In vitro study of the mode of action of antidepressants in cell culture models.

Comparison of the effects of *Hypericum perforatum L.* extracts and classical synthetic compounds on the β-adrenergic signal pathway.

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Dekan

To my parents

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Keywords

Hypericum perforatum L., St. John's wort, hyperforin, tricyclic antidepressants, selective serotonin reuptake inhibitors, cell culture, β-adrenoceptor down-regulation, cAMP, phospholipids, membrane fluidity

Summary

The clinical effectiveness of the plant extract of *Hypericum perforatum L.* in treating mild to moderate depression is well established. The extract shows a more favourable side effect profile than other antidepressant drugs, like tricyclics and selective serotonin reuptake inhibitors. Until now, the mode of action of antidepressant drugs is not well understood and it is possible that immediate and adaptive effects contribute to their effectiveness of treating depression. Reuptake inhibition of neurotransmitters, an immediate effect of antidepressant drugs, occurs after drug exposure in minutes to hours. Adaptive effects, like changes in the number of neurotransmitter receptors, phospholipid (PL) accumulation and alterations of cell membrane properties, need a prolonged, chronic exposure of several days to weeks to develop.

In the present work, influences of chronic exposure of *Hypericum perforatum* extracts to rat C6 glioblastoma cells (C6 cells) and human skin fibroblasts were investigated. Changes on the cellular level including cell shape, morphology and proliferation rate and on the β-adrenergic signal pathway including membrane properties, number of β-adrenoceptor and accumulation of the second messenger cAMP were monitored. These effects were compared with those of the selective serotonin reuptake inhibitor (SSRI) fluoxetine and the tricyclic antidepressant desipramine (DMI).

Chronic exposure of C6 cells and fibroblasts to a Hypericum extract, fluoxetine or DMI led to a change in the cell morphology. The cell body broadened and vesicular inclusions were observed. These changes were more pronounced, especially in C6 cells, after chronic exposure to fluoxetine or DMI. The effects were not due to cell hypertrophy since the DNA/protein ratio remained constant under each treatment condition. In contrast to fluoxetine and DMI, Hypericum extract exposed cells showed a distinct cell growth inhibition.

Chronic exposure of fibroblasts and C6 cells to the Hypericum extract led to change in membrane properties. Fibroblasts exposed to the plant extract showed an accumulation of the total amount of PL, as observed after exposure to fluoxetine or DMI. Moreover, the membrane fluidity of C6 cells and fibroblasts was increased after Hypericum extract treatment, whereas exposure to DMI or fluoxetine did not influence the membrane fluidity.

Complex changes in the relative PL content of the cellular and plasma membrane composition were observed after chronic exposure of C6 cells to Hypericum extract or DMI. The most pronounced and consistent effect was the increase of phosphatidylinositol after both treatments.

The β-adrenoceptor number and its signal pathway were affected after chronic exposure of C6 cells to Hypericum extract, fluoxetine or DMI. All treatment conditions resulted in a down-regulation of the β-adrenoceptor number at varying degrees. The cAMP accumulation after chronic exposure to all drugs was reduced.

Our results showed that the *in vitro* effects of the Hypericum extract were comparable to those of the well-established tricyclic antidepressant DMI and the SSRI antidepressant fluoxetine and they might, therefore, share a common mode of action.

Hyperforin, a constituent of the *Hypericum perforatum* extract, has gained more attention in recent years. It has been shown that mainly hyperforin is responsible for interactions with co-administered drugs that may lead to severe conditions. Thus, using a hyperforin-"free" and hyperforin-rich fraction of the Hypericum extract, the significance of hyperforin on the *in vitro* antidepressant effects in C6 cells was investigated. Down-regulation of the β-adrenoceptor number could be observed in C6 cells after chronic exposure to the hyperforin-"free" fraction. This reduction was comparable to that obtained with the Hypericum extract. Contrarily, chronic exposure of C6 cells to a hyperforin-rich fraction did not reduce the β-adrenoceptor number. Interestingly, exposure of C6 cells to both fractions inhibited the cAMP accumulation comparable to that level observed after exposure to Hypericum extract.

It was shown that a hyperforin-"free" fraction induced changes on the β-adrenoceptor number and on the cAMP accumulation similar to a Hypericum extract, the tricyclic DMI and the SSRI fluoxetine. Consequently, it is tempting to point out that a hyperforin-free Hypericum extract might posses the same potential as a hydroalcoholic Hypericum extract in treating mild to moderate depression, but without inducing metabolic interactions with co-administered drugs.

Abbreviations

5-HT	serotonin, 5-hydroxy-tryptamine
AC	adenylate cyclase
AD	antidepressant
BDNF	brain derived neurotrophic factor
B_{max}	maximal binding capacity
BSA	bovine serum albumin
C6 cell	rat C6 glioblastoma cell
CAD	cationic amphiphilic drug
cAMP	cyclic AMP, cyclic adenosine 3',5'-monophosphate
$CHCl_3$	chloroform
COMT	catechol-O-methyltransferase
CREB	cAMP response element binding protein
CRF	corticotrophin-releasing factor
CSF	cerebrospinal fluid
CYP450	cytochrome P450
DABA	diaminobenzoic acid dihydrochloride
DAG	diacylglycerol
DIC	differential interference contrast
DMI	desipramine
ECT	electroconvulsive therapy
ECV	endosomal carrier vesicles
G protein	guanine-nucleotide binding protein
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GRP	G protein-coupled receptor specific phosphatase
GTP	guanosine triphosphate
H_2O_{dd}	double distilled water
HBS	Hanks balanced solution
HPA-axis	hypothalamic-pituitary-adrenal-axis
IBMX	3-isobutyl-1-methyl-xanthine
IL	interleukin

IMI	imipramine
INF	interferon
IP3	inositol-3-phosphate
K_D	equilibrium dissociation constant
LSD	lysergic acid diethylamide
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitors
MEM-Eagle	minimum essential medium Eagle
MeOH	methanol
NMDA	N-methyl-D-aspartate
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PKA	proteinkinase A
PL	phospholipid
PLC	phospholipase C
PMV	plasma membrane vesicles
PS	phosphatidylserine
RespF	response factor
SPH	sphingomyelin
βARK	β-adrenoceptor kinase
SSRI	selective serotonin reuptake inhibitors
TCA	tricyclic antidepressant
TLP	theoretical lower phase
TMA-DPH	trimethylamino-diphenylhexatriene
$Tris_{hypo}$	hypotonic Tris buffer
$Tris_{iso}$	isotonic Tris buffer
TUP	theoretical upper phase

1 Introduction

1.1 Depression

Depression is characterised primarily by changes of mood, rather than by thought disturbances. Depressive disorders are common, approximately 15% of the population experience a depressive episode at some point of life. It may range from a very mild condition to severe depression, accompanied by hallucinations and delusions. The depressive symptoms include emotional and clinical components:

- misery, apathy and pessimism
- low self-esteem: feelings of guilt, inadequacy and ugliness
- indecisiveness, loss of motivation
- retardation of thought and action
- loss of libido
- sleep disturbances and reduced appetite

Two types of depressive illness can be distinguished, namely unipolar depression, in which the mood swings are always in the same direction, and bipolar affective disorder, in which depressive episodes alternate with mania. These two types are separated diagnostically only and no preferential effect of any single antidepressant agent is known. In three fourths of the cases, unipolar depression is clearly associated with stressful life events and is thus termed reactive depression. In about one fourth of the cases, the depression shows an inherited pattern that is unrelated to stressful events and is, therefore, termed as endogenous depression. Bipolar depression usually appears in early life, between 20 and 30 years of age. This disorder is characterised by alternating depression and mania over a period of a few weeks. There is good evidence for a genetic predisposition to bipolar disorder. Genetic linkage studies in affected families and comparative studies in monozygotic twins indicate an autosomal dominant transmission. A continuous depression of mood for a minimum of 2 weeks is commonly referred to as major depression.

1.2 The monoamine hypothesis of depression and its limitations

The monoamine hypothesis of depression was first formulated 40 years ago by Schildkraut (1965) and Bunney & Davis (1965). The hypothesis proposes that there is an underlying biological basis for depression: a deficiency of the monoamine neurotransmitters in the brain, particularly of norepinephrine (NE). Coppen (1972) extended the monoamine hypothesis to serotonin (5-HT). On the basis of this hypothesis, various classes of antidepressant agents have been developed that act to increase levels of monoamines within the cleft of synapses in the brain. This increase is achieved by inhibition of neurotransmitter degradation, by blockade of their reuptake into the presynaptic axons or by an increased neurotransmitter release from presynaptic storage vesicles caused by a blockade of presynaptic autoreceptors.

The monoamine hypothesis was formulated on the basis of a number of key observations that were made during the 1950s. It was noted that lysergic acid diethylamide (LSD) blocked peripheral 5-HT receptors (Woolley & Shaw, 1954), and this raised the question of whether LSD might also have similar actions in the brain, since the behavioural effects of LSD were well known. It was concluded that central 5-HT might have a role in the aetiology of mood disorders. Another observation was that the antihypertensive agent reserpine occasionally led to depression in hypertensive patients (Muller et al., 1955). It was noted that reserpine, an alkaloid from the root of *Rauwolfia serpentina*, depleted brain 5-HT stores (Shore, 1955) by interfering with vesicular storage of 5-HT and NE.

Further evidence came from the observation that iproniazid, an antimycobacterial agent, improved mood in tubercular patients with depression (Crane, 1956; Kline, 1961). It was found that iproniazid inhibits monoamine oxidases (MAO) thus preventing the degradation of 5-HT and NE (Zeller et al., 1952). This led to the development of other monoamine oxidase inhibitors (MAOI) as antidepressants.

In 1957, the Swiss psychiatrist Kuhn reported that the tricyclic compound imipramine (IMI) was effective in the treatment of depression, hereby initiating the development of a very important new class of antidepressant agents; the tricyclic antidepressants (TCA). IMI was originally developed as a neuroleptic for use in psychotic patients, but was ineffective as such. It was shown that IMI inhibits the reuptake of NE and 5-HT,

peripherally and centrally (Herting et al., 1961; Carlsson et al., 1968). This led to the development of a number of TCA inhibiting 5-HT and NE reuptake to varying degrees.

Due to the adverse effects of the TCA such as drowsiness, sedation, hypotension, dry mouth, constipation and arrhythmia, non-TCA reuptake inhibitors which do not display such adverse effects have been developed. Fluoxetine, sertraline, paroxetine, and fluvoxamine are selective inhibitors of the 5-HT uptake transporter and reboxetine represents the first non-TCA selective NE reuptake inhibitor.

There are several major issues that the monoamine hypothesis does not address:

- A delay in the onset of the antidepressant effect is common to all antidepressant drugs. The reuptake of monoamines due to inhibition of the monoamine transporters occurs within hours, whereas it takes usually 2 to 3 weeks before the clinical antidepressant effect becomes apparent.
- Not all NE reuptake inhibitors, e.g. cocaine and amphetamine, show antidepressant activity.
- Substances that do not alter the neurotransmitter concentrations are suitable for the treatment of depression, e.g. iprindole and trazodone.

Due to the shortcomings of the monoamine hypothesis of depression, research has attempted to determine other systems that may be involved in depression. One finding is that in depressed patients the hypothalamic-pituitary-adrenal (HPA)-axis is hyperactive (Slattery et al., 2004).

Despite these limitations, the monoamine hypothesis is still the best basis for understanding the action of antidepressant drugs and all the common antidepressant agents have been developed according to it.

