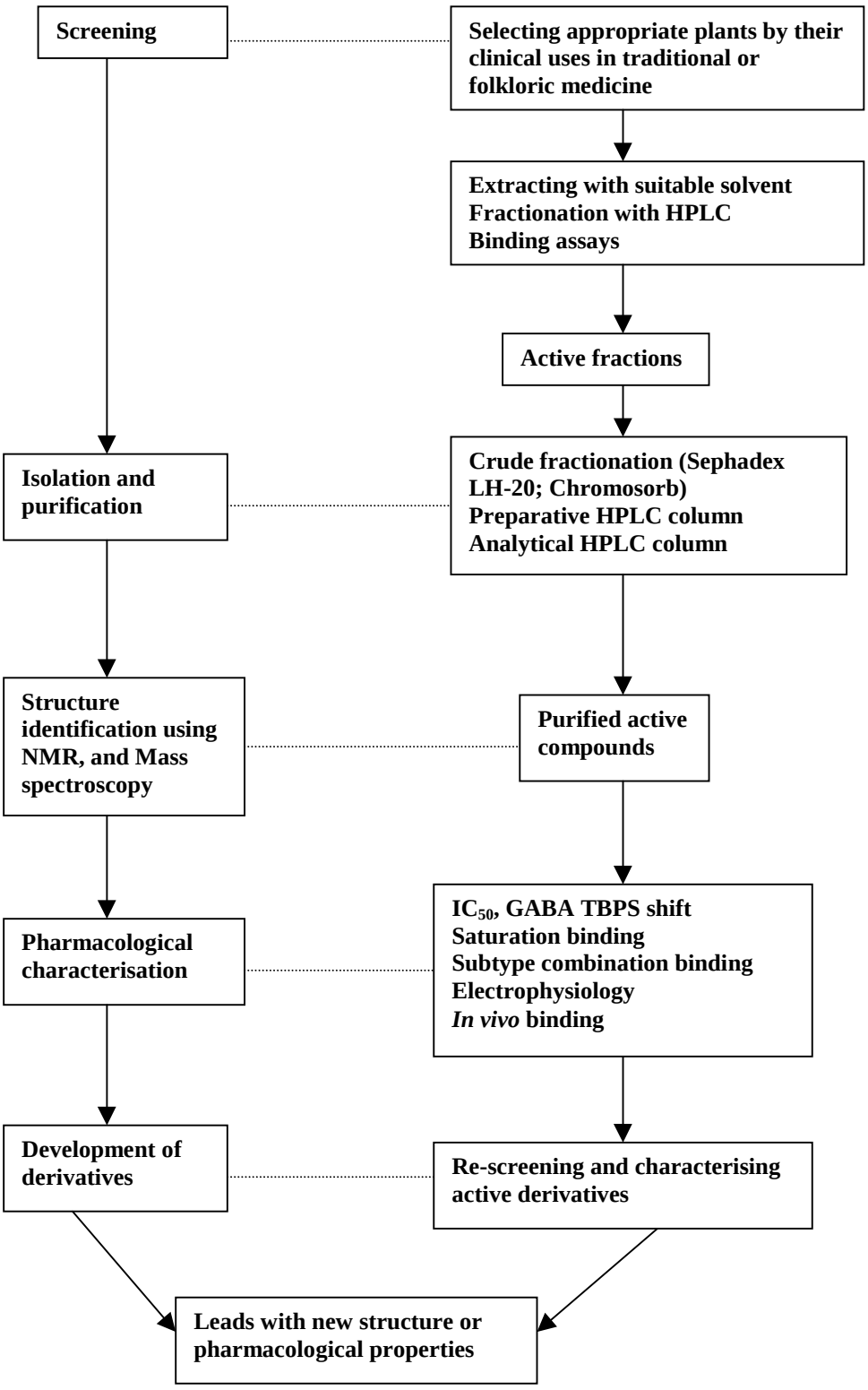


Figure 16. The general procedure for identification of $GABA_A$/BZD receptor ligands from plants.

4.2. Materials and Methods

4.2.1. Materials

Isotopes and chemicals: Radioactive ligands [^{3}H]diazepam (methyl-[^{3}H], 83 Ci/mmol), [^{3}H]Ro 15-1788 (N-methyl-[^{3}H], ethyl 8-fluoro-5, 6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate, 87 Ci/mmol), [^{3}H]muscimol (methylene-^{3}H (N), 20 Ci/mmol), [^{35}S]TBPS (t-butylbicyclophosphoro-[^{35}S]-thionate), [^{3}H]SCH 23390 (N-methyl-^{3}H, 71.3 Ci/mmol) and [^{3}H]QNB ([^{3}H]quinuclidinyl benzilate (L-[benzilic-4, 4'-3H(N)], 43.5 Ci/mmol), and [^{3}H]kainic acid (62.5 Ci/mmol) were from New England Nuclear, Dupont (Boston, MA, U.S.A.).
All HPLC solvents were from Rathburn Chemical (HPLC grade). Tested flavonoids were from Extrasynthese, Genay, France. Honokiol and magnolol were from Nacalai Tesque Inc. Japan and The Institute of Drug Identification, Beijing, China.
GABA and Diazepam were purchased from Research Biochemicals International, Natick, U.S.A..
Plants: Plants were purchased from The pharmacy of China Academy of TCM in Beijing, and identified by Dr. S.J. Xu.

4.2.2. Methods

***Preparation of rat brain membranes*:** The radioreceptor binding assays were carried out as previously described [Nielsen et al., 1988; Witt and Nielsen, 1994; Dekermendjian et al., 1996; Ai et al., 1997a, c; Ai et al., 1999]. In brief, male Wistar rats (weighing about 200 g) were decapitated, the cortex, hippocampus and cerebellum were rapidly excised and homogenised using an Ultra Turrax homogeniser in Tris-citrate buffer (50 mM, pH 7.1), followed by centrifugation (30,000 × g, 10 min). This washing procedure was repeated 3 times. The last pellets were stored at -24^o C until use. After thawing, the pellet was resuspended in Tris-citrate buffer, subjected to one additional washing step and diluted to a concentration of 2 mg original tissue/ml for the binding assays.

[^{3}H]diazepam, [^{3}H]flunitrazepam and [^{3}H]Ro 151788 binding assays: Aliquots of 1 ml membrane preparation were incubated with [^{3}H]diazepam (2 nM), [^{3}H]flunitrazepam (1

nM) or [^{3}H]Ro-151788 (1.2 nM) for 40 min at 40 min at 0-4^o C. For saturation assays, 6 to 9 concentrations of [^{3}H]Ro 15-1788 (0.05-15 nM) or [^{3}H]diazepam (0.2 – 10 nM) were used. Non-specific binding was determined by addition of midazolam (10 μM) or Ro-151788 (100μM).

[^{3}H]muscimol binding assays: Assays was conducted on washed-frozen-washed membrane preparation. Aliquots of 1 ml membrane preparation were incubated with [^{3}H]muscimol (10 nM) for 30 min at 0-4^o C. In saturation experiments 6 to 9 concentration of [^{3}H]muscimol (0.2 – 25 nM) was used. Non-specific binding was determined by addition of GABA (100μM) in the assay.

[^{35}S]TBPS binding assays: For [^{35}S]TBPS binding, the pellet containing cortical membranes was washed once with Tris-citrate buffer (50 mM, pH 7.1) and centrifuged. The pellet was resuspended in Tris-citrate buffer containing NaCl (1M) to a concentration of 10 mg original tissue/ml and assayed (0.5 ml assay volume) for specific [^{35}S]TBPS (0.9 nM) binding. Non-specific binding was obtained by incubation of 50 μM picrotoxinin. For TBPS on-rate experiments, incubations were done at 25 ^{o}C varying the incubation time from 20 to 240 min.

[^{3}H]QNB binding assays: In the [^{3}H]QNB (1.5 nM) binding assays, rat brain cortical membrane was prepared and used in the same way as in [^{3}H]muscimol binding assays. Unspecific binding was determined by including 10 μM atropine. Samples were incubated at 37 °C for 60 min.

[^{3}H]SCH 23390 binding assays: [^{3}H]SCH 23390 (0.25 nM) binding assays were performed on membrane preparation of rat brain striata as previously reported (Dekermendjian, et al., 1997). Briefly, Striata were dissected and rapidly homogenised (Ultra Turrax, 10 s) in 50 mM KH_2PO_4 buffer pH 7.4 and centrifuged at 30,000 g for 10 min. The pellet was resuspended in 50 mM KH_2PO_4 buffer pH 7.4 at a concentration of 1 mg tissue/ml, [^{3}H]SCH 23390 was added to aliquots of 1 ml homogenate and incubated for 60 min at 30°C.

[^{3}H]kainic acid binding assays: The binding assays were carried out as previously described (Andersson et al., 1995). Briefly, [^{3}H]kainic acid (2 nM) was added to aliquots of 0.5 ml membrane preparation (final concentration of 20 mg original tissue/ml), and incubated for 60 min at 0 – 4 °C. Non- specific binding was determined by adding L-glutamate (600 μM).

All binding assays were terminated by vacuum filtration through Filter Max filters using Semiautomatic Cell Harvester (Skatron Instrument, UK), or through Whatman GF/C glass filters. Filters with bound ligand were washed twice with ice-cold Tris-citrate buffer (50 mM, pH 7.1). Radioactivity on the filters was measured in 3 ml of scintillation fluid. Specific binding was calculated by subtracting non-specific binding from total binding. All binding assays were done in duplicate.

$GABA_A$/BZD receptor expression in Sf-9 insect cells for receptor binding: The expression of recombinant $GABA_A$/BZD receptor subtypes using the Sf-9/baculovirus expression system was previously reported [Witt et al., 1996]. In brief, Sf-9 insect cells were grown in Sf 900 medium at 27 ^{o}C to a cell density of approximately 1 x 10^6 cells/ml (200 ml batches) and infected with baculovirus containing cDNA for various α (α_1, α_2, α_3 and α_5) and the β (β_2 and β_3) and γ_{2S} subunit with a MOI (Multiplicity Of Infection) value of 1:1:1. Cells were harvested by centrifugation approximately 45 hours post infection for receptor binding assays or kept as pellets at -80^o C until use.

Preparation of Sf-9 insect cells for binding assays: Sf-9 cells (30 x 10^6 /ml) were homogenised in 5 ml Tris-citrate buffer (50 mM, pH 7.1) using an Ultra-Turrax homogeniser and centrifuged (30,000 x g, 10 min). The pellet was resuspended in 1.5 ml of Tris-citrate buffer, incubated at 37^o C for 30 min and centrifuged, followed by two further washes in Tris-citrate buffer. The final pellet was resuspended in Tris-citrate buffer to give a concentration of 10^6 cells/ml and used for binding assays. The determination of non-specific binding, the incubation conditions and the termination of the assays for different radio-ligands were carried out in the same way as described above.

Data analysis: All binding data was analysed using linear regression and statistic significance was obtained using student's t-test.

Electrophysiology: The experimental set-up and solutions used in electrophysiological experiments are described in detail in **Appendix II**. In electrophysiological experiments, all compounds were dissolved in the external solution just before use.

Selection of plants and compounds: There are two ways to start the screening. One way is to select plants by their confirmed effectiveness as sedative, anxiolytic, anti-convulsive and anti-epilepsy according to the TCM clinical literature. Although these plants were chosen as a single plant for the screening work, most of them are the main elements of known prescriptions which possess the above mentioned clinical properties. For example, *Pseudostellaria heterophylla* was chosen for screening from a known Chinese prescription named Kangdianling (Panacea of anti-epilepsy). *Angelica dahurica* was identified from a secret anti-epilepsy prescription containing 10 plants donated by a known TCM doctor in Beijing.
Another way to identify compounds from TCM plants is to screen compounds (or plants containing the compounds) which were reported to have CNS activities *in vivo*. Due to various reasons, e.g. the general lack of sensitive detecting systems like the receptor or receptor subtypes selective radio-ligand binding assay, a large number of studies have used purified or semi-purified compounds in *in vivo* studies rather than *in vitro* experiments. Since most of these data is published exclusively in Chinese language, it is not available for the scientists in western countries. In the book of Jiang and Xiao (1986) alone, there are more than one hundred compounds showing analgesic, anticonvulsive, anxiolytic, sedative and hypnotic effects. We did not conduct the investigation in this way yet, but it would potentially be a rewarding source to seek active compounds for specific CNS receptor systems.

Extraction and preliminary separation of plant extracts by HPLC: All plants (5 g) were extracted with 50 ml 50% ethanol (volume/volume) for 24 hrs in room temperature (ca. 23 ^{o}C). Plants were cut to small pieces and homogenised by using Ultra Turrax homogeniser. The homogenate was extracted for another 24 hrs by standing in room

temperature. The extractions were terminated by a gravity filtration (Whatman no. 1 filter paper). The extracts (equivalent to ca. 500 mg original dry plants per tube) were lyophilised at reduced pressure.

The dried extracts were re-dissolved in 5 ml acetonitrile (CH_3CN)/water (25%/75% v/v), and 0.5 ml of the solution was applied to Waters HPLC, C_{18} Delta-Pak 3.9×150 mm analytic reverse phase column at flow 1.2 ml/min using linear gradient from 25% to 100% acetonitrile (75% to 0% of water) within 50 minutes under UV detection (210 nm). A linear gradient from 0% to 100% acetonitrile (Water run, 100% to 0% of water) were used for the fractions, which came out at the very beginning of the previous HPLC and was found active in the [^{3}H]muscimol binding. Usually, these fractions are considered as GABA it self. 1 min fractions were collected and lyophilised for binding assays. 25 µl 93% ethanol were added to fractionated plant samples as well as control samples in the screening binding assays. Because the drugs determining the non-specific binding were dissolved in 93% ethanol, 25 µl Tris-citrate buffer were added to these samples in the binding assays.

4.2.3. Approaches for biochemical and pharmacological characterisation of active compounds

***IC_{50}*:** IC_{50} is the concentration of compounds causing 50% inhibition of radioligand specific binding to the receptors. The IC_{50} values is determined by including a range of concentrations of a compound (e.g. 10 nM to 100 µM) in the given receptor binding assay carried out with a fixed concentration of radioligand and membrane preparation. This value is used to evaluate the potency of a purified substance at the relevant receptor or binding sites.