It seems clear that the monoamine hypothesis has to be adapted and elaborated upon. Consequently, major research now focuses on long-term effects of antidepressant treatments to explain the late onset of the antidepressant activity. Secondary, adaptive changes in the brain due to chronic treatment may be responsible for the antidepressant effect.

1.3 Types of antidepressant drugs

The main types of antidepressant drugs are TCA, selective serotonin reuptake inhibitors (SSRI), MAOI and atypical antidepressants. Lithium is used as mood stabiliser in manic-depressive illness (bipolar depression). It prevents the swings of mood and thereby reduces both the manic and the depressive phases. The mechanism of action is not understood, but lithium interferes with the inositol pathway and with the cyclic AMP (cAMP) formation. Plant extracts, like the extract of *Hypericum perforatum L*., play a major role in the treatment of depression.

TCA act by inhibiting the uptake of NE and 5-HT at monoaminergic nerve terminals. Typical members of this class are IMI, desipramine (DMI) and amitriptyline.

SSRI block the reuptake of 5-HT from the synaptic cleft into the presynaptic nerve terminal. This leads to an elevated concentration of 5-HT in the synaptic cleft.

MAOI inhibit one or both forms of brain MAO, thereby increasing the cytosolic stores of NE, dopamine and 5-HT in the nerve terminals. Phenelzine, tranylcypromine and iproniazid inhibit both types of MAO irreversibly, whereas the newer MAO inhibitor moclobemide is selective for the MAO type A. MAO-A has a substrate preference for 5-HT and NE and its inhibition correlates with the antidepressant effect, whereas MAO type B has a preference for dopamine.

The atypical antidepressants include compounds like nomifensine and maprotiline that act like the TCA, but have a different chemical structure. Other compounds with different pharmacological actions are mianserin, bupropion and trazodone.

1.3.1 *Hypericum perforatum L.*

Hypericum perforatum (Saint John’s wort) is a perennial herb indigenous to Europe, western Asia and northern Africa. It is a member of the family Clusiaceae of the order Theales (Cronquist, 1988). The genus Hypericum consists of more than 370 species and *Hypericum perforatum* itself has been divided into four subspecies that are distinguished by the size of their sepals (Schütt & Schulz, 1993). The crude drug, Hyperici herba, consists of the above ground parts of *Hypericum perforatum*

collected just before or during flowering time. Consequently, the constituents found in the drug are from flowers, leaves and stems and their concentrations vary within species and according to ecological factors, time of harvest and processing of raw material. Its leaves show a characteristic dotted pattern, hence the name "perforatum" (Figure 1C). *Hypericum perforatum* possesses sedative and astringent properties, and has been used traditionally for the treatment of excitability, neuralgia, fibrosis, sciatica, menopausal neurosis, anxiety, depression, as a nerve tonic and in topical preparations for the treatment of wounds (Newall et al., 1996; Schaffner et al., 1992). St. John's wort is used extensively in herbal products as well as in homoeopathic preparations. In 2004, Swissmedic had 13 medicaments with

Figure 1: Aerial parts of *Hypericum perforatum L.* (A) and a closer view of the blossom (B) and the leave (C) with the characteristic dotted pattern.

Hypericum perforatum and 5 with *Hypericum perforatum* in combination with other plant extracts on record.

Hyperforin and hypericin are considered as the major active constituents of the Hypericum extract, although many other biologically active constituents are present. The potentially important parts of the bioactive compounds can be classified into seven chemical groups. Phenylpropanes, flavonoids and oligomeric proanthocyanidins are biogenetically related and are the major constituents of the dry crude drug. Xanthones and naphthodianthrones occur in small amounts, usually less than 1%. Phloroglucinols can exceed 5% in the fresh herb and the volatile fraction (essential oil) makes up 0.2% (Nahrstedt & Butterweck, 1997).

Naphthodianthrones, namely hypericin and pseudohypericin (Figure 2A), are found in the flowering portions of the plant and their concentrations can range from 0.03 to 0.3%, depending on the developmental stage of the plant. Total hypericin content has been used for standardisation purposes by the pharmaceutical industry. The red naphthodianthrones possess phototoxic properties, but photosensitivity seems to be an extremely rare event with recommended doses of St. John's wort (Ernst et al., 1998). The daily amount of hypericins for man, when using Hypericum preparations was 30- to 50-fold below the dose that is phototoxic in calves (Siegers et al., 1993). In an *in vitro* receptor binding study, hypericin and pseudohypericin showed a high affinity for dopamine D_3- and D_4-receptor subtypes, but only hypericin significantly inhibited binding to the β_1- and β_2-adrenoceptor subtypes (Butterweck et al., 2002). Down-regulation of β_1-adrenoceptors has been commonly used as a biological marker of antidepressant efficacy (Hancock & Marsh, 1985; Honegger et al., 1986; Fishman & Finberg, 1987). Both hypericin and pseudohypericin showed antiretroviral activity *in vitro* and *in vivo* in mice (Meruelo et al., 1988).

Hyperforin is active against several viruses *in vitro* but it failed to show an effect in humans with chronic infection of hepatitis C virus (Jacobson et al., 2001). As pure compounds, hyperforin and hypericin are almost insoluble in water at room temperature, but more than 40% of the content is extractable from the crude drug when preparing a tea. This increase in solubility is due to the high temperature of extraction, but it is suggested that co-effectors in the drug are present which modify the solubility of the naphthodianthrones.

Hyperforin is the main phloroglucinol compound, beside the closely related adhyperforin that contains an additional methyl group (Figure 2B). Both compounds

occur exclusively in the reproductive parts of the plant (Nahrstedt & Butterweck, 1997). The total content of these compounds increases from about 2% hyperforin and 0.2% adhyperforin in the flowers to 4.4% and 1.8% in the ripe fruits, respectively

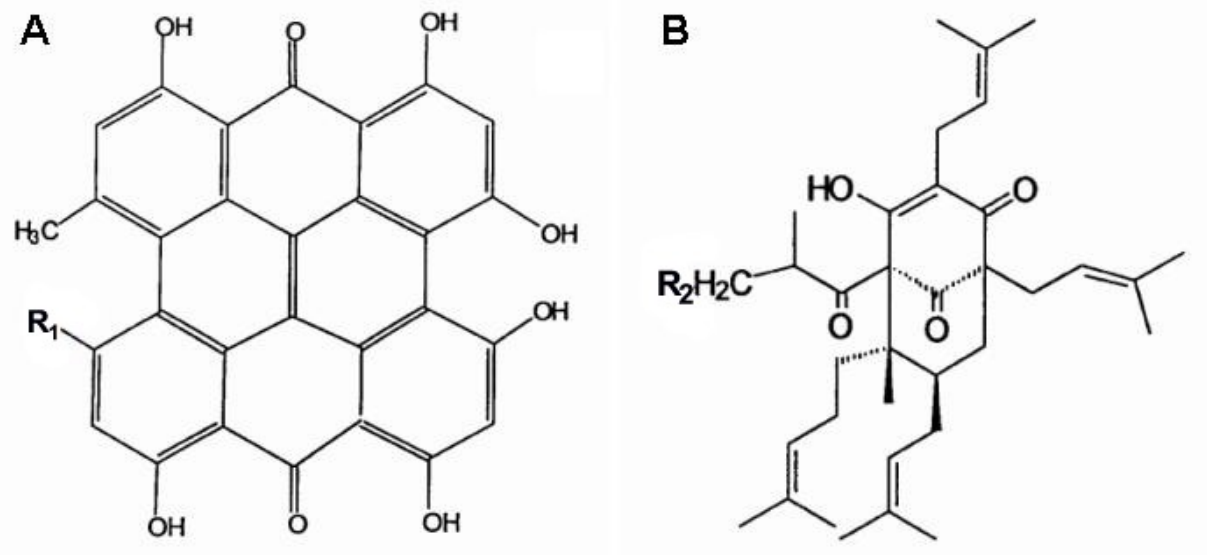

Figure 2: Structure of (A) naphthodianthrones (R_1 = CH_3, hypericin; R_1 = CH_2OH, pseudohypericin and (B) phloroglucinols (R_2 = H, hyperforin; R_2 = CH_3, adhyperforin).

(Maisenbacher & Kovar, 1992). The phloroglucinols are lipophilic. They are unstable against heat and light. One of the degradation products is 2-methyl-3-buten-2-ol, a component of the essential oil fraction. This tertiary alcohol is observed in hop plants and acts as a sedative in high doses (Wohlfart et al., 1983). Many pharmacological actions have been described for hyperforin. It was shown in synaptosomal preparations that hyperforin inhibits the uptake of 5-HT, dopamine, NE, γ-aminobutyric acid (GABA) and L-glutamate (Chatterjee et al., 1998; Wonnemann et al., 2000). Contrarily, in another study, using rat brain cortex slices, no inhibition of NE- or 5-HT-uptake could be detected either with pure hyperforin or hypericin, whereas the Hypericum extract Ze 117 inhibited the uptake of both neurotransmitters (Kientsch et al., 2001).

Hyperforin is reported to have an antibacterial activity against *Staphylococcus aureus*, multidrug-resistant *S. aureus*, *Streptococcus pyogenes* and *Corynebacterium diptheriae* (Schempp et al., 1999). It was emphasized that only high concentrations of hyperforin are antibacterially active. Furthermore, hyperforin can induce apoptosis by triggering activity of caspases in human malignant cell lines (Hostanska et al., 2003).

Chronic treatment of humans with Hypericum extract can lead to severe interactions with other co-administered drugs, like indinavir, theophylline, cyclosporine, warfarin or digoxin, by induction of cytochrome P450 (CYP) 3A4 enzyme (Markowitz et al., 2003; Wenk et al., 2004) and P-glycoprotein drug transporter. It has been shown by Moore et al. (2000) that treatment of human hepatocytes with Hypericum extract and hyperforin results in a marked induction of CYP3A4 expression and that hyperforin is a potent ligand for the pregnane X receptor, a nuclear receptor that regulates the expression of CYP3A4 monoxygenase. In line with these results is the study of Mai et al. (2004) in which the authors showed that a *Hypericum perforatum* extract with low hyperforin content does not influence the pharmacokinetic of cyclosporine but an extract with high hyperforin content decreases the cyclosporine plasma concentration, thus necessitating an increase in the cyclosporine dosage.

Flavonol glycosides with quercetin as the aglycon make up a major group of plant compounds in *Hypericum perforatum*, with a concentration of 2-4%. Only a small amount of free quercetin is detected. Other flavonoids are quercitrin, isoquercitrin, hyperoside, rutin and the biflavonoids biapigenin and amentoflavone (Nahrstedt & Butterweck, 1997). The chemical structure of flavonoids is similar to synthetic MAO inhibitors and it has been shown *in vitro* that quercetin inhibits the MAO activity and other flavonoids inhibit the catechol-O-methyltransferase enzyme (COMT). However, the level of flavonoids present in the Hypericum extract is too low for inhibition of these enzymes (Thiede & Walper, 1994). There is also *in vitro* evidence suggesting that some flavonoids, including hyperoside, quercitrin, isoquercitrin and amentoflavone, may elicit a sedative effect that could involve both benzodiazepine and GABA receptor agonism (Nielsen et al., 1988; Baureithel et al., 1997). In a recent receptor screening study performed by Butterweck et al. (2002), it was shown that amentoflavone exhibited affinity for the benzodiazepine-, 5-HT_{1D}-, D_3-, δ-opioid-receptor and for the dopamine transporter. The other flavonoids did not display such a broad receptor affinity. Moreover, in an animal study using the forced swimming test, it was suggested that a Hypericum extract fraction rich in flavonoids or naphthodianthrones shows an antidepressant activity comparable to that of the full extract (Butterweck et al., 1997). Nöldner & Schötz (2002) showed that a methanolic extract of *Hypericum perforatum* with a low level of rutin, in contrast to an ethanolic extract with a higher rutin level, showed no antidepressant activity in the forced swimming test paradigm. Only after addition of rutin to the methanolic extract,

resulting in a rutin concentration comparable to that of the ethanolic extract, it showed antidepressant activity. They concluded that rutin is essential for the antidepressant effect, because it increases the solubility of other compounds in the extract.

Xanthones appear to exhibit a strong MAO-A and MAO-B inhibition *in vitro* (Suzuki et al., 1984), but these substances are primarily found in the roots of the plant and only in trace amounts in the leaves and stems that are used for the manufacturing of the extract. Consequently, this group of compounds can not be responsible for the antidepressant activity.

The tannins in *Hypericum perforatum* appear to be oligomeric procyanidins that can make up 6 to 12% of the crude drug (Brantner et al., 1994). Proanthocyanidins exhibit many biological effects, including antimicrobial and antiviral activity (Scalbert, 1991; Kakiuchi et al., 1985), but no antidepressant effect has been reported. It was shown, however, that procyanidins increase the solubility of the naphthodianthrones hypericin and pseudohypericin leading to an enhanced activity in the forced swimming test (Butterweck et al., 2001).

The essential oil obtained from the dry leaves and petals of *Hypericum perforatum* by steam distillation accounts for 0.1 to 0.25% of the drug. Roth (1990) has published a list of 29 constituents making up about 65% of the steam distillate. The main constituents of the distillate are 2-methyloctane (16.4%) and α-pinene (10.6%). The sedative active methylbutenol constitutes for 0.2% of the distillate that is far below the concentration necessary for a sedative action.

Amino acids were detected in the crude drug in a concentration of about 10 mg/g drug (Nahrstedt & Butterweck, 1997). Among these compounds GABA, an inhibitory transmitter substance, was detected at about 0.7 mg/g of the crude drug. Because of this minimal concentration, there is no reason to suspect a significant contribution by GABA to the activity of the crude drug.

The antidepressant activity of the extracts of St. John's wort has been confirmed in several clinical trials. Linde et al. (1996) concluded in their meta-analysis of trials of St. John's wort extracts that there is good evidence that St. John's wort is superior to placebo in treating mild to moderate depression, despite small sample sizes and a lack of trials comparing St. John's wort with conventional antidepressants. Since then, several double-blind, randomised, controlled trials have been published that

have indicated the efficacy of St. John's wort in depression (Vorbach et al., 1997; Laakmann et al., 1998; Schrader, 2000; Woelk, 2000; Linde & Mulrow, 2000). Contrarily, a recent large-scale study by Shelton et al. (2001) raised concerns about the effectiveness of the plant extract. Patients with severe depression were included in that study, however, an indication for which St. John's wort is not approved in any country. The Hypericum Depression Trial Study Group (2002) addressed some of the unanswered questions, but found neither St. John's wort nor the SSRI sertraline to be superior to placebo treatment in severe depression. In a recent meta-analysis including randomised, double-blind and placebo or standard antidepressant controlled studies, it was concluded that the whole plant extract of *Hypericum perforatum L*. is as active as synthetic antidepressant drugs against mild to moderate depression and has a favourable side-effect profile (Röder et al., 2004).

1.3.2 Alternative antidepressant therapies and possible targets for new antidepressants

Electroconvulsive therapy (ECT), first introduced for the treatment of schizophrenia and epilepsy, is as effective as antidepressant drugs in treating depression. The response rates range between 60% and 80% but the main disadvantage is that it may cause confusion and memory loss lasting for days to weeks (Fink, 2001).

As described earlier, a hyperactivity of the HPA-axis is a common finding in depressed patients. This hyperactivity is demonstrated by increased cortisol levels, enlargement of the pituitary and adrenal glands and decreased glucocorticoid receptor sensitivity (Rubin et al., 1996; Krishnan et al., 1991; Evans & Nemeroff, 1987; Axelson et al., 1993). It is believed that these alterations are secondary to a hypersecretion of the corticotrophin-releasing factor (CRF). This hypersecretion is indicated by increased concentrations in cerebrospinal fluid (CSF) of patients with major depressive disorder (Bánki et al., 1992) and increased CRF mRNA levels in the paraventricular nucleus of the hypothalamus (Raadsheer et al., 1995). After successful antidepressant treatment, the elevated CRF concentrations in the CSF are decreased and normalised (Nemeroff et al., 1991; De Bellis et al., 1993).

Consequently, CRF receptor antagonists may represent a novel type of antidepressants.

Another common finding (in animal studies) is the elevated concentrations of the cAMP response element binding protein (CREB) and its active, phosphorylated form pCREB after chronic antidepressant treatment in the hippocampus and cerebral cortex (Thome et al., 2000). CREB is a downstream component in the cAMP cascade system and pCREB induces brain derived neurotrophic factor (BDNF) expression and this leads to neurogenesis, neuronal survival and neuronal plasticity (Walton & Dragunow, 2000). Post mortem and brain imaging studies have revealed atrophy or loss of neurones in the prefrontal cortex and hippocampus of depressed patients (Gurvits et al., 1996; Shah et al., 1998; Sheline et al., 1996) and some of these alterations may be reversed by antidepressants (Czeh et al., 2001). It was shown in mice that neurogenesis was required for the antidepressant action (Santarelli et al., 2003). Thus, drugs that lead to the up-regulation of any of the factors in the CREB and BDNF cascade may have potential antidepressant activity.

Other potential targets for antidepressants are cytokines, neurokinins and N-methyl-D-aspartate (NMDA) receptors. A number of alterations of the immune system are observed in major depression. Particularly increased levels of pro-inflammatory cytokines can be detected (Maes et al., 1997). It is unclear, however, whether this is a consequence of depression or a causal effect. In favour of a causal relationship, treatments with interleukin (IL)-2 and interferon (INF)-α used in patients with hepatitis C and some cancer forms frequently produce depressive symptoms (Schaefer et al., 2002). After chronic treatment with antidepressant drugs, serum IL-1β and serum IL-6 are reduced to normal levels in depressed patients (Sluzewska et al., 1996). In one study, an increased production of IL-1-receptor antagonist mRNA in specific regions of the rat cortex could be detected (Suzuki et al., 1996). This raises the possibility that drugs normalising the levels of cytokines (IL-1, IL-6) and IL-1-receptor antagonists could be a novel therapeutic strategy for the treatment of depression, but possibly the side-effects may outweigh the antidepressant effect.

Clinical trials demonstrated that neurokinin-1 (NK-1) receptor antagonists were surprisingly effective in treating depression (Stout et al., 2001) and a selective NK-2-receptor antagonist was shown to display antidepressant-like properties in

guinea pigs (Steinberg et al., 2001). This raises the question if neurokinin receptor antagonists could be potential new antidepressants.

Animal studies have shown that NMDA competitive and allosteric inhibitors reduce the immobility time in the forced swimming test (Trullas & Skolnick, 1990), an animal model for antidepressant activity. In addition, these inhibitors display antidepressant-like activity in a chronic mild stress model of depression (Papp & Moryl, 1994). Therefore, competitive and allosteric NMDA-inhibitors could be a further new approach for treating depressive states in humans.

1.4 Effects of chronic treatment with antidepressants

1.4.1 Effects on pre- and postsynaptic receptors

Chronic antidepressant treatment results in a number of changes in the pre- and postsynaptic neurones. It was found that most antidepressant drugs down-regulate the postsyntaptic 5-HT_2-receptors in the rat frontal cortex (Peroutka & Snyder, 1980). This antidepressant-induced down-regulation can lead to a normalisation of the 5-HT_2-receptor number that is increased in frontal cortices of depressed suicide victims and unmedicated depressed patients (Stanley & Mann, 1983; Mann et al., 1986; Yates et al., 1990). Antidepressant treatments do not consistently alter postsynaptic 5-HT_{1A}-receptor number, either no change (Green, 1988) or an increase in number is found (Burnet et al., 1994; Klimek et al., 1994). Electrophysiological studies have shown that most TCA and ECT increase postsynaptic 5-HT-mediated responses in the hippocampus after chronic treatment, however, and it is thought that 5-HT_{1A}-receptors are responsible for this change in responsiveness (Owens, 1996). In contrast, presynaptic 5-HT_{1A}-autoreceptors are desensitised after repeated exposure to 5-HT reuptake inhibitors (Chaput et al., 1991). Some antidepressants decrease dopamine D_1-receptor densities, whereas the D_2-receptor number remained unchanged in rat brain (Nowak et al., 1991; Klimek & Nielsen, 1987). Paul et al. (1994) reported that chronic treatments with antidepressants of every major class decrease the functional activity of NMDA receptor in the frontal cortex.

Furthermore, it was shown that antidepressants could influence $GABA_B$-receptors in the rat brain. The effect of antidepressant drugs on α_2-adrenergic autoreceptors appears complex. IMI down-regulates the α_2-adrenoceptors, whereas DMI up-regulates the same receptors (Baldessarini, 1996). Sacchetti et al. (2001) showed that long-term treatment with DMI induces adaptive changes involving desensitisation of the α_2-autoreceptor on noradrenergic neurones without changing their number.

One of the most consistent observations is that antidepressants desensitise postsynaptic NE receptor-coupled adenylate cyclase (AC) in the limbic brain regions, such as the cerebral cortex and the hippocampus (Vetulani & Sulser, 1975). This decreased responsiveness is attributed to down-regulation of postsynaptic β-adrenoceptors (Banerjee et al., 1977). Riva & Creese (1989) showed that after chronic treatment with DMI, the number of the β_1-adrenoceptors was decreased, whereas the β_2-adrenoceptor number remained unchanged. The adaptive response, down-regulation of β_1-adrenoceptors, follows repeated treatment with various types of antidepressants, including tricyclics, some 5-HT reuptake inhibitors, MAOI and electroshock in animals (Sulser & Mobley, 1980). In studies using postmortem tissue of depressed suicide victims, it was shown that the β-adrenoceptors are up-regulated in the frontal cortex (Mann et al., 1986; Biegon & Israeli, 1988; Sastre et al., 2001). Papp et al. (1994), using chronic-mild stress as a model of depression in animals, showed that in stressed rats the β-adrenoceptor number was increased and that the same rats show signs of depression. Chronic treatment with IMI decreased the β-adrenoceptor number and the symptoms of depression were reduced.

Until today, the mechanism of β-adrenoceptor down-regulation has not been fully explained. Since most of the antidepressant agents do not show any high affinity for β-adrenoceptors, this change in number is not due to a direct interaction. It has been suggested that the serotoninergic system may be involved in the down-regulation of β-adrenoceptors (Asakura et al., 1987; Gillespie et al., 1988). Conversely, it was shown that chronic antidepressant treatment of rat C6 glioblastoma cells and human fibroblasts down-regulate the β-adrenoceptors in the absence of a presynaptic input (Honegger et al., 1986; Fishman & Finberg, 1987; Fowler & Brännström, 1990). It seems that the down-regulation of β-adrenoceptors is a direct effect of

antidepressants on postsynaptic cells, leading to an inhibition of receptor synthesis (Hosoda & Duman, 1993) and/or increased receptor degradation (Kallal et al., 1998). A decrease in the number of cell surface receptors could also be due to an antidepressant-induced alteration of receptor trafficking routes, as shown by Bürgi et al. (2003): chronic treatment of rat C6 glioblastoma cells with the TCA DMI inhibited recycling of GFP-tagged β_1-adrenoceptors back to the cell surface.