***GABA-ratio*:** Due to the allosteric interaction, GABA and $GABA_A$ receptor agonists can modulate the binding affinity of BZD site ligands as tested by [^{3}H]BZD binding assays. GABA enhances, has no effect or reduces the affinity of BZD site agonists, antagonists and inverse agonists, respectively. Accordingly, GABA produces a shift of the dose-response curve to the left, no change or to the right for BZD site agonists, antagonists and inverse agonists, respectively. Thus, GABA-ratio (or GABA shift) is considered as a indicator for the efficacy of BZD site (receptor) ligands. The GABA-

ratio is defined as the IC_{50} value of compound inhibiting the [^{3}H]BZD specific binding in absence of GABA divided by the IC_{50} value in presence of GABA (usually 100 μM) in the binding assays. In general, full agonists give a ratio ≥ 2, partial agonists have a ratio between 1 and 2, antagonists have a ratio around 1, partial inverse agonists give a ratio between 1 and 0.5, while full inverse agonists have a ratio ≤ 0.5.

***Scatchard plot analysis*:** In order to determine the nature of action (competitive or non-competitive) for a substance with a binding site, saturation binding experiments are done in the presence or absence of the compound. Scatchard plot is a standard method for deriving equilibrium binding parameters (Affinity binding constant, K_D; and maximal concentration of binding sites, B_{max}) from saturation binding data. A range of radio-ligands (e.g. 0.2 – 10 nM for [^{3}H]diazepam, 0.05 – 15 nM for [^{3}H]Ro 15 1788 and 0.2 – 25 nM for [^{3}H]muscimol binding) are included in the binding assays. Protein was estimated spectrophotometrically using the Bio-Rad D_C Protein Assay Kit, using bovine serum albumin as standard.

***Determination of the selectivity*:** Different subtype of the $GABA_A$Rs has a distinct distribution in the brain. The primary tests on different brain region will show whether a purified compound will have potential receptor subtype selectivity. With this information, the subsequent tests on the different subunit combinations of the recombinant $GABA_A$Rs will further verify its selective activity.

***Functional characterisation*:** Functional characterisation of the active compounds can be achieved by directly measuring the Cl^- currents induced by the compounds on baculovirus/Sf9 cell system expressing recombinant $GABA_A$Rs using the whole-cell patch- clamp technique. Combining and comparing these data with data from the receptor binding assays, it is possible to thoroughly characterise the active compounds *in vitro*.

4.3. Results and Discussion

4.3.1. Results of the isolation and characterisation of active compounds from TCM plants

4.3.1.1. An overview of the screening results

Table 12. Summary of plants screened for CNS activity *in vitro* ∗

Chinese Name	Latin name	[^{3}H]flunitrazepam	[^{3}H]muscimol	Compounds purified
Baijiang	*Patrinia villosa*	4–10 min 40% ↓	0	
Baijuihua	*Chrysanthemum morifolium*	12, 15 min ↓	×	Acacetin (IC_{50} ~ 20 μM)
Baizhi	*Angelica dahurica*	↓↓	0	Phellopterin byakangelicol imperatorin (**Appendix III**)
Cangzhu	*Atractylodes chinensis*	25 min 50% ↓	25 min 207%↑	
Chaihu	*Bupleurum chinense*	0	0	15, 20, 25 min ↓ [^{3}H]kainic acid Saikosaponin A Saikogenin G (IC_{50} ~ 50 μM)
Dahuang	*Rheum palmatum*	0	Water run 3, 4, 6 min ↓	
Gualou	*Trichosanthes kirilowii*	4, 10, 15, 20 min ↓↓	×	
Houpo	*Magnolia officinalis*	15, 20 min ↓ 23 min ↑	15, 20 min ↑ 23 min ↑	Honokiol, magnolol (**Appendix IV**)
Shengma	*Cimicifuga foetida*	0	0	Cimicidanol ([^{35}S]TBPS ↓) IC_{50} ~ 4 μM
Taizishen	*Pseudostellaria heterophylla*	Ca. 20 active compounds ↓↓↓	0	βCCE, βCCM
Wuzhuyu	*Evodia rutaecarpa*	22 – 27 min ↓	0	Rutaecarpine (IC_{50} ~ 1μM)
Xinyi	*Magnolia liliflora*	×	6 min 300% ↑	
Yanhusuo	*Corydalis yanhusuo*	0	20-22 min 50% ↓ Single peak	It could be bicuculline

∗ 0 = no effect; × = no assay; ↓, ↓↓, ↓↓↓ = representing 40 to 60%, 60 to 80%, and 80 to 100% inhibition of the specific binding in the corresponding binding assays, respectively. ↑ = enhancing effect. The retention time was from the HPLC running linear gradient from 25% to 100% acetonitrile (75% to 0% of water) or from 0% to 100

% acetonitrile (Water run) within 50 minutes using UV detection (210 nm) on Waters HPLC, C_{18} Delta-Pak 3.9 × 150 mm analytic reverse phase column at flow 1.2 ml/min.

It has been generally realised that if the selection of plants were made on the grounds of their traditional usage, the chances for finding positive activity would be greater (Brito, 1996). In our current screening project, totally ca. 50 TCM plants were screened mainly for their activity to the various binding sites (GABA, BZD and TBPS binding sites) on the $GABA_A$Rs using *in vitro* radioligand-receptor binding assays (except some of the plants have also been tested against kainate receptor). Activities were found in 13 plants. 12 active compounds from 7 plants were purified and chemically elucidated (see Table 12). Because of the lower affinity found in the primary experiment, some of the active compounds (acacetin, rutaecarpine) were not investigated further.

Table 13. Plants with no activity found ∗

Chinese Name	Latin Name	Chinese Name	Latin Name
Huoxiang	*Agastache rugosa*	Longdanchao	*Gentiana scabra*
Gaoliangjiang	*Alpinia officinarum*	Zaojia	*Gleditsia sinensis*
Binglang	*Areca catechu*	Baimaogen	*Imperata cylindrica*
Madouling	*Aristolochia contorta*	Nuzhenzi	*Ligustrum lucidum*
Yinchenhao	*Artemisia capillaris*	Roudoukou	*Myristica fragrans*
Xixin	*Asarum heterotropoides*	Hangbai	*Phellodendron amurense*
Shayuanzhi	*Astragalus complanatus*	Huhuanglian	*Picrorhiza Kurrooa*
Chaojueming	*Cassia tora*	Biba	*Piper longum*
Guizhi	*Cinnamomum cassia*	Jiegeng	*Platycodon grandiflorum*
Rouchongrong	*Cistanche salsa*	Heshouwu	*Polygonum Multiflorum*
Shechuangzi	*Cnidium monnieri*	Qiancaogen	*Rubia cordifolia*
Yiyiren	*Coix lachryma-jobi*	Dingxiang	*Syzygium aromaticum*
Xiaohuixiang	*Foeniculum vulgare*		

∗ No activity are found in radioligand-receptor binding assays of [^{3}H]muscimol, [^{3}H]flunitrazepam and [^{35}S]TBPS binding to rat cortical cortex membrane preparation.

4.3.1.2. The characterisation of phellopterin

Angelica dahurica is used as an antipyretic and analgesic for cold, headache, and toothache in traditional Chinese medicine and it is known to contain a large number of coumarins and furanocoumarins. The furanocoumarin derivatives phellopterin, byakangelicol and imperatorin were purified from *Angelica dahurica* (see **Appendix III**). Phellopterin, the most potent one (determined as comparing of the IC_{50}s) among the three derivatives, was found inhibiting [^{3}H]diazepam and [^{3}H]Ro 15-1788 binding to the BZD binding site of the rat brain $GABA_AR$ *in vitro* with IC_{50} values of 400 and 680 nM, respectively. The GABA ratios of 1.3 for [^{3}H]diazepam binding and 1.7 for [^{3}H]Ro 15-788 binding indicate that phellopterin is a partial agonist at the BZD site. This finding was supported by TBPS shift experiments. Scatchard plot analysis showed that phellopterin increased the K_D without changing the B_{max} in [^{3}H]diazepam binding to various rat brain membrane preparations, which indicate a competitive inhibition of phellopterin. As discussed in Section 1, the partial agonist is more favourable for the development of new generation of anxiolytics as compared to full agonist for their lower intrinsic efficacy. This partial agonistic nature makes phellopterin a more interesting lead for developing active derivatives at the BZD site.

4.3.1.3. The isolation and characterisation of honokiol and magnolol

Two parts of work have been done on the characterisation of honokiol and magnolol. The first part of the work present here was done in this laboratory. The second part of work was done in collaboration with Dr. Squires in USA (**Appendix IV**).

The isolation and purification of honokiol and magnolol: Batches of the root from Magnolia officinalis were extracted by 10 volumes of ethanol 50% (volume/volume) (e.g. 5 g plant in 50 ml ethanol) for ca. 24 hours at room temperature and homogenised by an Ultra-Turrax homogeniser. The homogenate was further extracted for 24 hours at room temperature. The extractions were terminated by a gravity filtration and the ethanol extracts were lyophilised at reduced pressure. Samples (corresponding to ca. 500 mg original plant/tube) were re-dissolved in a mixture of acetonitrile/water (CH_3CN/H_2O, 25%/75%). Compounds in the extract were applied (in amounts corresponding to ca. 50 mg original plant material) and separated on a HPLC, C_{18} Nova-Pak reverse phase column (3.9×150 mm) at flow rate of 1.2 ml/min using linear

gradient (25% to 100% of CH_3CN/75% to 0% of H_2O) within 35 min using UV detection (210 nm). Fractions with retention times of 20 min and 22 min contained compounds that showed enhancing effect of [^{3}H]muscimol binding a membrane preparation from rat cortex. The fractions were pooled separately and applied to the same HPLC column at flow 1.2 ml/min using 42% isocratic ratio of CH_3CN/ H_2O, and two fractions with retention times of 12 min, 19 min were collected containing enhancing activity of [^{3}H]muscimol binding. The chemical structures of purified compounds were identified as honokiol and magnolol using NMR spectroscopy and Mass spectrometry by Prof. Olov Sterner in Division of Organic Chemistry 2, Lund University, Sweden.

Pharmacological characterisation of honokiol and magnolol: After the structure elucidation of the purified honokiol and magnolol, the work of pharmacological characterisation was done using pure honokiol and magnolol obtained from Nacalai Tesque Inc. Japan and The Institute of Drug Identification, Beijing, China (the purity from different batches of honokiol and magnolol was checked by HPLC, corresponding to more than 98% of the compounds).

Honokiol and magnolol (0.02 ~ 0.6 mM), in a concentration dependent manner, showed enhancement of [^{3}H]muscimol binding various membrane preparations (Figure 17a, 17b, 17c and 18a, 18b, 18c). At concentration higher than 2mM, both honokiol and magnolol fell out of assay binding buffer solution, therefor at these high concentration the compounds may have unspecific inhibition of [^{3}H]muscimol or [^{3}H]flunitrazepam binding to rat brain membrane preparations as well as to various human recombinant $GABA_A$ receptor subunit combinations expressed in Sf-9 insect cells. Thus, the maximum enhancing effects of honokiol and magnolol are not available.

Honokiol and magnolol have similar enhancing effects on [^{3}H]muscimol binding to cortical and hippocampal membrane preparations; both substances show no enhancing effect on [^{3}H]muscimol binding to cerebellar membrane preparation. (Figure 17a and 18a).

In the binding assays using membrane preparation of various human recombinant $GABA_A$ receptor subunit combinations, honokiol and magnolol show a significant enhancing effect on [^{3}H]muscimol binding to all the six subunit combinations tested ($\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_2$, $\alpha_2\beta_3\gamma_{2S}$, $\alpha_2\beta_3$, $\alpha_3\beta_3\gamma_{2S}$, $\alpha_5\beta_3\gamma_{2S}$; Fig. 17b, 17c and 18b, 18c). Within

these six $GABA_A$ receptor subtypes, a more potent enhancing effects of honokiol and magnolol was found on [^{3}H]muscimol binding to the subunit combinations containing the α_2 subunit ($\alpha_2\beta_3\gamma_{2S}$ and $\alpha_2\beta_3$); while a less potent enhancing effect was found on [^{3}H]muscimol binding to the subunit combination of $\alpha_5\beta_3\gamma_{2S}$ (Figure 17b, 17c and 18b, 18c).

Saturation binding experiments showed that honokiol (200 μM) significantly increased both the binding affinity (decrease in affinity constant, K_D) and the number of maximum binding sites (B_{max}) of [^{3}H]muscimol binding to subunit combinations containing α_1 subunit (Table 14). Honokiol markedly increased the maximum binding sites (B_{max}), but not the affinity (K_D) on [^{3}H]muscimol binding to subunit combinations containing α_2 subunit (Table 14).

The data of [^{3}H]flunitrazepam binding to membrane preparations of rat brain cortex, cerebellum and hippocampus was shown in Fig. 19a and 19b. Honokiol showed inhibitory effects on [^{3}H]flunitrazepam binding to all the three membranes tested (IC_{50} ~ 200 μM). Magnolol, in the concentration range of 20 μM to 100 μM, showed enhancing effects on [^{3}H]flunitrazepam binding to the membranes tested. Saturation binding experiments showed that honokiol (200 μM) significantly decreased binding affinity (3-5 folds) with slight increase (ca. 1.2 fold) or without changing B_{max}, magnolol (60 μM) slightly increased both the binding affinity (1.5 fold) and B_{max} (ca. 1.2 fold) of [^{3}H]flunitrazepam binding to membrane preparations of rat brain cortex, cerebellum and hippocampus.

In the preliminary electrophysiological experiments, Sf-9 insect cells expressing human recombinant $GABA_A$ receptors were used. Honokiol (10 μM) showed a similar potency as compared to diazepam (10 μM), in potentiation of GABA-induced whole-cell chloride currents in all the subtypes tested $\alpha_x\beta_{2/3}\gamma_{2S}$ (X=1,2,3).

In the experiments of [^{3}H]SCH 23390 binding to membrane preparation of rat brain striata, both honokiol (IC_{50} of 22 ± 1 μM from three independent experiments) and magnolol (IC_{50} of 32 ± 2 μM from three independent experiments) showed inhibitory effects. In the experiments of [^{3}H]QNB binding to membrane preparation of rat brain cortex, both honokiol (IC_{50} of 83 ± 10 μM from three independent experiments) and

magnolol (IC_{50} of 76 ± 9 μM from three independent experiments) showed inhibitory effects.

Figure 17a.