1.4.2 Effects on membrane properties

Most antidepressant drugs can induce phospholipidosis after chronic treatment *in vitro* as well as *in vivo*. This is due to an excessive accumulation of phospholipids (PL) in the cell. PL are structural components of mammalian cell membranes and organelles. Affected cells may appear vacuolated when examined light microscopically and acquire a multilamellar morphological appearance detectable by electron microscopy (Lüllmann-Rauch, 1979; Reasor, 1989; Horn et al., 1996). Except for antidepressants, other substances belonging to different chemical classes and with different therapeutic effects can induce phospholipidosis. These drugs, termed cationic amphiphilic drugs (CADs), are characterised by a hydrophilic portion consisting of one or more primary or substituted nitrogen groups and a hydrophobic domain consisting of an aromatic and/or aliphatic ring. CADs include antidepressants, neuroleptics, antiarrhythmics, macrolide antibiotics, antimalarial agents, local anaesthetics and others (Kodavanti & Mehendale, 1990; Schneider et al., 1997). CAD-induced phospholipidosis is characterised by four principle features: excessive accumulation of PL in cells; ultrastructural appearance of membranous lamellar inclusions, predominantly of lysosomal origin; trapping of the inducing drug in charged form and in association with the increased PL in acidic compartments; and reversibility of alterations after discontinuation of drug treatment (Lüllmann-Rauch, 1979). In many animal studies, phospholipidosis has been observed following CAD administration far above concentrations used clinically. In humans, only amiodarone, chloroquine (Müller-Höcker et al., 2003), perhexiline, fluoxetine (Gonzalez-Rothi et al., 1995), gentamicin, and 4,4'-diethylaminoethoxyhexestrol have been reported to induce phospholipidosis (Reasor & Kacew, 2001).

Intracellularly, CADs accumulate principally in lysosomes. This phenomenon has been described by De Duve et al. (1974) as lysosomotropism. *In vivo*, CADs accumulate in lysosome-rich tissues, e.g. lung, liver and kidney. There are two possible mechanisms explaining this accumulation of the CADs in lysosomes. First, CADs, in their non-protonated, lipophilic form can permeate freely through the cell membrane into the lysosomes. Due to the acidic pH in lysosomes, the CADs are protonated. In this hydrophilic, charged form they can not permeate through membranes and are trapped in the lysosomes. Second, CADs bound to plasma membrane PL can be endocytosed and the endocytic vesicles may be directed to lysosomes. These two mechanisms lead to CAD-accumulation in this cellular organelle and inhibition of PL degradation, as has been shown for DMI, a TCA and CAD, in cell cultures. Honegger et al. (1983) examined the influence of the pH-gradient between the culture medium and the lysosomes on the cellular accumulation of DMI in human fibroblasts. A decrease in the extracellular pH led to less accumulation of DMI in human fibroblasts, due to a lower pH-gradient. In addition, an increase in PL, especially of phosphatidylinositol, has been detected by Fauster et al. (1983). This increase in PL could be explained by several mechanisms. CADs inhibit the lysosomal phospholipases either directly (Pappu & Hostetler, 1984; Kubo & Hostetler, 1985) or indirectly by increasing the lysosomal pH (Ohkuma & Poole, 1981). Furthermore, binding of CADs to PL can result in complexes that are indigestible for lysosomal phospholipases (Drenckhahn et al., 1976; Lüllmann et al., 1978) or an increase or redirection of synthesis of PL may play a role due to lysosomal accumulation of PL (Pappu & Hostetler, 1984). It is likely that to some extent each of the above-mentioned mechanisms contributes to the induction of phospholipidosis and it is also possible that the mechanism of PL accumulation is not identical for each CAD.

Chronic exposure of human fibroblasts and rat C6 glioblastoma cells to DMI lead to an increased concentration of PL and to a reduction of functionally active β-adrenoceptors (Zühlke, 1990; Zbinden, 1997). In addition, this treatment increased the membrane fluidity and changed the cellular and the plasma membrane PL composition (Zühlke, 1990). In the presence of α-tocopherol, the uptake of DMI was reduced and showed a vitamin E concentration dependency. Neither a change of the cellular PL pattern nor an alteration of the membrane fluidity could be detected nor a

reduction of functionally active β-adrenoceptors was observed after chronic treatment of cells with DMI in the presence of α-tocopherol (Scuntaro, 1995).
It is possible that a receptor-ligand complex shows changed mobility in the membrane due to changes of the membrane PL composition and membrane fluidity. Hirata et al. (1979) reported that an increased membrane fluidity enhanced the interaction of β-adrenoceptors with G proteins, thereby possibly increasing the intracellular signals.

1.5 β-Adrenergic signal transduction

β-Adrenoceptors belong to the group of G protein-coupled receptors (GPCRs) that is also referred to seven membrane-spanning receptors (Pierce et al., 2002). The protein is predicted to contain seven transmembrane-spanning (7TM) helices that is based on a homology with rhodopsin. In rhodopsin, the presence of an anticlockwise bundle of 7TM α-helices could be confirmed by determining its crystal structure (Palczewski et al., 2000). Oligosaccharides can be bound to the extracellularly located N-terminus, whereas the C-terminus is located intracellularly. The function of GPCRs is primarily to transmit extracellular stimuli into intracellular signals.
Agonist activation of the receptors induces conformational changes which are poorly understood, but which seem to involve at least rearrangements of membrane helices 6 and 3 and possibly of the intracellular loops. The activated receptor interacts and binds to a heterotrimeric guanine-nucleotide binding protein (G protein) (Figure 3). This results in the activation of the G protein by exchanging bound GDP with GTP. It is assumed that the activated heterotrimeric G protein dissociates into the βγ-subunit ($G_{\beta\gamma}$) and the α-subunit (G_{α}) with the bound GTP. β-Adrenoceptors activate α-subunits ($G_{\alpha s}$) that bind to the regulatory subunits of the AC enzyme, leading to its activation and to the production of the second messenger cAMP by hydrolysis of ATP. To prevent overstimulation of the cells, the cytoplasmic C-terminus of the β-adrenoceptor is phosphorylated by the specific β-adrenoceptor kinase (β-ARK). This phosphorylation results in subsequent binding of arrestins to the receptor and to disruption of the functional coupling between β-adrenoceptors and $G_{\alpha s}$. The GTP

bound $G_{\alpha s}$ activates not only AC, but also its own intrinsic GTPase and thus hydrolysis GTP into GDP. This inactivation of $G_{\alpha s}$ leads to a discontinuation of the AC activity. $G_{\alpha s}$ and $G_{\beta\gamma}$ are associating again to form the inactive trimeric G protein. cAMP is inactivated by phosphodiesterases leading to adenosine-5'-monophosphate (5'-AMP). cAMP itself activates the cAMP dependent proteinkinase A (PKA) which phosphorylates specific serine- and threonine-rich sites of proteins. Serines and threonines in the intracellular C-terminal tail of β-adrenoceptors are also phosphorylated by PKA, leading to desensitisation.

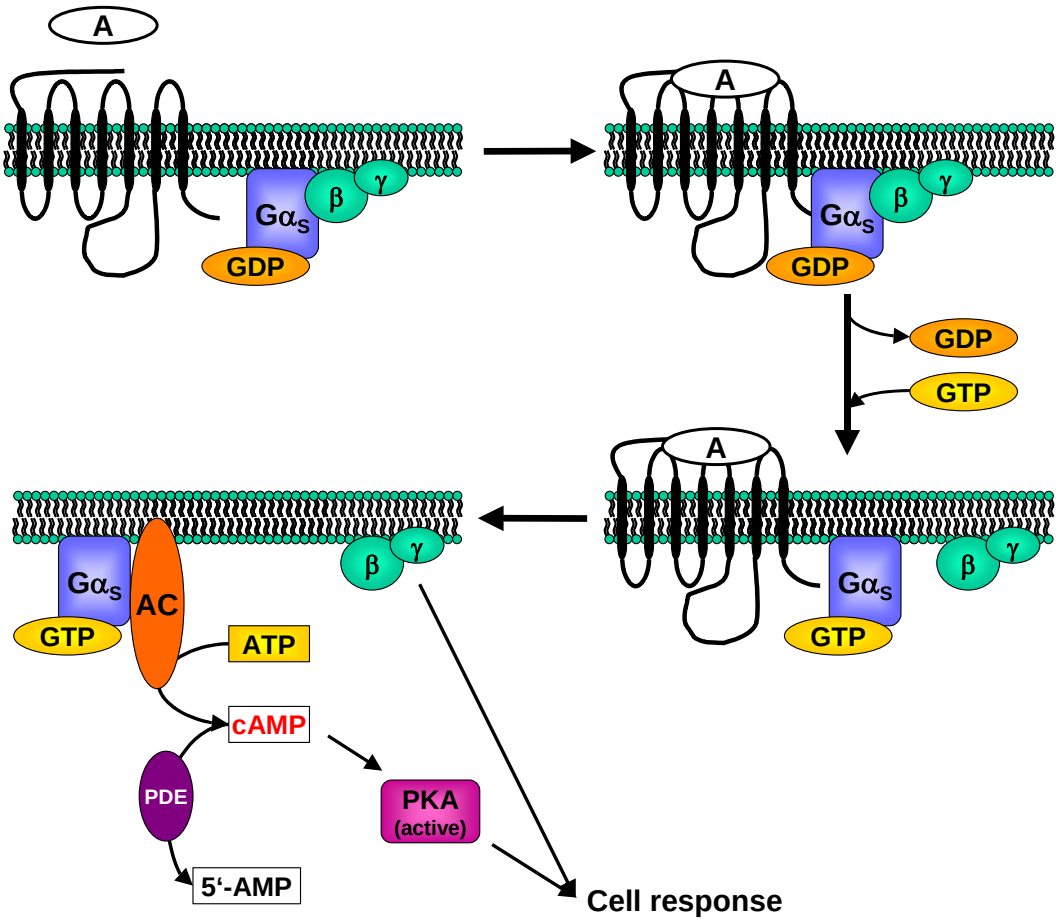

Figure 3: Schematic representation of the β-adrenergic signal transduction. Receptor activation after the binding of agonist (A) promotes the binding of the heterotrimeric G protein to the receptor. The G protein is activated by replacing guanosine diphosphate (GDP) with guanosine triphosphate (GTP), leading to the dissociation of the stimulatory G protein α-subunit ($G_{\alpha s}$) and G protein βγ-subunit. $G_{\alpha s}$ activates AC that hydrolyses adenosine triphosphate (ATP) into the second messenger cAMP. cAMP binds to the regulatory subunit of the cAMP dependent protein kinase A (PKA), resulting in enhanced activity of the enzymatic subunit. This leads to phosphorylation of different proteins and promotes various cell responses. The βγ-subunit of the G protein also mediates diverse cell responses. cAMP is deactivated by hydrolysis into 5'-adenosine monophosphate (5'-AMP) by phosphodiesterases (PDE).

Furthermore, PKA activates the cAMP response element binding protein (CREB) by phosphorylation of one serine. This activated pCREB binds to regions of genes that contain a cAMP-response element (CRE) and initiates a cascade of gene expression that persists long after the original stimulating cAMP has been degraded (Yin & Tully, 1996).