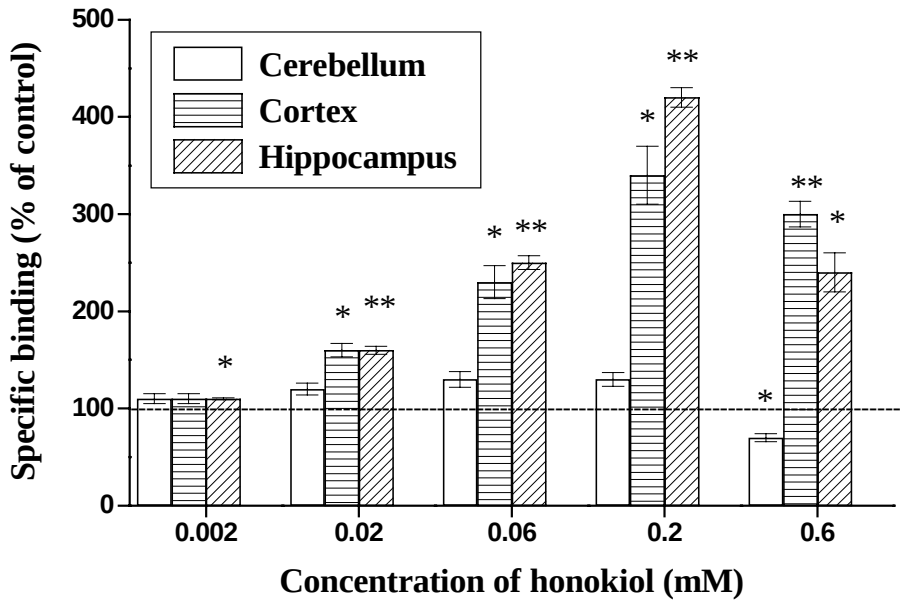


Figure 17a. Effects of honokiol on [^{3}H]muscimol (10 nM) binding to membrane preparations of rat brain cortex, cerebellum and hippocampus.

* $P < 0.05$, ** $p < 0.01$ compared to control value (100%).

Figure 17b.

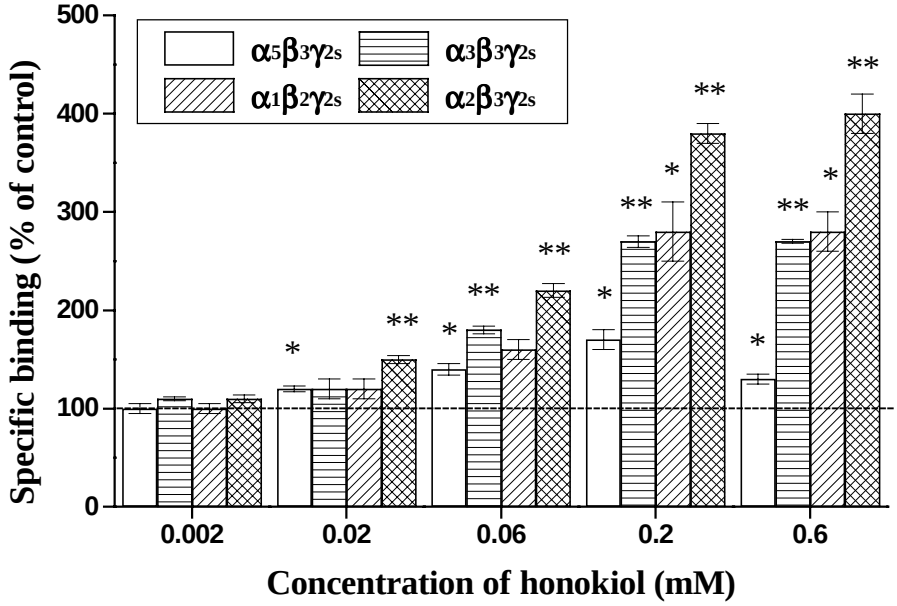


Figure 17b. Effects of honokiol on [^{3}H]muscimol (10 nM) binding to various human recombinant $GABA_A$ receptor constructed by α, β and γ subunit combinations expressed in Sf-9 insect cells by baculovirus.

* $P < 0.05$, ** $p < 0.01$ compared to control value (100%)

Figure 17c.

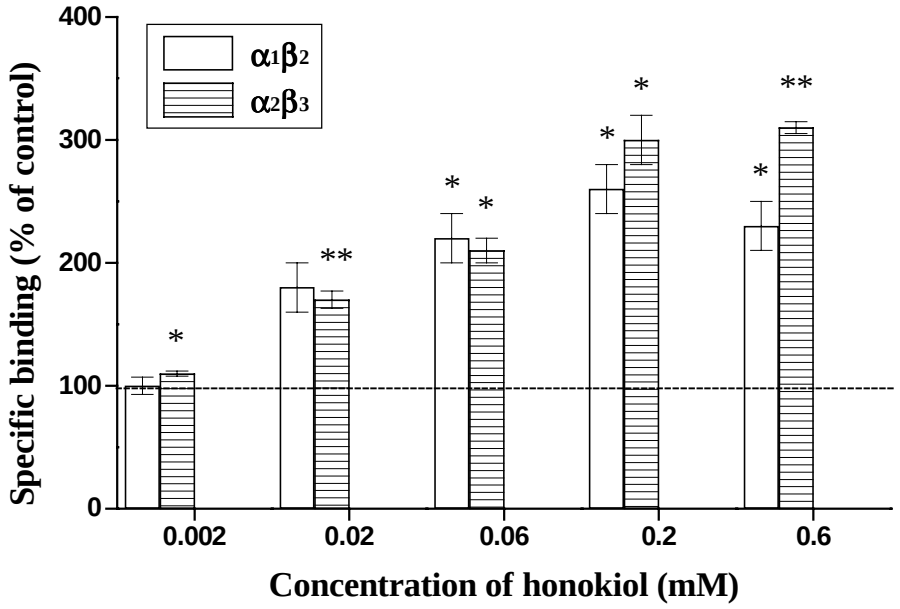


Figure 17c. Effects of honokiol on [^{3}H]muscimol (10 nM) binding to human recombinant GABA$_A$ receptor subunit combinations of $\alpha_1\beta_2$, $\alpha_2\beta_3$, expressed in Sf-9 insect cells by baculovirus.

* P < 0.05, ** p < 0.01 compared to control value (100%).

Figure 18a.

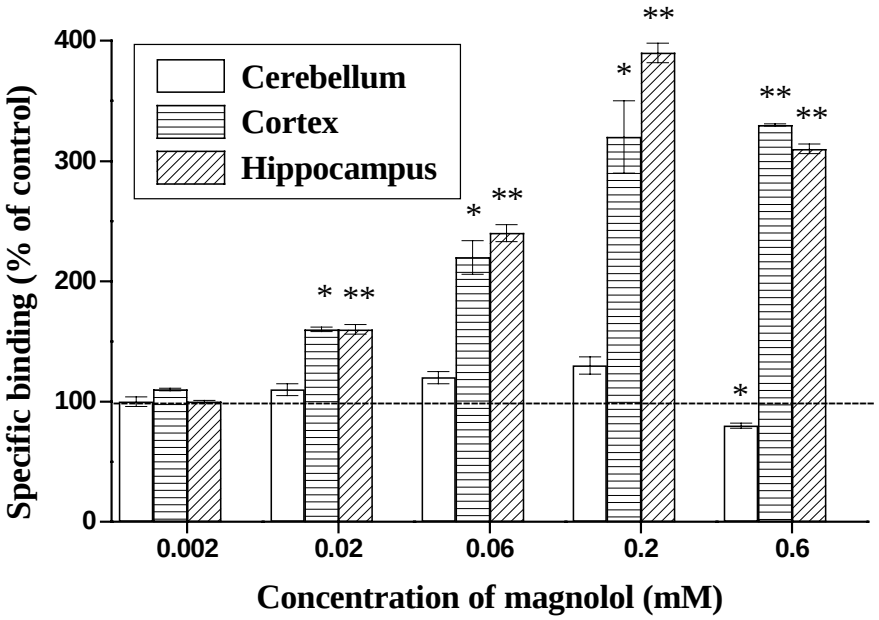


Figure 18a. Effects of magnolol on [^{3}H]muscimol (10 nM) binding to membrane preparations of rat brain cortex, cerebellum and hippocampus.

* $P < 0.05$, ** $p < 0.01$ compared to control value (100%).

Figure 18b.

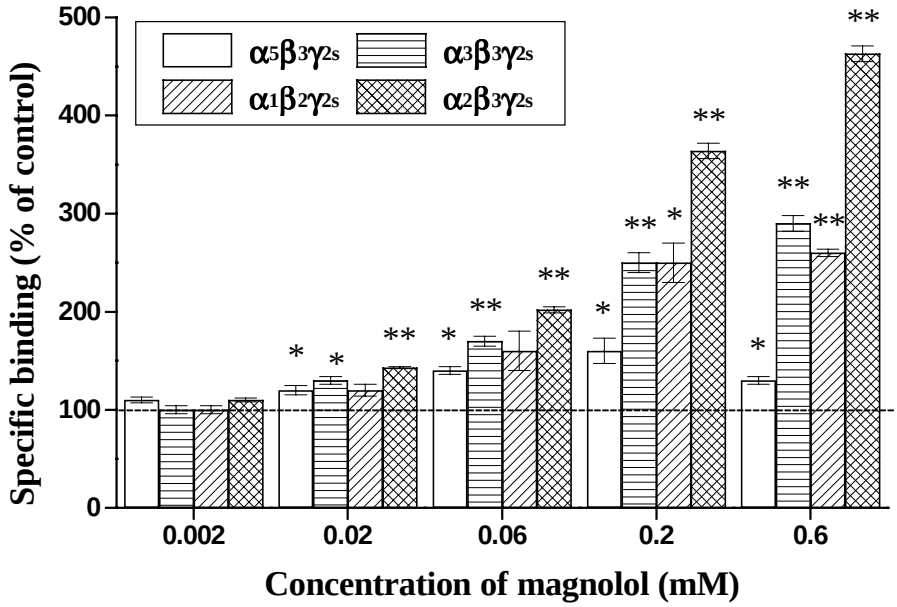


Figure 18b. Effects of magnolol on [^{3}H]muscimol (10 nM) binding to various human recombinant $GABA_A$ receptor constructed by α, β and γ subunit combinations expressed in Sf-9 insect cells by baculovirus.

* $P < 0.05$, ** $p < 0.01$ compared to control value (100%).

Figure 18c.

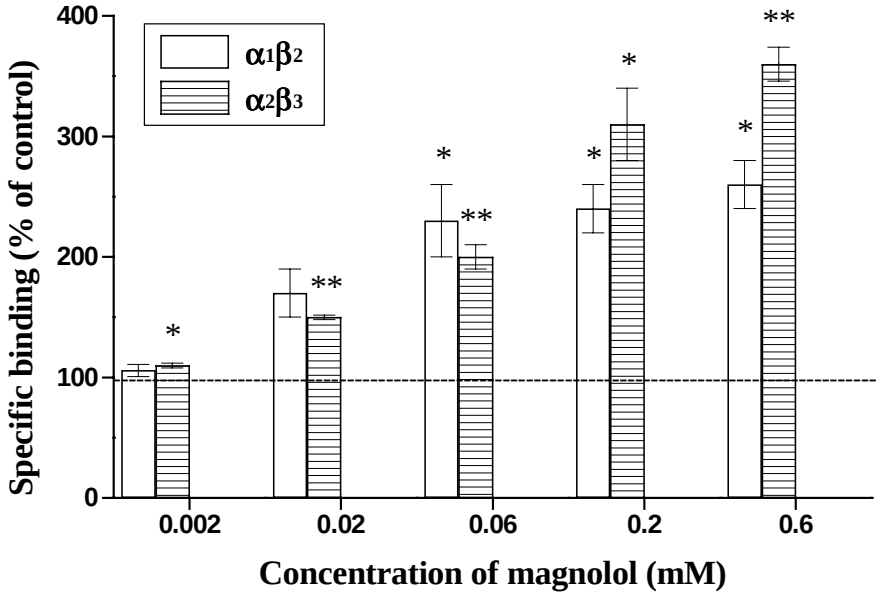


Figure 18c. Effects of magnolol on [^{3}H]muscimol (10 nM) binding to human recombinant GABA$_A$ receptor subunit combinations of $\alpha_1\beta_2$, $\alpha_2\beta_3$, expressed in Sf-9 insect cells by baculovirus.

* $P < 0.05$, ** $p < 0.01$ compared to control value (100%).

Figure 19a.

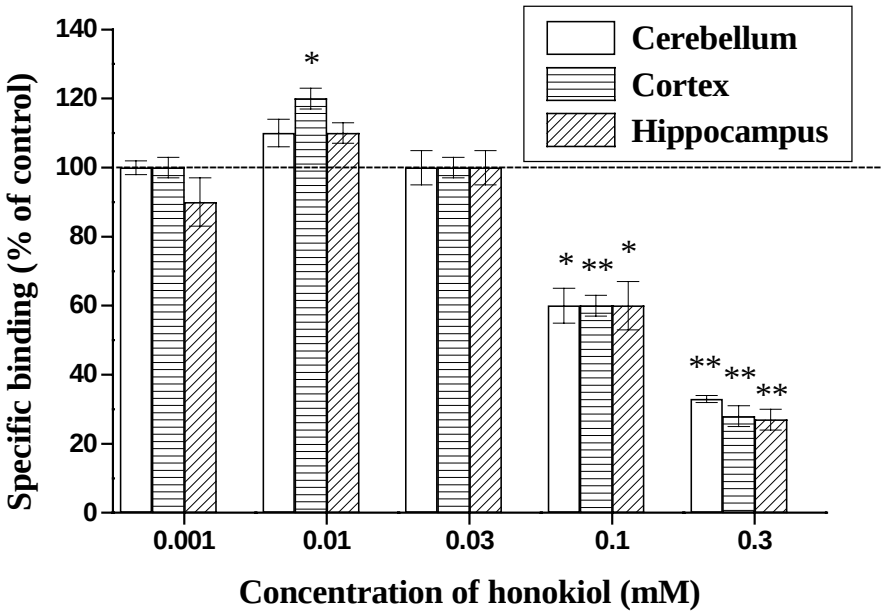


Figure 19a. Effects of honokiol on [^{3}H]flunitrazepam (1 nM) binding to membrane preparations of rat brain cortex, cerebellum and hippocampus.

* $P < 0.05$, ** $p < 0.01$ compared to control value (100%).

Figure 19b.

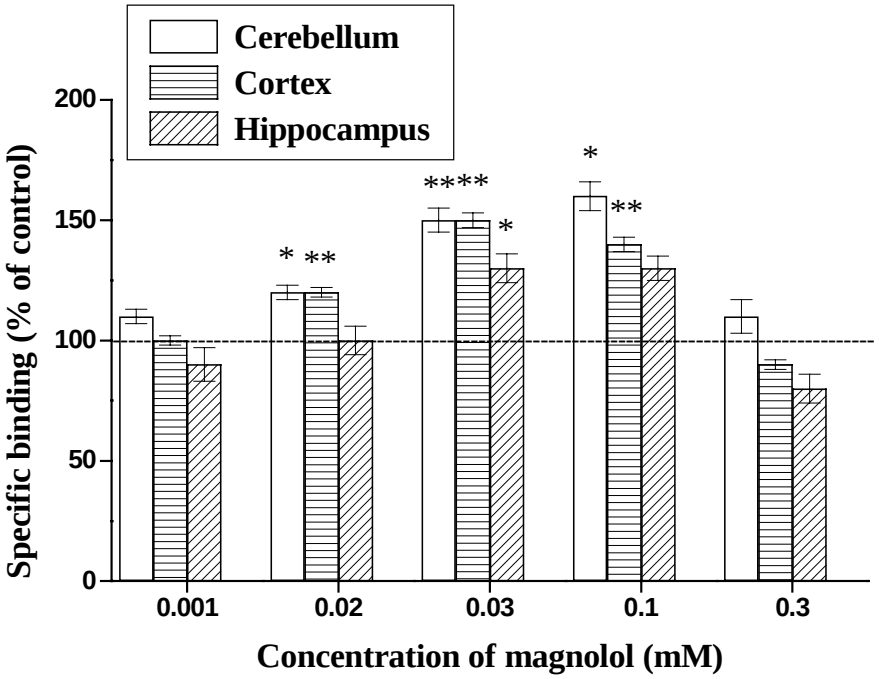


Figure 19b. Effects of magnolol on [^{3}H]flunitrazepam (1 nM) binding to membrane preparations of rat brain cortex, cerebellum and hippocampus.

* $P < 0.05$, ** $p < 0.01$ compared to control value (100%).

Table 14. Scatchard Plot analysis of the effect of honokiol on [^{3}H]muscimol binding to various human recombinant subunit combinations of $GABA_A$ receptor expressed in Sf-9/baculovirus system

Subunit combinations	Control		Honokiol (200 μM)	
	K_D (nM)	B_{max} (pmol/g protein)	K_D (nM)	B_{max} (pmol/g protein)
$\alpha_1\beta_2$	13 ± 1	2140 ± 340	5 ± 1 [a]	3670 ± 330 [a]
$\alpha_1\beta_2\gamma_{2S}$	25 ± 2	970 ± 10	10 ± 2 [a]	1750 ± 100 [a]
$\alpha_2\beta_3$	15 ± 3	1270 ± 150	11 ± 2	3220 ± 410 [a]
$\alpha_2\beta_3\gamma_{2S}$	18 ± 5	932 ± 160	16 ± 5	2970 ± 410 [a]

Values are expressed as mean ± S.E.M. from 3 estimations on various batches of the different subunit combinations. K_D is the affinity constant of [^{3}H]muscimol binding, the higher value means the lower affinity. B_{max} is the maximum binding sites on the various subunit combinations for [^{3}H]muscimol binding.

[a] $p < 0.05$ compared to the relative values in control experiments.

The bark of the root and stem of *Magnolia* species has been used as a folk medicine in China for the relief of fever, headache, anxiety and nervous disturbance (Fujita et al., 1972). The two neolignan compounds honokiol ($C_{18}H_{18}O_2$) and magnolol ($C_{18}H_{18}O_2$), main components of Chinese medicinal plant *Magnolia officinalis*, were reported to have many pharmacological activities, such as anti-platelet, anti-microbial, anti-tumour, insecticidal, anti-asthmatic, anti-oxidant, anti-emetic actions (For review, see Sarker, 1997). Most interestingly, these two compounds also showed some central depressant effects such as: sedation, ataxia, muscle relaxation as well as having anxiolytic activity in animal experiments, but the mechanism of action remains unknown (Watanabe et al., 1983; Maruyama et al., 1998, Kuribara et al., 1998).

The central depressant effects of honokiol and magnolol were first reported by Watanabe et al. (1983). Magnolol and honokiol produced sedation, ataxia, muscle relaxation in mice and a dose-dependent loss of the righting reflex in young chickens. Pre-treatment of mice with magnolol (100 mg/kg) inhibited tonic extensor convulsions and death produced by an intracerebroventricular injection of penicillin G potassium. It was concluded that magnolol causes a depression of the ascending activating systems as well as of the spinal cord. To study the possible mechanism, through which honokiol and magnolol elicit their central depressant effects, Tsai et al. (1995a) examined the influence of these two phenolic compounds on 25 mM K^+-stimulated release of [^{3}H]acetylcholine from hippocampal slices of rats. Honokiol, but not magnolol, elicited a concentration-dependent enhancement of K^+-evoked acetylcholine release. Since the addition of tetrodotoxin, pilocarpine, or methoctramine had no effect on the action of honokiol, the authors concluded that honokiol enhanced K^+-evoked acetylcholine release directly on hippocampal cholinergic terminals via receptors other than the M2 cholinergic subtypes. The same authors (Tsai et al., 1995b) showed that magnolol (1 ~ 100 μM) had no effect on 5-HT release from rat hippocampal slices but elicited a dose-dependent inhibition of 5-HT release from cortical slices. The inhibitory effect of magnolol on K^+-stimulated 5-HT release from the cortex was not affected by either antagonists (metergoline, propranolol and cyproheptadine 0.01 ~ 10 μM) of various 5-HT receptor subtypes or by the voltage-dependent sodium channel blocker tetrodotoxin (1μM). It was concluded that the suppression of brain 5-HT release by magnolol is site-specific, and the suppression of cortical 5-HT release by magnolol is not mediated by the

5-HT autoreceptors at the 5-HT terminals. Recently, it was shown that honokiol and magnolol are the active agents in extracts of Saiboku-to, an oriental herbal medicine. Mice were treated separately with honokiol and magnolol for 7 days and evaluated by means of elevated plus-maze test. Honokiol (0.19 mg/kg) is an active anxiolytic agent in mice and ca. 2 fold more effective than magnolol (0.49 mg/kg). Honokiol is at least 5000 times more potent than Saikoku-to in reducing anxiety in mice as evaluated by elevated plus-maze (Maruyama et al., 1998). In another study by means of elevated plus-maze test, honokiol (20 mg/kg) was found having anxiolytic effect, without changing motor activity (measured by a tilting-type ambulometer with bucket-like Plexiglas activity cage) or disruption of traction performance (measured by horizontal wire test). The effect of honokiol was inhibited by flumazenil, (+)bicuculline and caffeine. It was concluded that honokiol, as compared to diazepam, selectively induces an anxiolytic effect with less liability of eliciting motor dysfunction and sedation (Kuribara, et al., 1998), physical dependence, central depression and amnesia (Kuribara et al., 1999).
In our experiments, honokiol and magnolol showed similar effects on [^{3}H]muscimol binding to various membrane preparations. The comparable enhancing effect of honokiol and magnolol on [^{3}H]muscimol binding to $\alpha_1\beta_2\gamma_{2S}$, $\alpha_2\beta_3\gamma_{2S}$ subunit combinations as well as $\alpha_1\beta_2$, $\alpha_2\beta_3$ subunit combinations, suggests that the presence of γ subunit in the $GABA_A$ receptor complex is not necessary for the action of honokiol and magnolol on the GABA site.
The significant enhancing effect of honokiol and magnolol on [^{3}H]muscimol binding to rat brain cortical and hippocampal membranes, as compared to the no enhancing effect on cerebellar membrane suggested a possible selectivity of these two compounds to $GABA_A$ receptor subtypes. The effect was verified by binding assays of [^{3}H]muscimol binding to various human recombinant $GABA_A$ receptor subtypes, in which a stronger enhancing effect on [^{3}H]muscimol binding to $\alpha_2\beta_3\gamma_{2S}$, a significant weaker enhancing effect to $\alpha_5\beta_3\gamma_{2S}$ subunit combinations were seen. In the saturation binding assays, a differential effect of honokiol and magnolol on [^{3}H]muscimol binding to different subunit combinations were also observed. Honokiol changed the number of binding sites on $GABA_A$ receptors constructed by $\alpha_2\beta_3\gamma_{2S}$ and $\alpha_2\beta_3$ subunit combinations, suggested a non-competitive allosteric modification of the GABA binding site within the $GABA_A$ receptor complex. The changes of both the number of binding sites and the

binding affinity of [^{3}H]muscimol binding to receptor complexes of $\alpha_1\beta_2\gamma_{2S}$ and $\alpha_1\beta_2$ subunit combinations, suggested a mixture of competitive and non-competitive action of honokiol. A matching effect was also observed with magnolol (data not shown).
Although honokiol and magnolol showed similar effects on [^{3}H]muscimol binding to various membrane preparations, differential effects were seen for honokiol as compared to magnolol on [^{3}H]flunitrazepam binding to membrane preparations of rat brain cortex, cerebellum and hippocampus. Honokiol markedly decreased the affinity but with slight increase or without changing the B_{max} suggesting mixture action of competitive and non-competitive modulation on the BZD binding site on the $GABA_A$ receptors. For magnolol there is no competitive action was observed. These differences indicated a different mechanism of action of these two isomers on the $GABA_A$ receptor complex. This could possibly explain the different anxiolytic effects of honokiol and magnolol observed by Maruyama et al. (1998).
In whole-cell patch clamp experiments, the potency of 10 μM honokiol potentiating GABA-induced chloride current was comparable to that of diazepam with the same concentration. In binding assays conducted on membrane preparations of rat brain tissues and the recombinant human $GABA_A$ receptor subtypes expressed in Sf-9 insect cells, 20 μM honokiol and magnolol showed almost no effects. This discrepancy could be due to differences between the experimental conditions used in binding assays and functional assays (e.g. the Cl^- ion), suggested that honokiol and magnolol could be more potent interacting with the $GABA_A$ receptors *in vivo* than *in vitro*.
Since GABA predominantly mediates the inhibition of neuronal transmission, and our data showed that honokiol and magnolol, by allosteric modification, enhanced the function of GABA, this could partially explain the central depressant and anxiolytic effects of honokiol and magnolol. The different behaviour effect of honokiol compared to diazepam could be that honokiol preferentially interact with some subtypes of $GABA_A$ receptor (e.g. α_2 containing receptors), but not the others (e.g. α_5 containing receptors).
Since honokiol and magnolol also interacted with dopamine, serotonin (Tsai et al., 1995b), acetylcholine (Tsai et al., 1995a) and possibly some other neurotransmitter receptor systems as well, the in vivo central depressant and anxiolytic effect of them

could be an overall effects derived from the interaction with the different neurotransmitter receptor systems.

It would be interesting to determine and compare the differential effect of honokiol and magnolol on the different binding sites (e.g. GABA sites, BZD sites TBPS site etc.) presented on the different $GABA_A$ receptor subtypes. It is also important to further investigate the interaction of honokiol and magnolol with other neurotransmitter receptors.

The effects of honokiol and magnolol on the $GABA_A$ receptors have been characterised using another membrane preparations (EDTA/water dialyzed rat brain membranes), and the data is being published on Neurochem. Res. (**Appendix IV**)

4.3.1.4. The isolation and purification of β-carboline derivatives from *Pseudostellaria heterophylla*

The proconvulsant β-carboline derivatives were discovered in attempts to isolate endogenous ligands for BZD receptors. The ethyl ester of β-carboline-3-carboxylic acid (βCCE) was originally isolated from human urine (see Section 1.2.2.3.). In the screening of Chinese medicinal plants for compounds having GABAA receptor affinity *in-vitro*, we isolated and identified βCCM and βCCE from the root of *Pseudostellaria heterophylla*. Although β-carbolines are well known as BZD receptor ligands, this is to our knowledge the first report demonstrating a plant origin of these β-carboline-3-carboxylic acid derivatives.

Batches of totally 10 kg dried roots of *Pseudostellaria heterophylla* were extracted with water for ca. 24 hours at room temperature. The extraction was discarded. This procedure was repeated twice and followed by adding 10 times ethanol (50% v/v) to the plant root (v/w) and homogenised by an Ultra-Turrax homogeniser. The mixture was extracted for at least 48 hours. The extractions were terminated by a gravity filtration (Whatman no. 1 filter paper), followed by a rotary evaporation to remove the ethanol and water phase to 1/15 of the original volume. The water solution was applied and gently shook with Chromosorb for 30 min at room temperature. After washing of Chromosorb with water and ethanol (10% v/v), the [^{3}H]flunitrazepam binding inhibitory activity was eluted with ethanol (50% v/v) and ethanol (93% v/v). These two ethanol extracts were evaporated at reduced pressure. The first active fraction (from the ethanol

(50% v/v) elution of the Chromosorb extraction) were re-dissolved in ethanol (50% v/v), and applied in batches of 10 ml to a Sephadex LH-20 column (5 × 100 cm). The Sephadex LH-20 column was first eluted with ethanol (25% v/v) at a flow rate of 1.5 ml/min for 12 hours containing no inhibitory activity. The ethanol elute was discarded. The column was further eluted in 13.5 ml fractions with ethanol (40% v/v) for 12 hours and ethanol (50% v/v) for 12 hours. The elute yielded four fractions containing active compounds on displaying [^{3}H]flunitrazepam binding. Fractions showing inhibition of [^{3}H]flunitrazepam binding were pooled and freeze-dried. Fraction No. 1 was re-dissolved in acetonitrile (CH_3CN)/water (25%/75% v/v). Compounds were separated on a Waters HPLC, C_{18} Delta-Pak PrepPak 25 × 100 mm plastic reverse phase column, at flow rate 7 ml/min under linear gradient 25% to 50% CH_3CN/75% to 50% water within 50 min using UV detection (210 nm). Fractions with retention times of 22-25 min, 28-31 min, 36-37 min and 39-41 min contained compounds that showed inhibition of [^{3}H]flunitrazepam binding. The fractions were pooled and applied to Waters HPLC, C_{18} Delta-Pak 3.9 × 150 mm analytic reverse phase column at flow 1.2 ml/min using various isocratic ratios of CH_3CN/water. The first two active fractions (F1 and F2) were purified and the chemical structure of these compounds were identified as the methyl and ethyl ester of β-carboline-3-carboxylic acid, respectively. The final separation of F1 yielded a single peak at 7 min using isocratic CH_3CN/water (28%/72% v/v), and F2 at 10 min using isocratic CH_3CN/water (30%/70% v/v). A total of approximate 25 μg of each of these two active compounds was purified for the structure elucidation. The chemical structures of purified compounds were identified using NMR spectroscopy and Mass spectrometry by Prof. Olov Sterner in Division of Organic Chemistry 2, Lund University, Sweden.

The identical retention time obtained from βCCM and βCCE with F1 and F2, respectively, under the same condition of the final HPLC step of F1 and F2, supported the structural identification.