1.5.1 Classes of adrenoceptors

Adrenoceptors are cell-surface proteins that bind epinephrine and/or NE with high affinity, thereby triggering intracellular changes. The two major classes of adrenoceptors, alpha and beta, were originally separated by their different cellular actions but are now distinguished by their relative affinity for characteristic synthetic ligands. α-Adrenoceptors are blocked by phentolamine, whereas β-adrenoceptors are blocked by propranolol and activated by isoproterenol. Alpha-adrenoceptors are further subdivided into two subclasses, α_1- and α_2-adrenoceptors, based on studies of endogenous and cloned receptors. α_1-Adrenoceptors are pharmacologically separated from α_2-adrenoceptors by their high affinity for the agonist phenylephrine and the antagonist prazosin. They are widespread, with clinically important densities in the liver, the heart, vascular, intestinal, and genito-urinary smooth muscles, and the central and peripheral nervous systems. Its stimulation leads to the activation of phospholipase C by G_q proteins and the production of inositol-3-phosphate (IP3) and diacylglycerol (DAG) as second messengers. α_2-Adrenoceptors have a high affinity for the agonist clonidine and the antagonist yohimbine. They are found on pancreatic beta cells, platelets, vascular smooth muscle and in both pre- and postsynaptic membranes in the central and peripheral nervous systems. Stimulation of α_2-adrenoceptors leads to an inhibition of the cAMP production by the inhibitory $G_{\alpha i}$-subunits.

Beta-adrenoceptors are subdivided into β_1-, β_2- and β_3-subtypes. The beta-1 type is equally sensitive to epinephrine and NE and binds the agonist dobutamine and the antagonist metoprolol with high affinity. It is found in the heart, juxtaglomerular cells, and in the central and peripheral nervous systems. Beta-2 adrenoceptors are more

sensitive to epinephrine than to NE and have a high affinity for the agonist terbutaline. They are widespread, with clinically important roles in skeletal muscle, liver, vascular, bronchial, gastrointestinal, and genito-urinary smooth muscles. The beta-3 subtype is the predominant subtype expressed in white and brown adipocytes and is involved in modulating energy metabolism and thermogenesis. It is more sensitive to NE than to epinephrine. The activation of β-adrenoceptors leads to an increase in cAMP by the stimulatory $G_{\alpha s}$.

As already described, chronic treatment with antidepressants leads to a decreased responsiveness of postsynaptic β_1-adrenoceptors, due to their down-regulation. This adaptive response, down-regulation of β-adrenoceptors, follows repeated treatment with various types of antidepressants, including tricyclics, SSRI, MAOI and even electroshock in animals.

1.5.2 Guanine-nucleotide binding proteins (G proteins)

G proteins are heterotrimeric complexes including an alpha-, beta- and gamma-subunit. The α-subunit is responsible for the GTP and GDP binding and for the GTP hydrolysis, whereas the β- and γ-subunits are associated with a tightly linked βγ-complex (Gilman, 1987). Today each of these subunits are known to be a member of a gene family and so far at least 18 different human α-, 5 types of β- and 12 types of γ-subunits have been cloned (Hermans, 2003). The heterotrimeric G protein complex binds with its α-subunit to a stimulated GPCR and GDP is released from the G protein and replaced by GTP. This leads to dissociation of the G protein complex into a α-subunit and a βγ-dimer which both activate several effectors. Aluminium tetrafluoride (AlF_4^-) together with Mg^{2+} can interact with the α-bound GDP to mimic GTP and thereby activate the α-subunit. G proteins are generally characterised by

their α-subunits and four distinct subfamilies are recognised:

- G_s proteins mediate stimulation of AC
- G_i/G_0 proteins mediate inhibition of AC as well as activation of G protein-coupled inwardly rectifying potassium (GIRK) channels
- G_q/G_{11} proteins mediate activation of phospholipase Cβ
- G_{12}/G_{13} proteins mediate activation of Rho guanine-nucleotide exchange factors (GEFs)

G proteins bind to the membrane by isoprene moieties of the βγ-subunit. Traditionally, the βγ-complex was viewed as a membrane localisation anchor and/or negative regulator of the α-subunit (Hepler & Gilman, 1992). Although the βγ-dimer localises and regulates the α-subunit, it was found by Logothetis et al. (1987) that βγ by itself activates K^+-channels. It is now accepted that both the α- and the βγ-subunits are capable of activating a variety of effector molecules, but there is also evidence that βγ mediates many of those responses which once were attributed solely to the α-subunit (Clapham & Neer, 1997).

Chronic treatment with antidepressant drugs facilitates the coupling of $G_{\alpha s}$ to AC in synaptosomal membrane preparations of rats as well as in cell membrane of cultured rat C6 glioblastoma cells (Newman & Lerer, 1989; Chen & Rasenick, 1995). This enhanced coupling was not due to an increased expression of the α-subunit. Direct activation of the G protein (with NaF or GppNHp) showed a reduced AC activity in brain membrane preparations from rats chronically treated with antidepressant and this effect was slower in onset than the reduction in β-adrenoceptor number (Tiong & Richardson, 1990). Furthermore, chronic exposure of C6 cells to tricyclics, non-reuptake inhibitors and SSRI antidepressants induced an enrichment of $G_{\alpha s}$ in less hydrophobic membrane fractions, whereas $G_{\alpha i}$ levels did not change. The same phenomenon was observed in membranes of rat cerebral cortex after chronic antidepressant treatment (Toki et al., 1999). Donati et al. (2001) showed in C6 cells that this enrichment of $G_{\alpha s}$ in less hydrophobic membrane fractions could be due to a redistribution of the α-subunit after chronic exposure.

1.5.3 Adenylate cyclases

The activation of AC is initiated by the binding of an agonist to cell surface receptors and results in the intracellular production of cAMP. cAMP is formed from ATP by the hydrolysis of two phosphorus groups. Mammalian AC are integral membrane proteins that appear to contain two sets of six membrane-spanning helices and these helices are separated by a large (~40 kDa) cytoplasmic loop (Krupinski et al., 1989). Cytosolic cyclase domains are responsible for the catalytic activity and most of the regulatory properties of the enzymes (Tang & Gilman, 1995).

$G_{\alpha s}$ interacts with one of the two cyclase domains of the AC in a GTP-enhanced manner and $G_{\alpha s}$ forms a relatively high-affinity complex that is further stabilised by the second cyclase domain (Sunahara et al., 1997). Mammalian AC can also be activated by the diterpene forskolin (Seamon et al., 1981) and inhibited by certain adenosine analogues and adenine nucleotides (Londos & Wolff, 1977). Certain AC are also regulated by Ca^{2+}, Ca^{2+}-calmodulin, and phosphorylation (Smit & Iyengar, 1998). By means of molecular cloning techniques, nine mammalian genes that encode membrane bound AC and one gene encoding a soluble form have been identified. The soluble isoform, sAC, is similar to cyclases found in cyanobacteria. Membrane-bound AC isoforms are generally, but not exclusively, found in neurones and muscles. Within the brain, the AC isoforms are localised in different, discrete brain regions. Most isoforms are widely expressed, but AC1 and AC3 are expressed only in the brain. The soluble cyclase is present predominantly in the testis (Sunahara & Taussig, 2002).

The activity of the AC can be regulated by phosphorylation, either by protein kinase A (PKA) or protein kinase C (PKC). PKA-mediated phosphorylation is thought to negatively regulate AC5 and AC6 activity (Iwami et al., 1995). It is thought that PKC regulates AC in an isoform-specific manner. After activation of PKC by the unspecific activator phorbol ester, AC1, AC2, AC3 and AC5 are stimulated, whereas AC4 and AC6 are inhibited. For AC2, AC5 and AC6 this regulation is due to direct phosphorylation by PKC (Sunahara & Taussig, 2002).

Surprisingly, almost no literature is available concerning the effects on AC by acute and chronic treatment with antidepressant drugs. Zbinden (1997) showed that acute exposure of C6 cells and fibroblasts to DMI diminished both the isoproterenol and the forskolin stimulated cAMP responses.

1.5.4 Regulation of β-adrenoceptors

One important feature of the GPCR-signalling system, including the β-adrenoceptor signalling system, is that short, prolonged or repeated agonist exposure are able to attenuate its response in order to prevent over-stimulation. Thus, activation of a receptor leads to a reduced responsiveness to subsequent stimulation. This process depends on several different mechanisms, including the uncoupling of heterotrimeric G proteins from the receptor in response to receptor phosphorylation (desensitisation) (Bouvier et al., 1988), the internalisation of cell surface receptors to intracellular membranous compartments (e.g. Oakley et al., 1999) and the down-regulation of the total cellular receptor number. Down-regulation of receptors might be due to a reduction of receptor mRNA and protein synthesis and lysosomal and plasma membrane degradation of pre-existing receptors (Hadcock & Malbon, 1988; Valiquette et al., 1990; Jockers et al., 1999). The time necessary for the desensitisation to occur, ranges from seconds for phosphorylation to minutes for sequestration/endocytosis and hours for down-regulation. Internalised receptors can undergo re-sensitisation through recycling back to the cell surface.

The β_2-adrenoceptor is the best-examined representative of GPCRs and serves as model-system for explaining the underlying mechanisms of GPCR de- and re-sensitisation. Since the β_1-adrenoceptor shows a high similarity to the β_2-type, it is assumed that the mechanisms of de- and re-sensitisation are similar. Consequently, all the following explanations use the β_2-adrenoceptor subtype as a model.

1.5.4.1 Phosphorylation

Attenuation of the receptor responsiveness is achieved through phosphorylation of serine and threonine residues within the carboxyl-terminal tail domain of the receptor. This receptor phosphorylation is executed by the recruitment of cytosolic β-adrenoceptor kinase (βARK) and of cytosolic second messenger dependent protein kinases, including cAMP dependent PKA and PKC (Hausdorff et al., 1989). Special proteins such as PKA anchoring proteins, are responsible for the membrane anchoring of PKAs. βARKs bind to $G_{\beta\gamma}$ after dissociation of the α-subunit from the heterotrimeric G protein.

Second messenger dependent protein kinases phosphorylate activated receptors, but also certain receptor proteins that are not stimulated. Consequently, PKA or PKC activated by stimulation of totally different receptor types can phosphorylate and diminish the responsiveness of β-adrenoceptors. This decrease in responsiveness is termed heterologous desensitisation. Homologous desensitisation is the activation-dependent regulation of receptors that leads to their phosphorylation by βARKs and a subsequently diminished responsiveness.

Receptor phosphorylation promotes the binding of arrestins and uncoupling of receptors from heterotrimeric G proteins, resulting in the discontinuation of AC activation. Arrestins are cytosolic proteins recruited to the plasma membrane receptor. They preferentially recognise phosphorylation sites on the receptor. The arrestin-receptor complexes lead to the enrichment of receptors into clathrin-coated pits, since arrestins bind to clathrin adapter protein AP2 and clathrin (Laporte et al., 1999).

1.5.4.2 Sequestration/endocytosis

Internalisation of GPCRs, also termed receptor sequestration or endocytosis, occurs more slowly than desensitisation over a period of several minutes after agonist exposure. Subsequent recycling of endocytosed adrenoceptors is required for re-sensitisation (Pippig et al., 1995).

Clathrin-coated pits form invaginations resulting in the formation of vesicles (Figure 4). The pinching off of the vesicles from the plasma membrane is regulated by the cytosolic GTPase dynamin that binds to the AP2-clathrin complex of the clathrin-coated pits (Zhang et al., 1996). The clathrin-coat of internalised vesicles is removed and vesicles bind to and fuse with sorting/early endosomes. This fusion is controlled by specialized proteins like Rab5 and SNAREs (soluble NSF attachment protein receptors) that are incorporated into clathrin-coated pits and vesicles.

Sorting/early endosomes play an important role in the redirection of internalised material back to the plasma membrane or to the degradation pathway into the lysosomes. The weakly acidic environment promotes the release of agonist from the receptor, due to its conformational change. Receptors interact more easily with the endosomal phosphatases, leading to their dephosphorylation by a G protein-coupled receptor specific phosphatase (GRP) (Krüger et al., 1997).

Substances that increase the pH of vesicles block or interfere with the endocytosis. Monensin was shown to inhibit endocytosis and the CADs were found to influence both endocytosis and recycling of membrane receptors (Hunt & Marshall-Carlson, 1986). Recently, Bürgi et al. (2003) showed that chronic DMI treatment of C6 cells impaired the recycling of β_1-adrenoceptors after stimulation with isoproterenol. It is possible that uncoupling of receptor bound agonists is partially impaired due to the elevated pH in endosomes, resulting in prolongation of the desensitised state of the receptor.

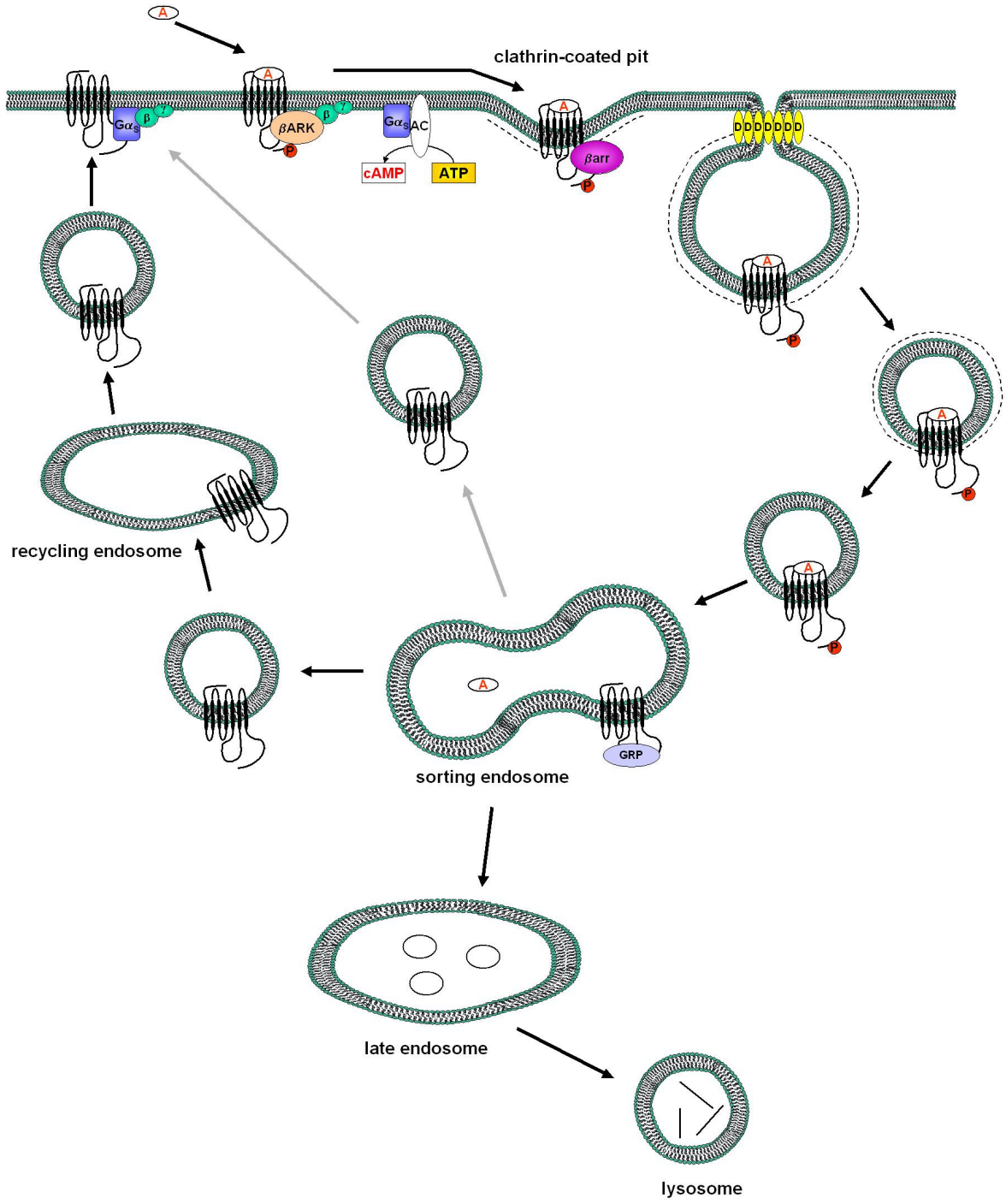

Figure 4: Pathways of internalised receptors. The binding of an agonist (A) results in receptor activation and subsequent dimerisation of the heterotrimeric G protein into $G_{\alpha s}$ and $G_{\beta\gamma}$. $G_{\alpha s}$ activates AC, resulting in the formation of the second messenger cAMP. Cytosolic β-adrenoceptor kinase (βARK) is recruited to the receptor, leading to the phosphorylation of the β-adrenoceptor. Receptor phosphorylation promotes binding of β-arrestin (βarr) and the receptor is recruited to clathrin-coated pits. The pinching off of clathrin-coated vesicles is promoted by the GTPase dynamin (D). The clathrin coat is removed and the endocytic vesicles fuse with sorting endosomes. Due to the weakly acidic pH in this compartment, the agonist is released from the receptor and the receptor is dephosphorylated by a G protein receptor specific phosphatase. Receptors are recycled back to the cell membrane either directly (grey arrows) or via recycling vesicles. Proteins determined for degradation are transferred via late endosomes to lysosomes.

1.5.4.3 Lysosomal degradation / Down-regulation

The mechanisms and signals by which receptors and other cellular components are sorted, either for recycling, or for degradation, are not well understood. Receptors selected for degradation in lysosomes show sorting signals in their carboxyl-terminal tail, facilitating their selection by proteins of the sorting machinery (Kirchhausen, 1999). It is assumed that components targeted for degradation are either transported in endosomal carrier vesicles (ECVs) from sorting endosomes to late endosomes (Aniento et al., 1993) (Figure 4), or stay in sorting endosomes that undergo maturation towards late endosomes (Mukherjee et al., 1997). The mechanism by which late endosomes deliver their content to lysosomes is not yet understood. Mullock et al. (1998) proposed that late endosomes are fusing transiently with lysosomes. After digestion of the content, lysosomes are formed again out of these hybrid-organelles.

1.5.4.4 Recycling

Receptors without special sorting motifs and most of the membrane components, e.g. PL, recycle back to the plasma membrane either directly from sorting endosomes or via recycling endosomes (Figure 4) (Mukherjee et al., 1997). Specific Rab proteins that are small GTPases drive the targeting of the different vesicles. These proteins are localised in the membrane of the endosomes and show a distinct localisation pattern in the endosome population. For example, the membrane of sorting endosomes is enriched with Rab4 and Rab5 proteins, whereas recycling endosomes show prevalence for Rab4 and Rab11 (Sönnichsen et al., 2000). β-Adrenoceptors that are recycled back to the plasma membrane need several minutes to be re-sensitised and to regain their responsiveness (Pippig et al., 1995).

Aim of this work

The aim of this study was to investigate the *in vitro* antidepressant effects of plant extracts of *Hypericum perforatum L.* in cell cultures and to compare those with the effects of well-established synthetic antidepressant drugs. In addition, the significance of hyperforin in the antidepressant action of Hypericum extracts was explored, because it has been shown that this compound is the main cause for pharmacokinetic interactions of Hypericum extracts with co-administered drugs.

For the above purposes, rat C6 glioblastoma cell and human skin fibroblast cultures were chronically exposed to Hypericum extracts, the TCA desipramine or the SSRI antidepressant fluoxetine. The Hypericum extracts included a full hydroalcoholic extract of *Hypericum perforatum L.* and fractions of it with high and very low hyperforin content. Cellular changes after chronic exposure were examined by determination of the cell morphology and proliferation rate. Alterations in the β-adrenoceptor-sensitive signal pathway were monitored, including the determination of the membrane phospholipid composition and membrane fluidity as well as the measurement of the number of surface β-adrenoceptors and of the second messenger cAMP after direct and indirect activation of the adenylate cyclase.

2 Materials and Methods

2.1 Cell cultivation

2.1.1 Rat C6 glioblastoma cells (C6 cells)

Identical rat C6 glioblastoma cells from the European Collection of Animal Cell Cultures (ECACC Nr. 85040101) and from the American Type Culture Collection (ATCC CCL-107) were used. These cell lines originate from the source described in Benda et al. (1968).

Both cell lines were treated identically. They were cultured in Minimum Essential Medium Eagle (MEM-Eagle) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 44 mM $NaHCO_3$, 22.7 mM glucose, 10 ml/l non-essential amino acid solution, 100 U/ml penicillin G and 100 µg/ml streptomycin. The cells were cultivated in a 5% CO_2 incubator with a humidified atmosphere at 37°C.

2.1.1.1 Subcultivation

The cells were kept in 175 cm^2 culture flasks or 10 cm ∅ culture dishes and subcultivated once a week. The medium was discarded and the cell monolayer was rinsed once with Hanks' balanced salt solution (HBS). 10 ml or 3 ml trypsin 0.1%, in HBS was added. The cells were detaching from the surface of the culture flask or culture dish after an incubation of 5 to 10 minutes in the 5% CO_2 incubator at 37°C. The trypsin was inactivated with 10 ml or 3 ml medium, respectively, and the cell suspension was poured into a conical 50 ml centrifuge tube. The cell culture flask or dish was additionally rinsed twice with 5 ml or 2 ml medium and pooled in the centrifuge tube. After 2 minutes of centrifugation at 210 x g, the supernatant was discarded and the cell pellet was resuspended in 8 ml or 4 ml medium. 1 ml or 0.5 ml cell suspension solution was added into a 175 cm^2 cell culture flask prefilled with 29 ml medium or into a 10 cm ∅ culture dish preloaded with 9.5 ml medium. The cell

culture flask or dish was slightly shaken to homogenise the cells in the medium to obtain a homogenous cell monolayer. For the seeding a dilution of 1:8 was chosen.

2.1.2 Human skin fibroblasts

Dermal fibroblasts were obtained from the laboratory of Professor U.N. Wiesmann of the Children Hospital of the University of Bern. The fibroblasts were cultivated in MEM-Eagle containing 10% FCS, 2 mM L-glutamine, 26.2 mM $NaHCO_3$, 200 I.U. penicillin G, 10 µg/ml chlortetracyline and 10 ml/l non-essential amino acid solution. The fibroblasts were grown in 175 cm^2 tissue culture flasks. After one or two weeks of cultivation in a humidified incubator with 5% CO_2 at 37°C cultures were trypsinised. The medium was discarded, the cell monolayer rinsed with 10 ml HBS and 10 ml trypsin 0.1% in HBS was added. The cell monolayer was incubated for 15 minutes at 37°C in a 5% CO_2 atmosphere. After this treatment the cells were detaching from the culture flask. The trypsin was inactivated by the addition of 5 ml medium. The cell suspension was poured into a conical 50 ml centrifuge tube. The tissue culture flask was then rinsed twice with 5 ml medium and the solutions were pooled. The cell suspension was centrifuged at 210 x g for 2 minutes. The supernatant was discarded and the cell pellet was resuspended in 5 ml medium. 1 ml of the cell suspension was diluted in 29 ml medium. The flask was shaken cautiously to homogenise the cells in the medium to obtain a homogenous cell layer. The cells were seeded with a dilution of 1:5.