We chose to investigate *Pseudostellaria heterophylla*, because it was a main element from a well-known traditional Chinese medicinal prescription named *Panacea anti-epilepsy.* As seen by the separation of compounds on HPLC, there are at least 10 or more active substances that show inhibition of [^{3}H]flunitrazepam binding with similar potency *in-vitro*. Although the presence of βCCE could be argued as an artifact of the

isolation procedure with ethanol (In reality it may not be the case, because the condition for the formation of βCCE in urine was high temperature and lower pH (Nielsen et al., 1979; Schmiechen et al., 1993), that does not exist in the isolation of βCCE from *Pseudostellaria heterophylla*), the presence of βCCM can not be an isolation artifact. In addition, the HPLC fractions of extracts of *Pseudostellaria heterophylla* from acetonitrile, ethyl acetate, isopropanol and DMSO showed a similar activity (both retention time and potency) as compared to ethanol extract.

Both βCCM and βCCE are known as potent proconvulsant acting within $GABA_A$/BZD receptor complex. The plant is used in the reverse pharmacological direction as an anti-convulsant treatment in traditional Chinese medicine. This raised the intriguing possibility that some other β-carboline derivatives or other compounds having anti-convulsant property presence in this plant.

4.3.2 Characterisation of the semi-synthesised derivatives of nature-occurring compounds (Appendix II)

As we can see from Table 7, the synthetic derivatives of nature-occurring compounds take a great part of the total nature-originated drugs. Therefore, based on the available data of nature-occurring compounds to investigate their synthetic or semi-synthetic derivatives for a possible activity would be a natural consideration. The flavonoids are the most common constituent occurring in high plants with various biological and pharmacological properties. Many flavonoids have been purified from plants and were shown to bind to BZD receptors in the CNS *in vitro* (for an overview, see **Appendix I**). The first BZD receptor active flavonoid described, amentoflavone, as well as another two flavonoid (dinatin and skrofulein) were isolated from Africa medicinal plants in our laboratory. Chrysin, apigenin, kaempferol and four other active flavone derivatives were isolated from Latin American and New Zealand medicinal plants, respectively. In addition, two semisynthetic flavonoids (6,3'-dinitroflavone and 6-bromoflavone) were reported binding to BZD receptors with high potency.

In the screening of 32 commercially available flavonoids, seventeen structurally related flavonoid derivatives are found to inhibit the binding of [^{3}H]diazepam to $GABA_A$/BZD receptors. 6-methylflavone, the most potent one (IC_{50} value of 120 nM) was characterised by binding as well as functional assays. Saturation binding experiments

showed that 6-methylflavone is a competitive inhibitor of [^{3}H]Ro 15-1788 binding to rat cortical $GABA_A$/BZD receptors. Combined the data of the GABA ratios for [^{3}H]diazepam binding to various rat brain membrane preparations and the GABA ratios for [^{3}H]Ro 15-1788 binding to various recombinant human $GABA_A$/BZD receptor subunit combinations expressed in baculovirus/Sf9 insect cell system, with the data obtained from the whole-cell patch clamp on Sf-9 insect cells, we concluded that 6-methylflavone is most likely a antagonist on the BZD binding site. Meanwhile, based on available data, some general structure-activity relationship of flavonoids for their inhibitory effect on the $GABA_A$/BZD receptors have been discussed (**Appendix II**).

4.4. The blood-brain barrier and bio-availability of CNS active compounds

The blood-brain barrier (BBB) is a unique feature of the CNS, which isolates the brain from the external environment and prevents the access of the majority of circulating substances in the blood to the brain (for reviews, see Bonate, 1995; Abbott and Romero, 1996; Bradbury, 1997; Shah and Morradian, 1997; Pardridge, 1998; van de Waterbeemd et al., 1998; Jolliet-Riant and Tillement, 1999; Rubbin and Staddon, 1999; Saunders et al., 1999). The anatomic basis of BBB is the brain capillaries. They are formed by a complex cellular system including endothelial cells (brain endothelial cells adhere strongly to each other forming the specific structures called tight junctions, which prevent cell migration or molecule movement between endothelial cells), basal lamina (surrounding the endothelial cells on the CNS side, the basal lamina encloses contractile cells called pericytes which form an intermittent layer and probably play some roles in terms of phagocytosis activity and defence), astrocytic end feet (almost the entire surface of brain microvessels is covered with astrocytic feet processes, which build a continuous sleeve and maintain the integrity of the BBB by the synthesis and secretion of soluble growth factors essential for the endothelial cells), the perivascular macrophages and microglia cells.

It is generally accepted that compounds that are unionised at physiological pH, lipophilic and of low molecular weight (under a 400-600 Dalton threshold) can cross the BBB by lipid-mediated diffusion. Other compounds as AA, neuropeptides have specific carrier proteins, which either actively or passively transport these substances into the brain to ensure that the brain receives the nutrients, and metabolic substrates.

In general, there are two strategies for increasing the brain availability of drugs. The first one does not involve modification of the molecule of drugs, including 1). Direct injection of drugs into the CNS. It could be a useful way of introducing longer acting slow-releasing preparations, implanted devices etc. 2). Disruption of BBB function. This can be achieved by e.g. intracarotid arterial injection of a hypertonic solution mannitol or arabinose etc, which cause endothelial cell shrinkage and opening of the tight junctions for a period. This technique is only suitable for the acute delivery of drugs. 3). Reducing the drug efflux from the CNS. This can be achieved by inhibition of the active efflux mechanisms that are presented at the BBB transporting substances out of the brain. These efflux systems include the P-glycoprotein and the multidrug resistance-associated protein. They exhibit very broad substrate specificity and will pump a wide range of structurally unrelated compounds out of the brain, including some small molecular size or lipophilic substances. The second strategy for increasing the brain availability of drugs involves modification of the molecular structure of drugs. These include 1). Design drugs to utilise the active transport systems which brain uses for receiving the nutrients. However, because of the high substrate specificity and low capacity of these inward transport systems, this method may only be suitable for delivering small quantities of analogues of endogenous substrates (e.g. AA, nucleosides and peptides). 2). Increasing the lipophilicity of the drugs. Concerning the lipid pathway, there is a good correlation between *in vivo* BBB penetration and lipophilicity (The lipophilicity is classically expressed as its partition coefficient between an organic solvent, such as octanol, and water) of a drug. Other properties of the drug, such as the molecular volume, charge of the drug and the extent to which it makes hydrogen bonds in penetrating the membrane, are also factors contribute to the brain availability of the drug. Therefore, increasing the lipophilicity of the drug is an important strategy for increasing the brain availability of the drug. It has been shown that addition of hydrophobic groups to a molecule may help it to penetrate the brain BBB. The addition of methyl groups to a series of barbiturates improved their lipophilicity and brain penetration, leading to increased hypnotic activity. On the contrary, small molecule permeation through the BBB was decreased for each hydroxyl group added to the molecule.

In the current project, we solely characterise the active compounds by *in vitro* methods. However, some postulation could be made based on available data. For example, honokiol and magnolol have been shown having anxiolytic, central depressant effects, and our experiments showed that they interact with the $GABA_A$Rs *in vitro*. Therefore, these compounds might be able to pass the BBB to exert their CNS effects. It would be also be interesting to investigate the *in vivo* effects of phellopterin in the future experiments.

4.5. Advantages, pitfalls, challenges and the future

Despite of these advantages, there are some inherent pitfalls associated with plant-derived drug research that could hinder its progress. From our own experiences, there are several points should keep in mind when carry on the research on active compounds from plants. 1). Identity of the plants: At the very beginning of the project, when certain plants were chosen for the screening based on their traditional use, the identity of these plants could be a key factor determining the later successful isolation. For example, in TCM, it is quite common that the same name of the herbal medicine could include several botanically distinct species, e.g. Chinese Baizhi, includes *Angelica dahurica*, *Angelica anomala*, *Angelica formosana* and *Heracleum scabridum* four items. The active compounds of interest could only appear in one of these items. 2). Extraction and solubility: Since the starting material is a complex mixture of hundreds of compounds, a suitable solvent is very important. In our project, we usually used 50% ethanol in order to give consideration both to lipophilic and lipophobic compounds. During the purification, the solubility of the active compounds could cause some problems, especially when a trace amount of the active compounds appears in the plant. The similar solubility of compounds with similar chemical structure in the same plant (and that is always the case) sometimes can make the separation infeasible. 3). Trace amount of the active compounds: The isolation, purification as well as structural elucidation could be a time-consuming and labour-intensive process due to the trace amount of the interesting compound, moreover, the final isolated compounds could be worthless for the pharmacologist. It took us more than one year to purified 25 μg of two active compounds from more than 10 kg *Pseudostellaria heterophylla,* and the structural elucidation turn out to be βCCE and βCCM. Although it could be of interest for the

botanist, it was meaningless for us to go further. 4). Lost activity: It is not the case, the active compounds could be purified whenever they are found. Usually the screening started with a small sample of a plant, a large amount of plant is needed to purify compounds enough for the chemical elucidation. Even with a defined identity, components of the plant could vary from batch to batch because of the different processing, different collecting place or season etc. The activity being traced on could disappear. The biological activity could also be lost during the purification due to instability of the active compounds or the fact that the activity is a synergistic or additive effect between several structurally different compounds, whenever they are separated, the activity is no long there. 5). None interesting activity: Not all activities found are of interest. In our screening project, almost all the plants showed strong activity to the GABA binding site. However, since these activities appeared in the first several fractions from HPLC, we conservatively believe that it could be GABA itself. Despite of their possible pharmacological significance in their traditional use, we have to abandon any further investigation. One thing worth a try is to run HPLC under linear gradient from water, compounds other than GABA should give some more retention time from the HPLC (see Table 12). 6). False positive activity. Contaminated columns or other experimental instruments could yield false positive results. To avoid this happening, a separate set of columns solely used for plant screening would be helpful. Besides these practical difficulties, from pharmaceutical industry point of view, the patent protection is weaker for plant-originated drugs; quantitative long-term supply of botanically well-defined raw materials can be difficult to maintain, especially for imported items; the structural complexity of the active compound may preclude cost-effective synthesis (Bruhn, 1989; Nisbet and Moore, 1997). That is why, over the years, interest of the pharmaceutical industry in natural products as a source of potential, new chemotherapeutic agents has been deciding. The lead compound generation and drug discovery processes have been significantly impacted by emerging novel approaches such as advanced genomics, high-throughput screening, combinatorial chemistry and biology, and computer-assisted drug design. The role that natural products have played historically in lead generation is facing a serious challenge (Nisbet and Moore, 1997; Shu, 1998).

However, the possible application and integration of new developed technologies, such as high-throughput screening, combinatorial chemistry and genetic engineering etc., to natural products research (Nisbet and Moore, 1997), along with its intrinsic advantages, natural product research, especially these based on ethnopharmacological usage, will continue to be a active contributing force for new drug discovery, as well as novel biological tools.

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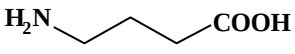
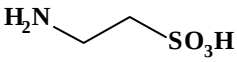
Chemical structures of compounds

$GABA_AR$ agonists (Table 2)

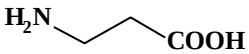
Endogenous



GABA

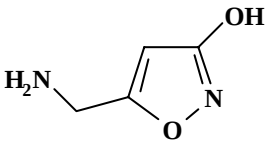


Taurine

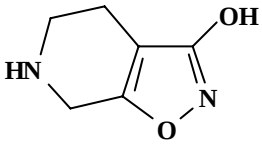


β-alanine

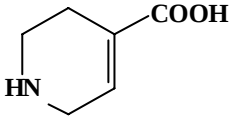
Exogenous



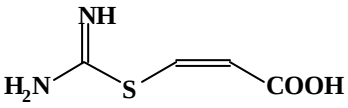
Muscimol



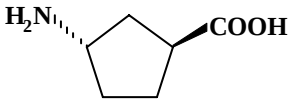
THIP



Isoguvacine

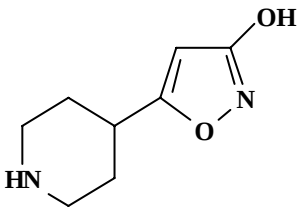


ZAPA

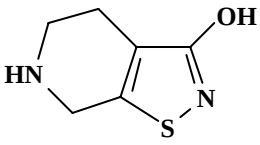


(+)-TACP

Partial agonists



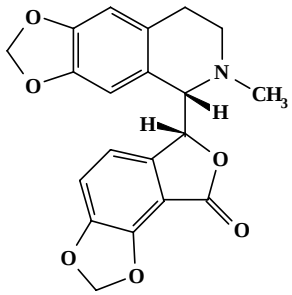
4-PIOL



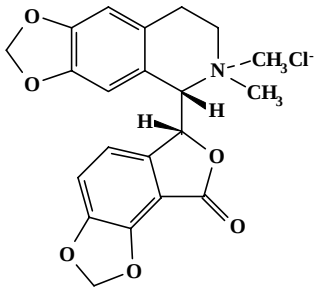
Thio-THIP

$GABA_AR$ antagonists (Table 2)