Substances and solutions for cell culture:

Chlortetracycline-HCl 5 g/ml (100 x)	Gibco BRL 15280-027
Desipramine	Ciba Geigy
Fetal calf serum	Invitrogen Gibco BRL 10270-106
Fluoxetine	Sigma F-132
Hanks' balanced salt solution (HBS)	Inselspitalapotheke
Hyperforin-"free" fraction and hyperforin-rich fraction	Professor M. Hamburger, Friedrich-Schiller-Universität in Jena
Hypericum-Lutrol extract	Zeller AG, Romanshorn
L-glutamine 100 x (200 mM)	Sigma G7513
Minimum Essential Medium Eagle Auto-Mod	Sigma M0769
Non-Essential Amino acid solution 100 x	Sigma M7145
Penicillin G lyophilised	Inselspitalapotheke
Penicillin G / Streptomycin stabilised	Sigma P4333

2.1.3 Chronic treatment

The cells were treated during 6 or 7 days with 10 µM desipramine (DMI), 10 µM fluoxetine, Hypericum-Lutrol extract corresponding to 5 µM hypericin and different concentrations of hyperforin-"free" and hyperforin-rich extracts obtained from the laboratory of Professor Dr. M. Hamburger in Jena. Medium supplemented with the same amount of ethanol was used as control. For the 6-day treatment regimen, the medium was changed at day 0, 2, 4 and 5 and for the 7-day treatment regimen at day 0, 2, 4, and 6. The cells were used for the experiment at day 6 or day 7.

2.2 Protein determination

2.2.1 Method according to Lowry et al.

The cells of a 10 cm ∅ petri dish were scraped and resuspended in a total of 6 ml HBS. This cell suspension was sonicated and 200 µl of the sonicated solution or the same volume of a bovine serum albumin (BSA) standard (20-100 µg) was mixed with 1 ml Biuret solution. After 10 minutes 200 µl of Phenol reagent was added, the solution was vortexed and 60 minutes later the absorption of the bluish solution was measured with a spectrophotometer at a wavelength of 750 nm against a blank solution (Lowry et al., 1951).

Biuret solution:	Lowry A 100 parts, Lowry B 1 part, Lowry C 1 part
	Lowry A: 3.0% Na_2CO_3 and 0.4% NaOH in H_2O_{dd}
	Lowry B: 4.0% KNa-Tartrate in H_2O_{dd}
	Lowry C: 2.0% $CuSO_4$ in H_2O_{dd}
Phenol reagent:	Folin-Ciocalteus-Phenol reagent diluted 1:1 with H_2O_{dd}
BSA-standard:	BSA (Serva) 0.5 mg/ml in HBS

2.2.2 BCA estimation

The cell suspension was diluted approximately to 50-150 µg protein/ml, a sample of 5 µl was taken and mixed with 1 ml of the BCA working solution. The samples were incubated for 1 hour in a water bath heated to 60°C. After cooling to room temperature the absorption of the solution was measured against a blank solution with a spectrophotometer at a wavelength of 562 nm. For the calculation of the protein content a standard curve with BSA concentrations of 2-20 µg was prepared analogical to the samples.

BCA working solution:	25 parts MicroBCA A, 24 parts MicroBCA B, 1 part MicroBCA C
BSA standard:	2.0 mg/ml in 0.9% NaCl and 0.05% Na-azide (Pierce MicroBCA Protein Assay Reagent Kit 23235)

2.3 Phospholipid analysis

2.3.1 Phosphorus concentration in phospholipids

All glassware used for this assay was washed with 0.1 N HCl and rinsed twice with doubly distilled water (H_2O_{dd}) to remove organic and inorganic phosphorus. 50 µl of a sonicated cell suspension was mixed with 1 ml of 1.2 M trichloroacetic acid in a phosphorus free borosilicate glass vial to precipitate proteins. The vial was shaken vigorously for 10 minutes. The lightly turbid solution was centrifuged at 5000 x g for 10 minutes and the supernatant was discarded. For a standard curve different volumes of a phosphatidylcholine (PC) stock solution (10 mM in theoretical lower phase (TLP)) equal to 1.5-18 nmol PC were transferred into borosilicate glass vials. The vials were placed in a water bath prewarmed to 35°C and the TLP was evaporated under an N_2-air stream. Samples and standards were mixed with 1 ml ashing reagent. The solutions were evaporated for 1 hour at 160°C and then for 2 hours at 230°C. After cooling to room temperature the residues were dissolved in

1 ml H_2O_{dd}, mixed with 200 µl of ammonium heptamolybdate solution and 20 minutes later mixed with 200 µl of malachite green solution. After 30 minutes the absorption of the solutions were measured with a spectrophotometer at a wavelength of 610 nm against a blank solution (Van Veldhoven & Mannaerts, 1987).

Ashing reagent:	5 ml 36 N H_2SO_4, 2 ml 70% $HClO_4$, 93 ml H_2O_{dd}
Ammonium heptamolybdate solution:	0.75% NH_3 x 4 H_2O
Malachite green solution:	0.035% malachite green, 0.35% polyvinyl alcohol 3000
TLP:	86 parts $CHCl_3$, 14 parts MeOH and 1 part H_2O_{dd}

2.3.2 Lipid extraction according to Folch et al.

An aliquot, equal to 200 µg phospholipids (PL), as determined by the phosphorus concentration (chapter 2.3.1), was taken from the sonicated cell suspension and transferred into a 10 ml PVC tube with lid. The volume of each sample was adjusted to 250 µl with H_2O_{dd}. 2.5 ml of a 1:1 solution of $CHCl_3$ and MeOH was added and the samples were shaken vigorously for 15 minutes. The tube was centrifuged for 5 minutes at 1500 x g and 4°C to sediment the proteins which were discarded. The supernatant was transferred into a new 10 ml PVC tube and 1.25 ml $CHCl_3$ and 0.8 ml 0.74% KCl was added. The biphasic system was vigorously shaken for 5 minutes. The upper aqueous phase was removed and the lower phase was washed with 0.75 ml theoretical upper phase (TUP). The biphasic system was shaken for 5 minutes and then centrifuged for 5 minutes at 1500 x g and 4°C. The two phases were separated and the upper phase was removed. The lower phase containing the PL was evaporated under a nitrogen air stream in a water bath preheated to 50°C. The obtained PL extracts were stored at –20°C until use (Folch et al., 1957).

TUP:	6 parts $CHCl_3$, 96 parts MeOH and 94 parts 0.74% KCl

2.3.3 Phospholipid thin layer chromatography

The dried PL-samples as well as a PL-standard containing 6.8% sphingomyelin, 49.5% phosphatidylcholine, 5.2% phosphatidylserine, 5.9% phosphatidylinositol and 32.5% phosphatidylethanolamine were dissolved in TLP at a concentration of 1 µg PL/µl and were kept on ice. The standard PL composition is reflecting the PL pattern of C6 cells. A CAMAG Linomat III was used to spray 15 µl of each PL solutions in triplicate on high performance thin layer chromatography (HPTLC) plates (Merck No. 5641, 10 x 20 cm). The solution was applied 1 cm above the lower rim of the plate with a rate of 1 µl/5s while the plate was move back and forth. A space of 7 mm was introduced between each 5 mm long band. The HPTLC plate was dried for 2 minutes in a desiccator. The one-dimensional chromatography was performed according to the method of Vitiello & Zanetta (1978). The plate was placed into a chromatography chamber that was equilibrated for at least 30 minutes with the mobile phase. During 40 minutes the plate was developed, dried for 5 minutes at 180°C and cooled to room temperature in a desiccator. The separated PL were detected using the method of Zühlke (1990). The thin layer plate was immersed for exactly 10 seconds into the detection reagent and then dried at 180°C for 6 minutes. The stained PL bands were measured densitometrically with a CAMAG Scanner II. The plates were scanned in absorption mode at a wavelength of 547 nm with a light ray width of 2 mm and a scan speed of 1 mm/s. The detected PL spots were integrated with the CAMAG Software Cats V.3.13. The peak area of a PL was used as a measure for the amount of the PL in the sample.

Mobile phase:	25 parts $CHCl_3$, 1-propanol, methylacetate, 10 parts MeOH and 9 parts 0.74% KCl
Detection reagent:	3% copper(II)acetate and 11% phosphorus acid in a 1:1 mixture of MeOH and H_2O_{dd}

2.3.4 Phospholipid analysis and quantification

On each HPTLC plate with 12 sample bands, 3 PL standard bands were included. The relative content of each PL in the extracts could be calculated with the help of the PL standards. To account for the varying sensitivity of the different PL to the detection reagent a so-called response factor (RespF) had to be calculated for each PL according to Equation 1. This is due to the fact that the colour intensity of each PL is depending of the saturation degree of the fatty acids in the PL (Spillman et al., 1983).

Equation 1: Calculation of the response factor for each PL in the standard PL preparation

$$\mathrm{RespF}_i = \frac{S_{i_{eff}} \times \sum_{i=1}^{n} S_{i_{\mathrm{det}}}}{S_{i_{\mathrm{det}}} \times 100}$$

RespF_i : relative response factor of a standard PL_i

$S_{i_{eff}}$: effective relative amount of a standard PL_i in percent

$S_{i_{\mathrm{det}}}$: peak area of a standard PL_i in percent of the total peak area of all standard PL

The Response Factor was then used for the calculation of the effective relative amount of a PL in the sample (Equation 2). The measured peak area of a PL in percent of the total peak area of all PL was corrected with the response factor.

Equation 2: Calculation of the effective relative amount of a PL in a sample PL preparation

$$PL_{i_{eff}} = \frac{\mathrm{RespF}_i \times PL_{i_{\mathrm{det}}} \times 100}{\sum_{i=1}^{n} PL_{i_{\mathrm{det}}}}$$

$PL_{i_{eff}}$: effective relative amount of a PL_i in percent

$PL_{i_{\mathrm{det}}}$: peak area of a PL_i in percent of the total peak area of all PL

2.4 Plasma membrane isolation

The medium of a 175 cm^2 tissue culture flask was removed and the cell monolayer was rinsed twice with 10 ml phosphate buffer (CMPBS) prewarmed to 37°C. 15 ml incubation buffer was added and the cells were incubated for 90 minutes at 37°C and room atmosphere. The incubation buffer induced protrusions of the plasma membrane. The buffer was removed carefully and replaced with 10 ml rinsing buffer. The cell monolayer was further incubated at room temperature for 5 minutes. The culture flask was shaken horizontally during 60 seconds to detach the formed plasma membrane vesicles (PMV) from the cell surface membrane. The rinsing buffer was collected in a conical 50 ml centrifuge tube. The rinsing step was repeated twice and all rinsing solutions with PMVs were pooled. The tubes were centrifuged at 500 x g for 5 minutes to separate detached cells and cell particles from the solution. The supernatants were transferred into 50 ml polypropylene centrifuge tubes and the PMVs were collected at 48000 x g and 4°C for 45 minutes. After the centrifugation the supernatant was discarded and the PMV pellets were resuspended in 2 ml isotonic Tris buffer ($Tris_{iso}$). The PMV suspensions were pooled and centrifuged for 45 minutes at 48000 x g and 4°C. The supernatant was removed again and the PMV pellet was resuspended in 5 ml hypotonic Tris buffer ($Tris_{hypo}$) to break up PMV and to remove enclosed soluble proteins. The suspension was centrifuged at 48000 x g and 4°C for 45 minutes, the supernatant was discarded and the pure PMV pellet obtained (Scott, 1976) was stored at -20°C until use. To generate sufficient PMV material, tissue cultures of six 175 cm^2 flasks were used.