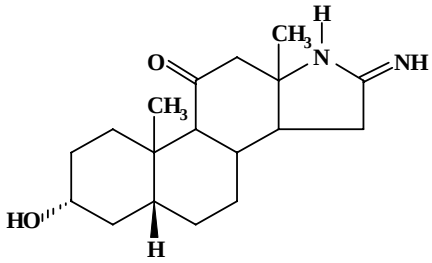
Competitive



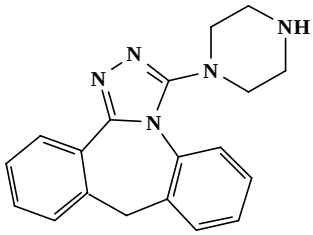
Bicuculline



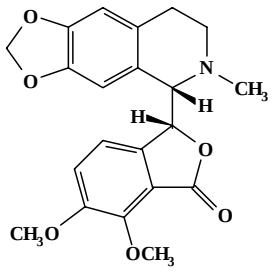
Bicuculline methochloride



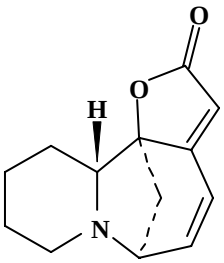
RU 5135



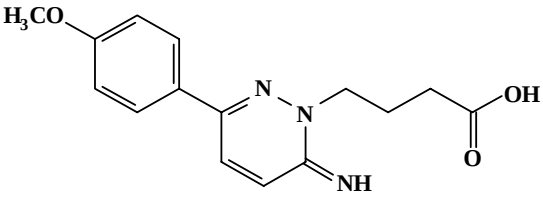
Pitrazepin



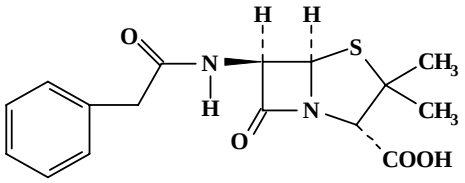
(+)-Hydrastine



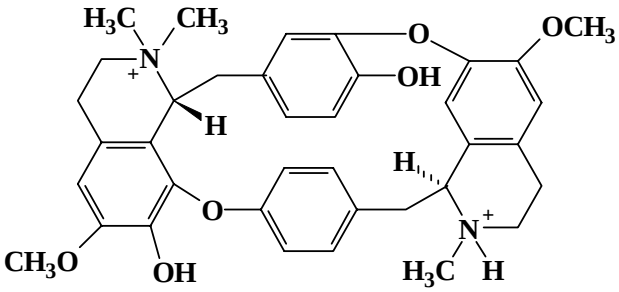
Securinine



SR 95531

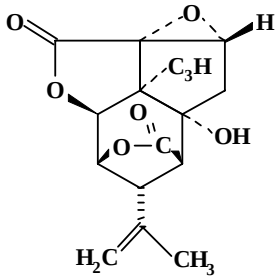


Benzylpenicillin

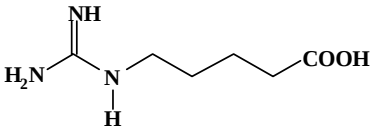


(+)-Tubocurarine

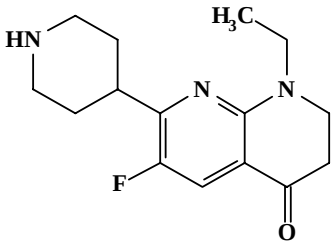
Noncompetitive



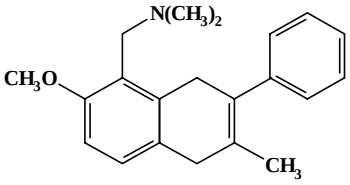
Picrotoxinin



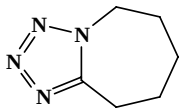
δ-guanidinovaleric acid



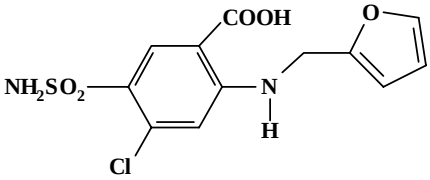
Enoxacin



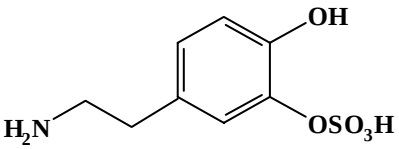
Dimefline



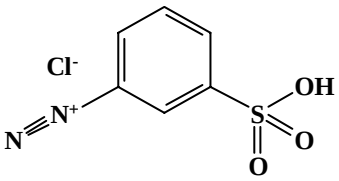
Pentamethylenetetrazole



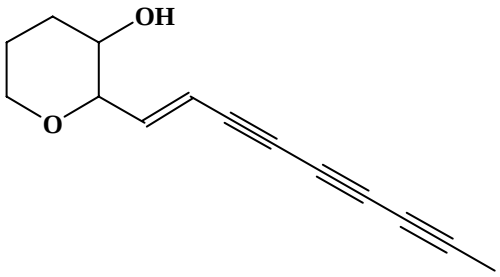
Furosemide



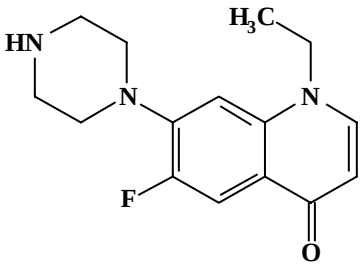
Dopamine 3-o-sulfate



m-Benzenesulfonium diazonium chloride

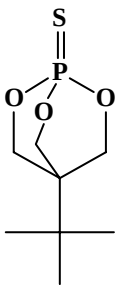


Cunaniol

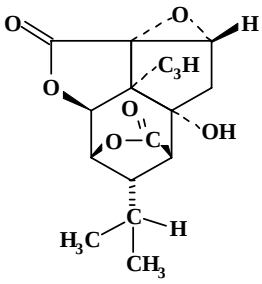


Norfloxacin

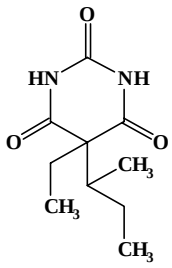
$GABA_AR$ allosteric sites ligands (Section 1.3. ~ 1.5.)



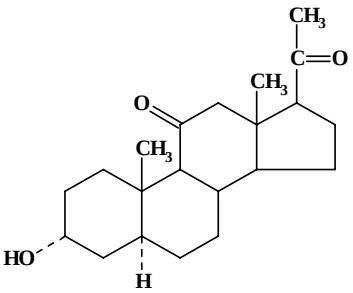
TBPS



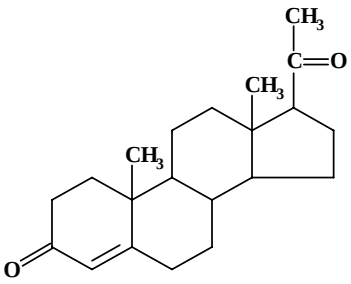
α-dihydro-picrotoxinin



Pentobarbital

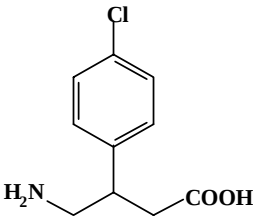


Alphaxalone

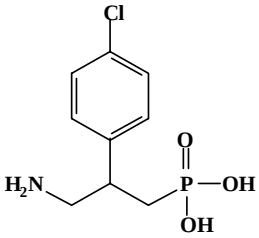


Pregesterone

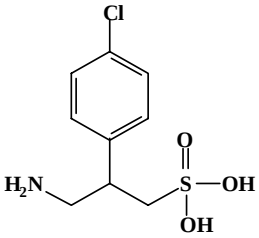
$GABA_B$ receptor ligands (Table 1)



Baclofen

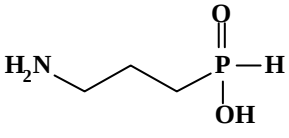


Phaclofen

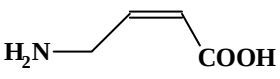


Saclofen

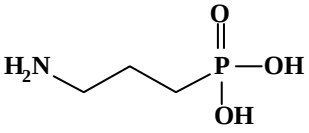
$GABA_C$ receptor ligands (Table 1)



3-APPA

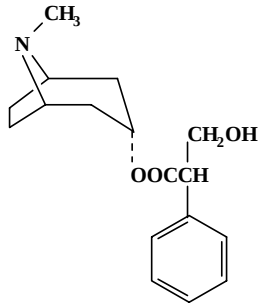


CACA

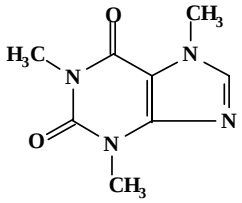


3-APA

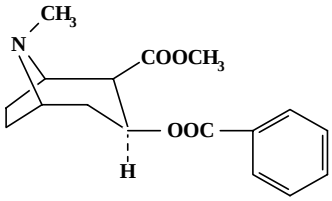
Drugs with plant origin (Table 8)



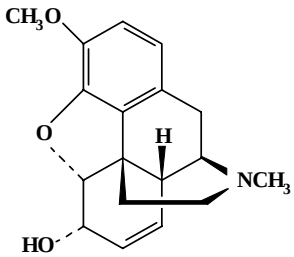
Atropine



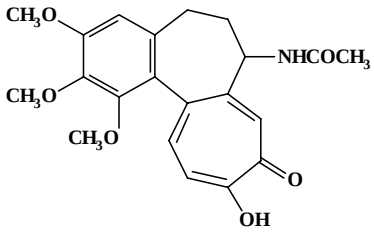
Caffeine



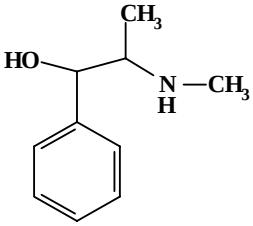
Cocaine



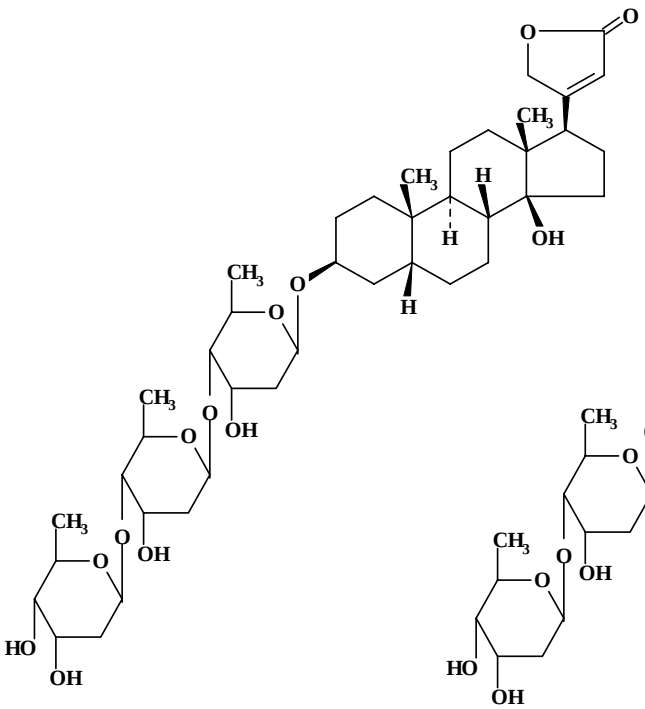
Codeine



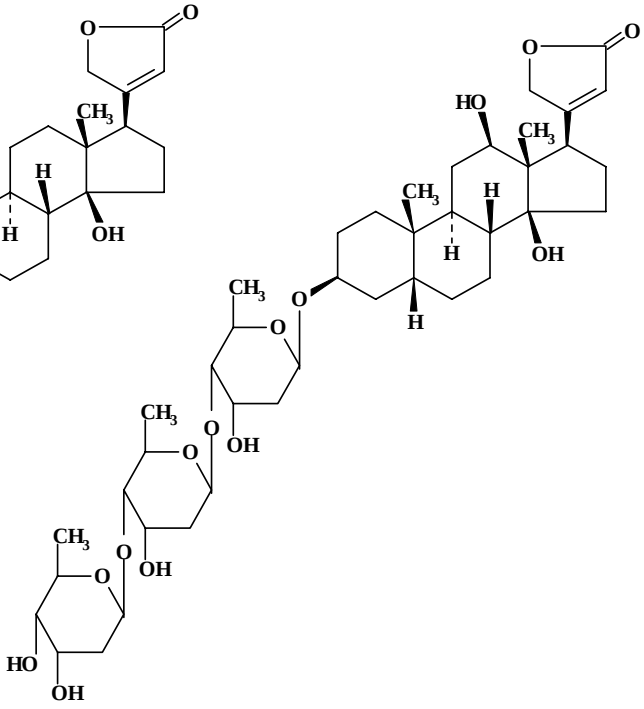
Colchicine



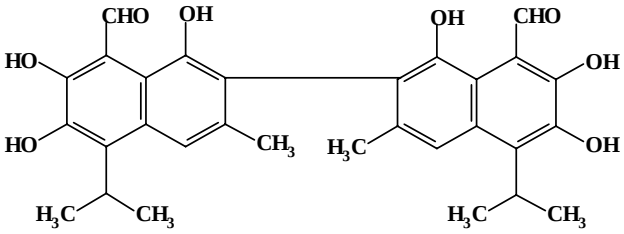
Ephedrine



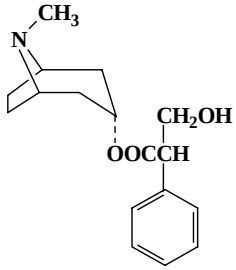
Digitoxin



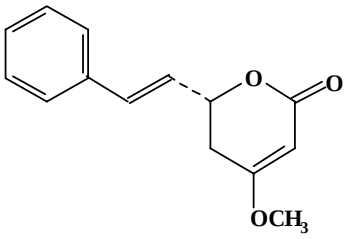
Digoxin



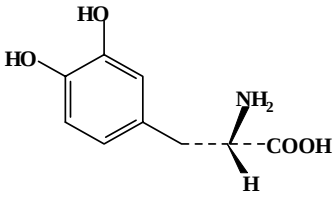
Gossypol



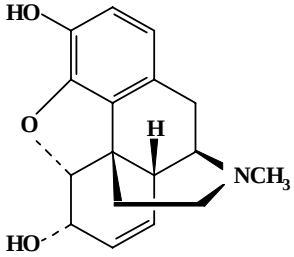
Hyoscyamine



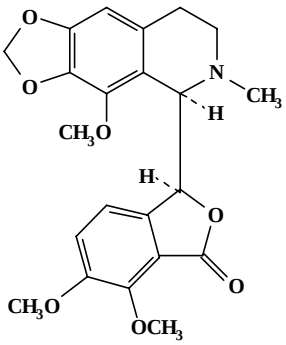
Kawain



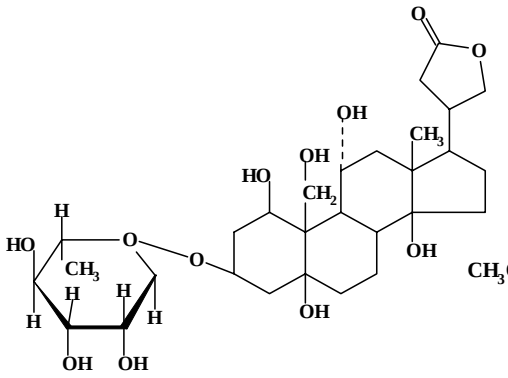
Levodopa



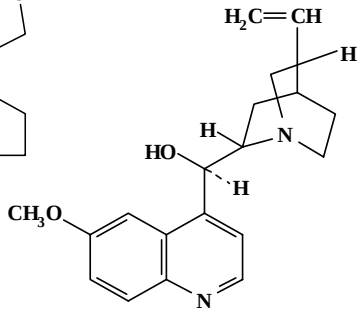
Morphine



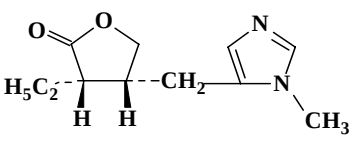
Noscapine



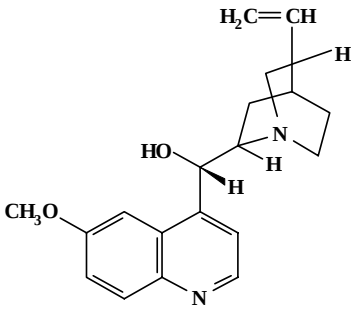
Ouabain



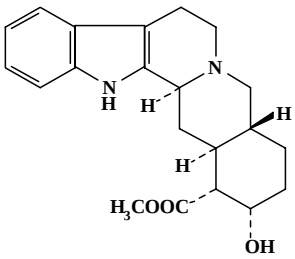
Qunine



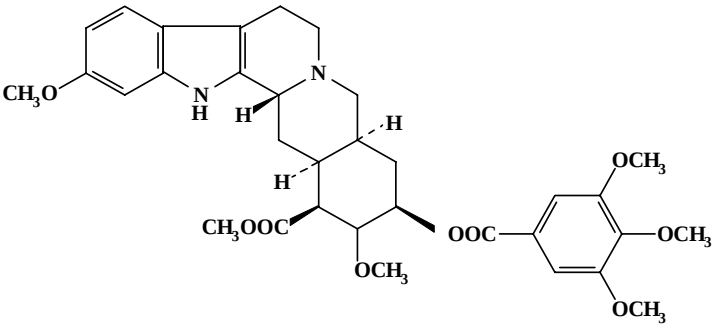
Pilocarpine



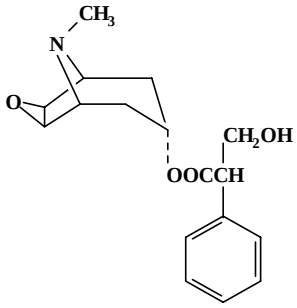
Quinidine



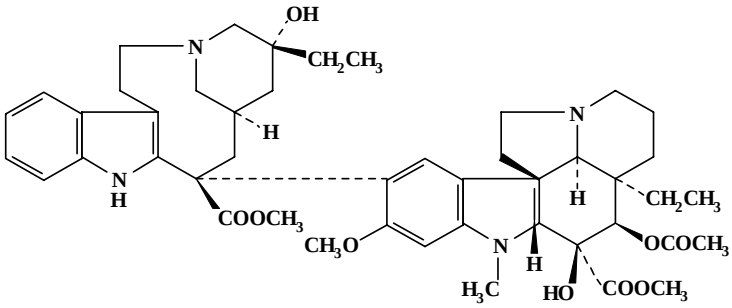
Yohimbine



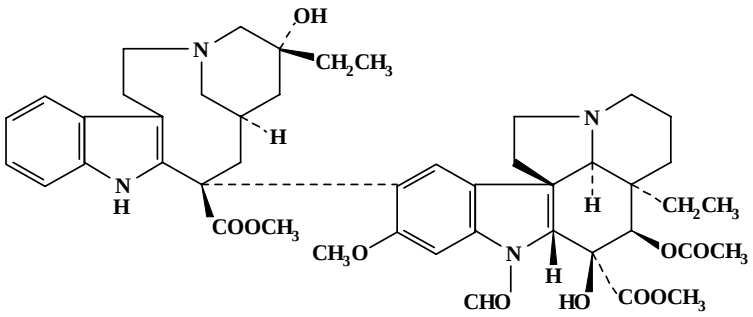
Reserpine



Scopolamine



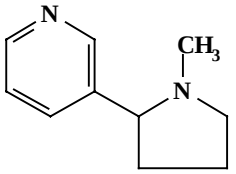
Vinblastine



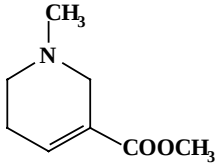
Vincristine

Compounds with natural origin as pharmacological tools (Table 9)

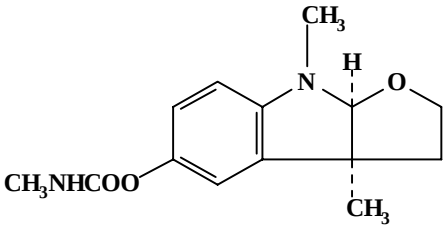
Nicotinic AchR ligands



Nicotine

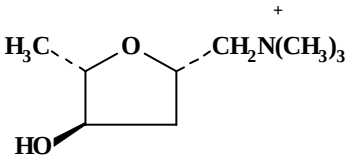


Arecoline

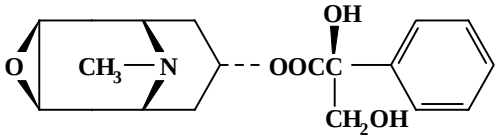


Physostigmine

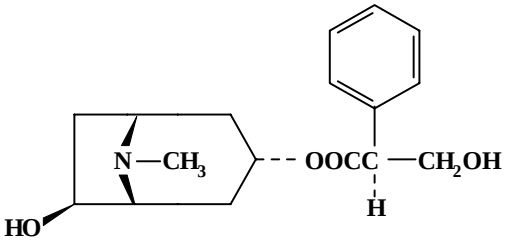
Muscarinic AchR ligands



Muscarine

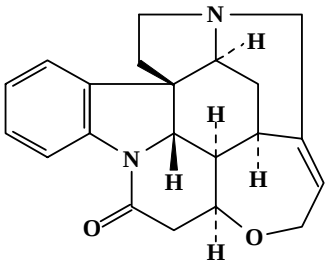


Anisodamine



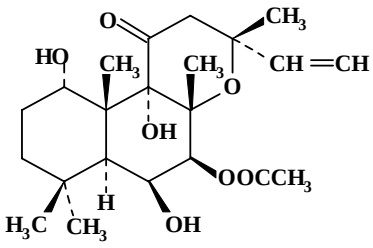
Anisodine

Glycine receptor ligand

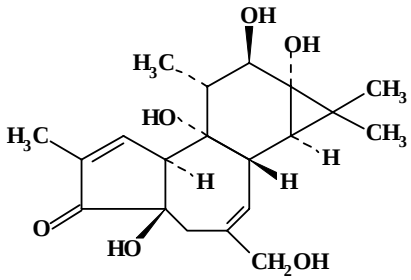


Strychnine

Adenylate cyclase and protein kinase C ligand

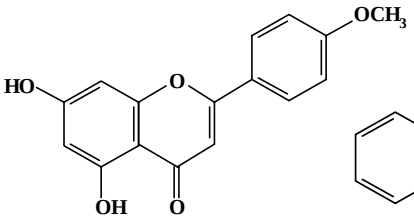


Forskolin

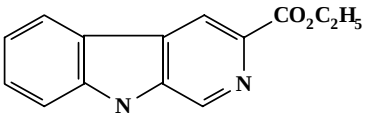


Phorbol

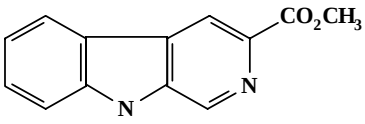
Compounds isolated from CMH in this project (Table 12)



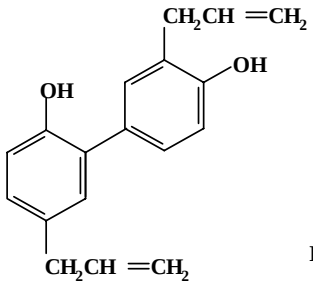
Acacetin



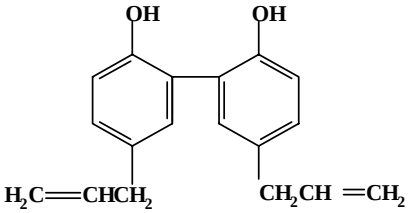
βCCE



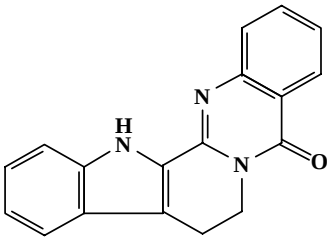
βCCM



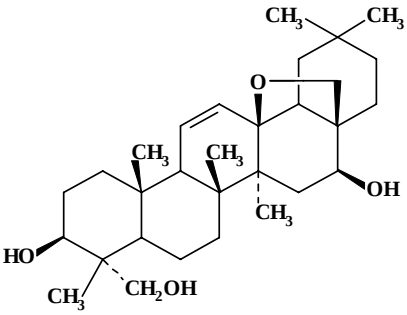
Honokiol



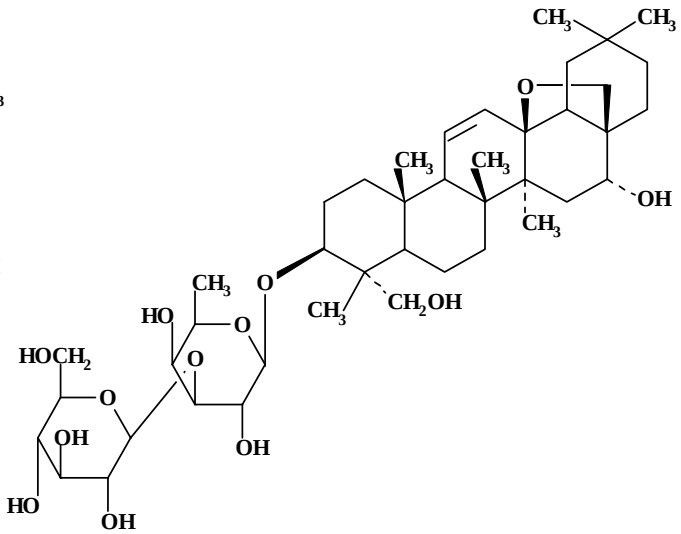
Magnolol



Rutecarpine

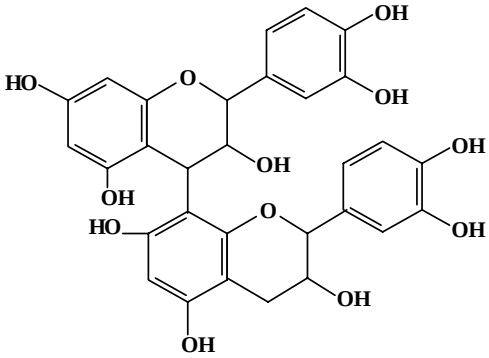


Saikogenin G

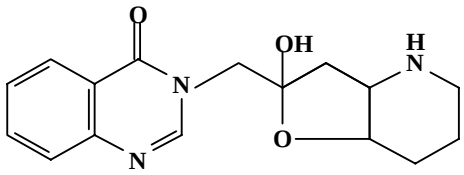


Saikosaponin A

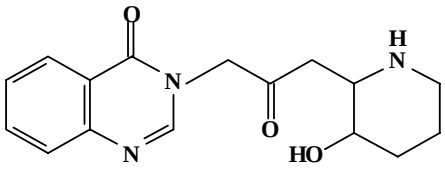
Drugs or drug candidates from Chinese plants (Table 11)



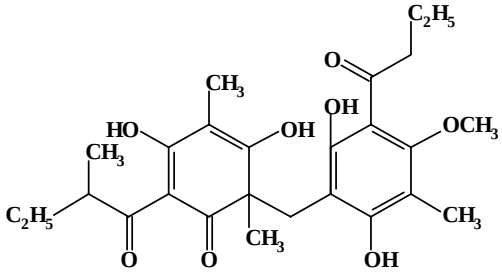
5,7,3',4'-tetrahydroxy-flavone-3-nol C_4-C_8 dimer



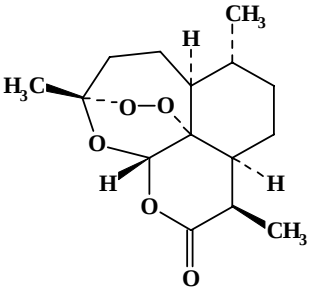
α-dichroine



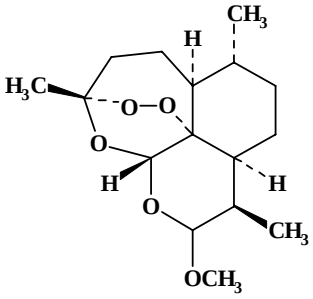
β-dichroine



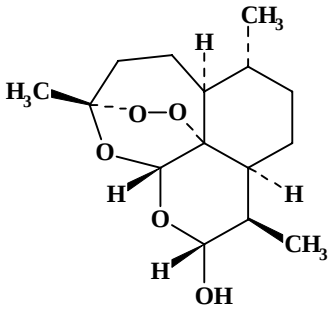
Agrimophol



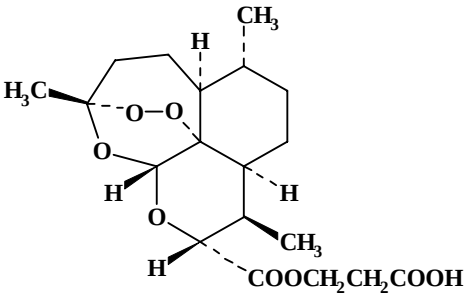
Artemisinin



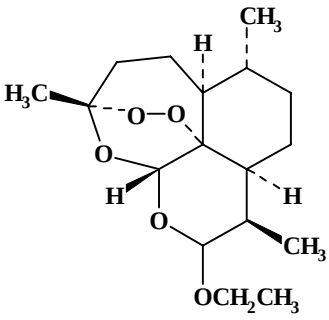
Artemether



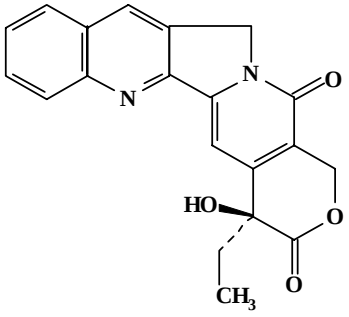
Dihydroartemisinin



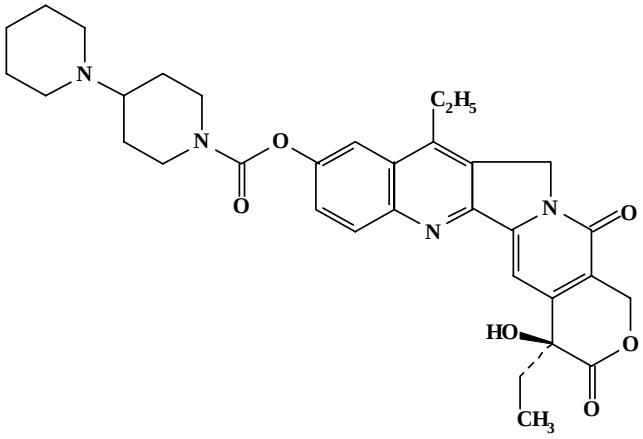
Artesunate



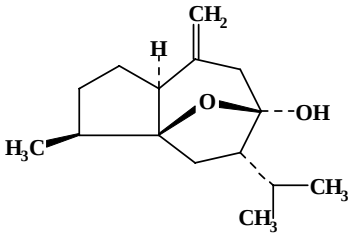
Arteether



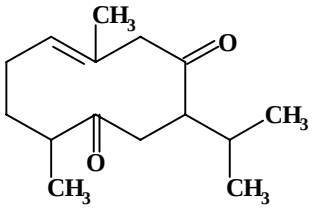
Camptothecin



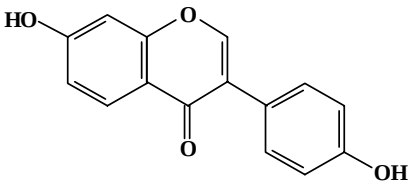
Ironotecan



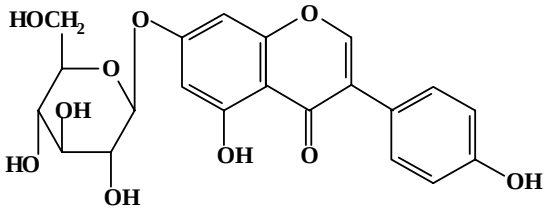
Curcumol



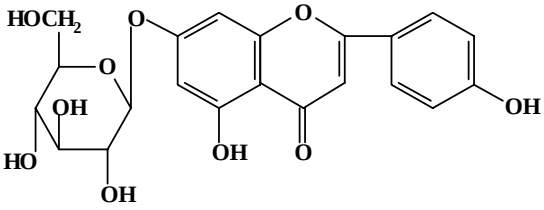
Curdione



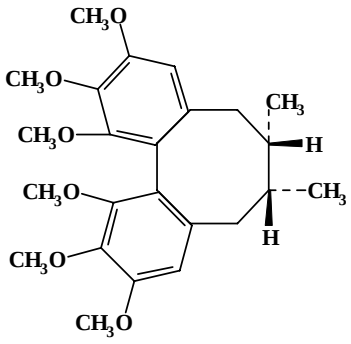
Daidzein



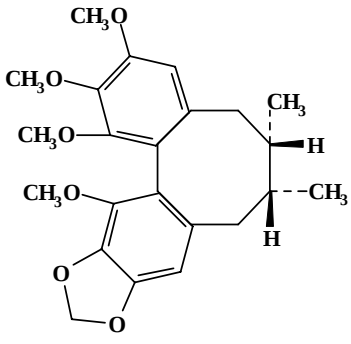
Daidzin



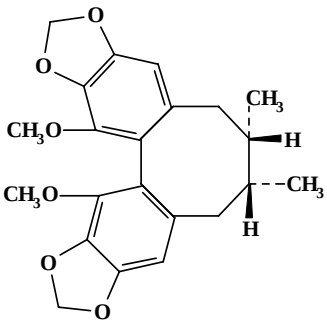
Genistin



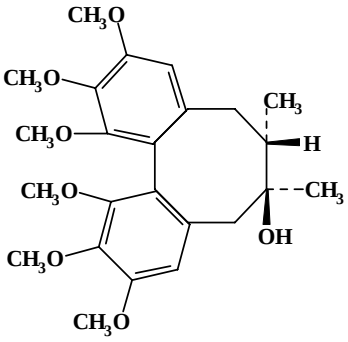
Schisandrin A



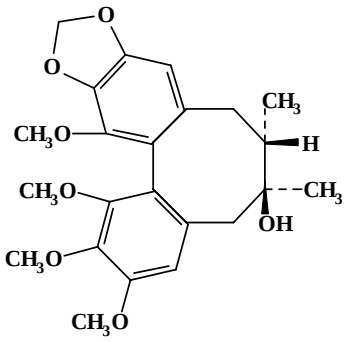
Schisandrin B



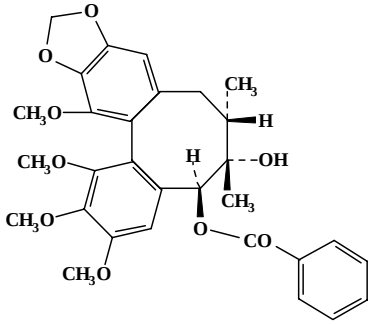
Schisandrin C



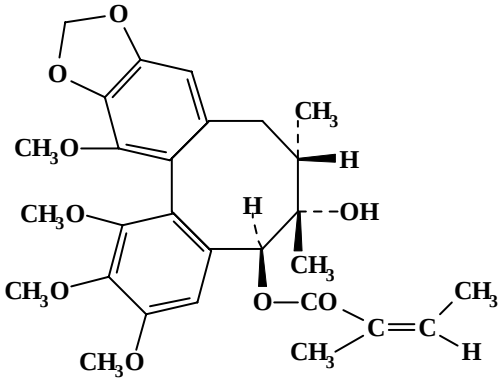
Schisandrol A



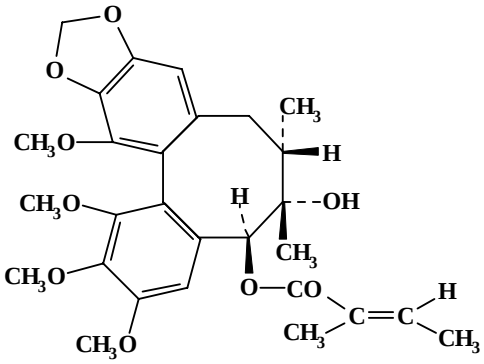
Schisandrol B



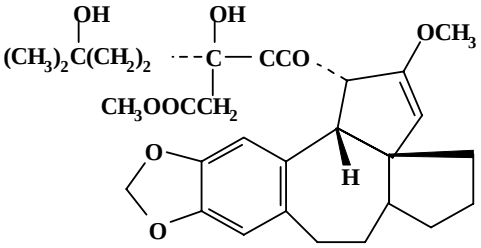
Schisantherin A



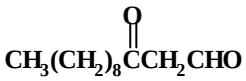
Schisantherin B



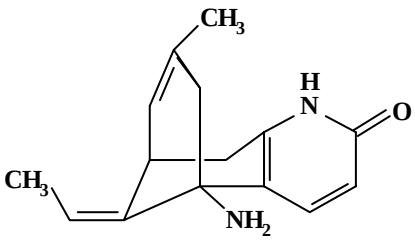
Schisantherin C



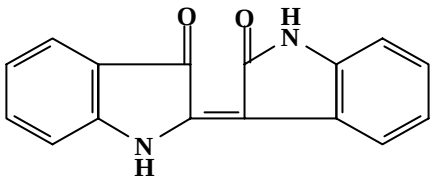
Homoharringtonine



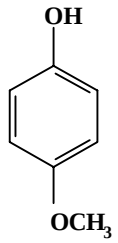
Houttynin



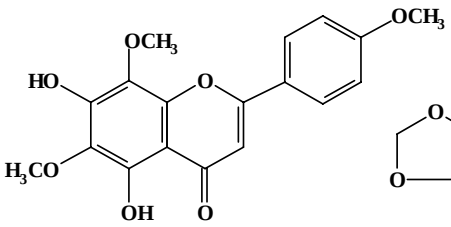
Huperzine A



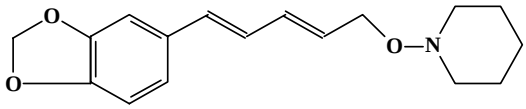
Indirubin



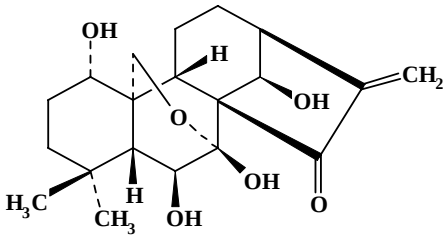
Methyl hydroquinone



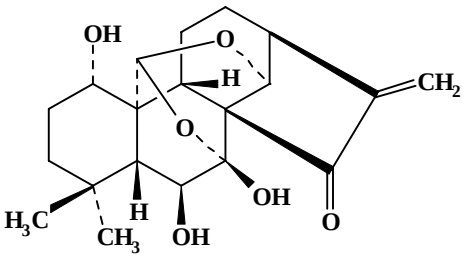
Nevadensin



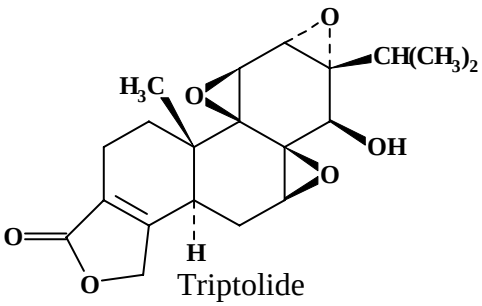
Piperine



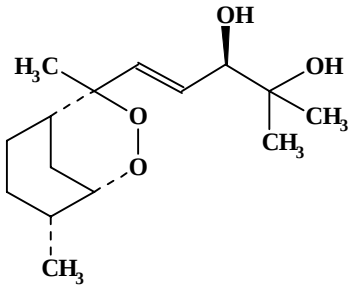
Rubescensin (oridonin)



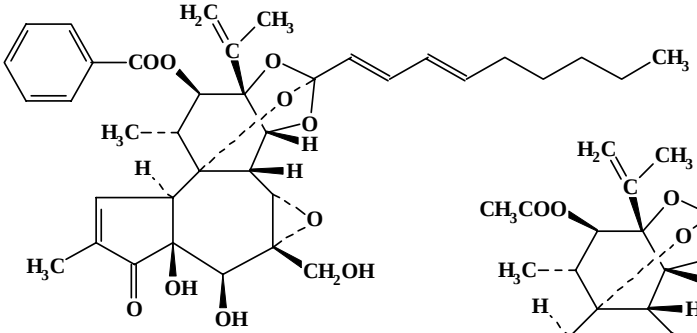
Pomocodin



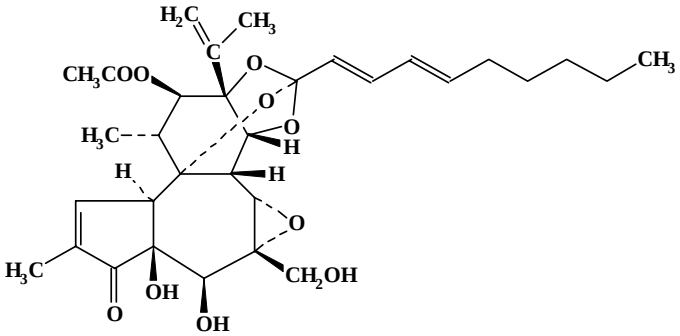
Triptolide



Yingzhaoshu A

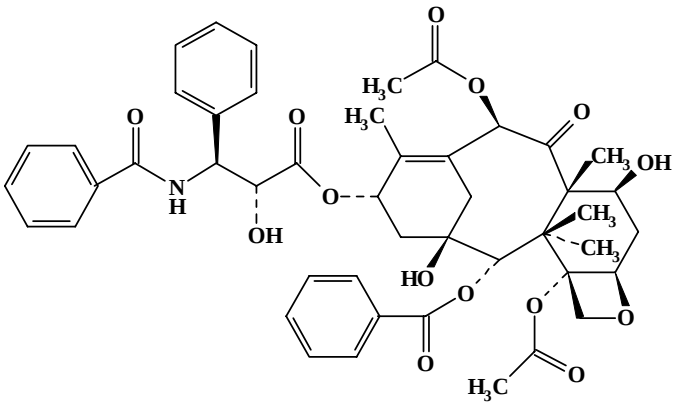


Yuanhucin A

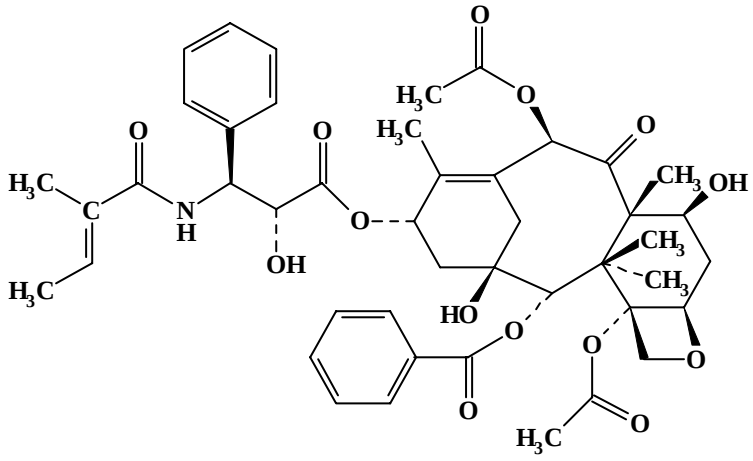


Yuanhucin B

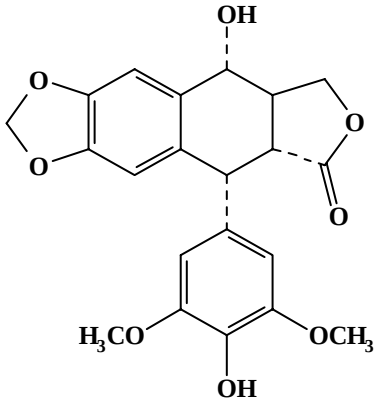
Anti-cancer drugs from plants (Section 3.1.)



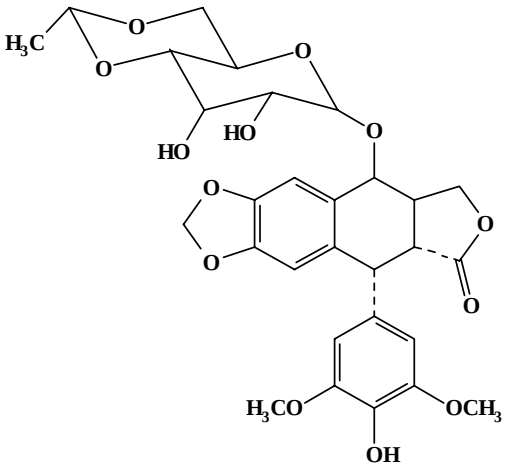
Taxol



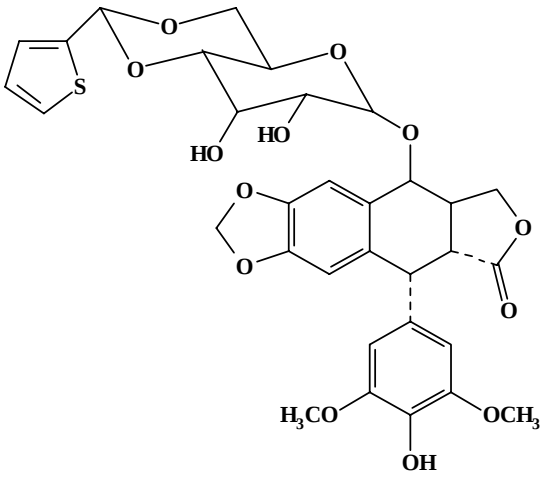
Taxotere



Podophyllotoxin



Etoposide



Teniposide