CMPBS:	0.68 mM $CaCl_2$ x 2 H_2O, 0.49 mM $MgCl_2$ x 6 H_2O, 4.1 mM Na_2HPO_4 x 2 H_2O, 1.47 mM KH_2PO_4, 170 mM NaCl, pH 7.4
Incubation buffer:	CMPBS with 25 mM formaldehyde and 2 mM dithiothreitol (DTT)
Rinsing buffer:	0.1% BSA, 10 mM Tris-HCl, 168 mM NaCl, pH 7.4
$Tris_{iso}$:	10 mM Tris-HCl, 168 mM NaCl, pH 7.4
$Tris_{hypo}$:	10 mM Tris-HCl, pH 7.4

2.5 Fluorescence anisotropy measurement with TMA-DPH

For the fluorescence anisotropy measurement with trimethylamino-diphenylhexatriene (TMA-DPH) cells were cultivated on glass cover slips (40 x 11 mm). Because of the temperature dependence of the fluorescence anisotropy all solutions and the carousel of the spectrofluorometer were prewarmed to 37°C. The cover slip was placed with a special support in a cuvet filled with 3 ml 5 µM TMA-DPH solution. The support ensured that the cover slip was placed in an angle of 30° to the monochromatic polarised excitation light to reduce light scattering. 10, 15 and 20 minutes after the cover slip was placed into the TMA-DPH solution, the fluorescent tracer was excited vertically and horizontally at a wavelength of 360 nm. Vertical and horizontal fluorescence emissions were recorded at a wavelength of 430 nm (Prendergast et al., 1981). The emitted light was detected with a SHIMADZU RF540 spectrofluorometer equipped with an automatically rotating polarisation filter. The anisotropy was calculated after Equation 3.

Equation 3: Calculation of the fluorescence anisotropy r(G)

$$r(G) = \frac{I_{vv} - G \times I_{vh}}{I_{vv} + G \times 2I_{vh}}$$

G: Correction factor for the optical system of the device (I_{hv} / I_{hh})
I_{vv}: Intensity of the vertical fluorescence emission after vertical excitation
I_{vh}: Intensity of the vertical fluorescence emission after horizontal excitation
I_{hv}: Intensity of the horizontal fluorescence emission after vertical excitation
I_{hh}: Intensity of the horizontal fluorescence emission after horizontal excitation

TMA-DPH solution:	5 µM TMA-DPH (Molecular probes) in HBS
HBS:	1.25 mM $CaCl_2$, 5.5 mM D(+)-Glucose, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.8 mM $MgSO_4$, 136.9 mM NaCl, 0.43 mM Na_2HPO_4

2.6 Cyclic AMP Analysis

2.6.1 Isolation of the cyclic AMP binding protein

The isolation of the cAMP binding protein was performed as described by Gilman & Murad (1974). All operations were performed at 0-4°C. Fresh bovine calf heart was obtained from a slaughterhouse and stored in tyrode solution supplemented with 10 I.U./ml heparin until it was processed. The heart muscle was grinded with a meat grinder and subsequently homogenised for 2 minutes in a Waring blender with the same weight of 4 mM Na-EDTA, pH 7.0. The resulting homogenate was centrifuged at 15000 x g for 45 minutes. The supernatant was adjusted to pH 4.8 by slow addition of 1 M acetic acid. After 15 minutes, the precipitate was collected at 15000 x g for 45 minutes and discarded. The supernatant was adjusted to pH 6.8 by the addition of 1 M KH_2PO_4, pH 7.2. Solid ammonium sulphate (0.33 g/ml) was added slowly. The solution was stirred for 30 minutes and the precipitate was collected at 15000 x g for 45 minutes. It was dissolved in approximately 5% of the original homogenate volume in 5 mM KH_2PO_4, pH 7.0, and dialysed overnight in 125 times the volume of the dialysate with buffer (SpectraPor Float-A-Lyzer MWCO 15000, 10 ml, Spectrum Labs 235106). The dialysed ammonium sulphate precipitate was applied to a column of DEAE-cellulose (Whatman DE52, pre-swollen anion exchanger), previously equilibrated with 5 mM KH_2PO_4, pH 7.0. The column was washed with 2 bed volumes of the same buffer. The column was eluted with a linear gradient of KH_2PO_4, pH 7.0, from 100 mM to 400 mM and fractions of 20 ml were collected. The binding capacities of the fractions for the cAMP were tested. 100 µl of each fraction and diluted fractions were mixed with 50 µl 0.25 µCi ^{3}H-cAMP (Amersham) and 50 µl cAMP standard (16 pmol cAMP / 50 µl, Amersham) for the detection of maximal competition capacity. The minimal competition capacity was determined by the addition of 50 µl Tris-EDTA buffer instead of 50 µl cAMP standard solution. The cAMP containing fractions were appropriately diluted with Tris-EDTA buffer containing 1 mg/ml BSA. The different fractions were pooled and a standard curve was determined. Samples of 20 ml were stored at –20°C until use.

Tyrode solution:	137 mM NaCl, 5.4 mM KCl, 2.4 mM $CaCl_2$, 2.2 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.6 mM Na_2HPO_4, 5.6 mM glucose, 5 I.U./ml heparin
Tris-EDTA buffer:	50 mM Tris-HCl, 4 mM Na-EDTA, pH 7.5

2.6.2 Cyclic AMP stimulation and extraction

The medium of cells cultivated in 6 cm ∅ tissue culture plates was discarded and the cell monolayer was rinsed twice with 2 ml HBS prewarmed to 37°C. The cell layer was pre-incubated with 2 ml 1 mM 3-isobutyl-1-methyl-xanthin (IBMX) in HBS for 15 minutes. IBMX an inhibitor of cytosolic and membranous phosphodiesterases was added to prevent the metabolism of the cAMP produced. The cells were stimulated for 10 minutes at 37°C with a maximal (10 µM) or submaximal (10 nM) concentration of isoproterenol, a β-adrenoceptor agonist, 100 µM forskolin, a direct AC stimulator, or 100 mM NaF, a stimulator of the G_s-protein. In each experiment at least one tissue culture plate was incubated with the solvent to determine the basal level of cellular cAMP. The reaction was terminated by withdrawal of the stimulation solution, rinsing of the cell monolayer twice with 2 ml ice-cooled HBS and the addition of 3 ml ice-cooled 0.1 N HCl. Deep-freezing of the cell culture plate at –20°C for 3 hours, followed by thawing, lysed the cells. This procedure released the cAMP from the cells and aliquots of 0.5 ml were transferred into 1.5 ml Eppendorf tubes. The solution was stored for 2 hours at -80°C. The frozen samples were lyophilised for 24 hours and stored at -20°C until use.

2.6.3 Cyclic AMP measurement

The lyophilised samples were dissolved in Tris-EDTA buffer (50 mM Tris-HCl, 4 mM Na-EDTA, pH 7.5) and were diluted in this buffer to a cAMP concentration of 2 - 12 pmol/50 µl. The dilution factor had to be estimated in a preliminary assay. 50 µl of a diluted sample was mixed with 50 µl 0.25 µCi ^{3}H-cAMP and 100 µl cAMP binding protein solution in a 1.5 ml Eppendorf tube. The mixed samples were incubated for 18 hours at 4°C. The unbound ^{3}H-cAMP was removed by the addition of 100 µl ice-

cooled charcoal suspension (520 mg charcoal and 400 mg fatty acid free BSA in 20 ml Tris-EDTA buffer). The sample was vortexed and centrifuged at 14000 x g and 4°C for 2 minutes to precipitate the charcoal-bound ^{3}H-cAMP. 200 µl of the supernatant was pipetted into a scintillation vial and 5 ml of liquid scintillation fluid (Ultima Gold, Perkin Elmer) was added. The vials were shaken vigorously and the bound radioactivity was counted in a liquid scintillation counter (Packard TriCarb TR2100). In each experiment standards (Amersham) with a concentration of cAMP of 0 – 16 pmol/50 µl were included. The determination of the cAMP is based on the competition of the cellular cAMP with the radiolabelled cAMP for the binding at the cAMP binding protein. The higher the concentration of the cellular cAMP, the lower the amount of radiolabelled cAMP bound to the cAMP binding protein. The amount of produced cAMP by the addition of a stimulator is calculated as the difference between the total amount of cAMP and the basal level of cAMP.

Charcoal suspension:	520 mg charcoal, 400 mg fatty acid free BSA in 20 ml of Tris-EDTA buffer
^{3}H-cAMP 0.25 µCi:	50 µl 1 mCi/ml ^{3}H-cAMP [Amersham TRK 304] in 10 ml Tris-EDTA buffer

2.7 Receptor binding

2.7.1 β-Adrenoceptor binding

For the determination of cell surface β-adrenoceptors, the hydrophilic β-adrenoceptor antagonist ^{3}H-CGP-12177 (Amersham) was used. The C6 cells of a 10 cm ∅ culture plate were scraped off and suspended in 6 ml HBS. 200 µl cell suspension was mixed with 50 µl radioligand solution resulting in end concentrations of 0.5 – 6 nM ^{3}H-CGP-12177. The radioligand/cell solutions were incubated for 60 minutes at 37°C in a shaking water bath. The incubation was stopped by the addition of 1 ml ice-cooled HBS. 1 ml of the diluted cell suspension was filtrated with the fully automated filtration machine HON1 (constructed by Prof. U.E. Honegger) through glass fibre filters (Whatman GF/C) at an exactly defined vacuum pressure. The filter was rinsed

three times with 5 ml ice-cooled physiological NaCl solution to remove unbound radioligand and put in a scintillation vial. 5 ml scintillation fluid (Ultima Gold, Perkin Elmer) was added to the filter and the radioactivity was measured in a liquid scintillation counter (Packard TriCarb TR2100). The unspecific binding of the radioligand was determined in the presence of 1 µM timolol, a β-adrenoceptor antagonist, which displaced the radioligand from the specific binding sites of the receptor. The specific binding was calculated as the difference between total binding (without timolol) and unspecific binding (with timolol in the incubation solution). The protein content of the cell suspension was determined according to the method of Lowry et al. (see chapter 2.2.1).

Physiological NaCl solution: 0.9% NaCl in H_2O_{dd}

2.8 DNA measurement

The cell monolayer of a 10 cm ∅ cell culture plate was rinsed twice with 2 ml HBS, the cells were scraped off with a "rubber policeman" in a volume of 6 ml HBS. The cell suspension was sonicated for 10 s and 100 µl was overlaid in a vial with an equal volume of a 40% diaminobenzoic acid dichloride (DABA) solution that was filtrated through activated charcoal before use. The solution was incubated for 45 minutes at 60°C and the reaction was stopped by the addition of 3.5 ml 1 N HCl. The solution was mixed and the fluorescence signal was measured at an excitation wavelength of 416 nm and an emission wavelength of 520 nm against standard curve solutions with a DNA amount of 0 to 20 µg/ml. For the standard DNA stock solution 400 µg DNA of calf thymus (Aldrich) was swollen for 10 minutes in 1 ml H_2O_{dd} and sonicated for 10 s (Hinegardner, 1971).

2.9 Microscopy

2.9.1 Light microscopy

Light microscopic pictures were made with a Nikon FE photo camera attached to a Nikon TMS phase-contrast reversal microscope on Ilford PanF50 Asa films. Cells cultivated in tissue culture dishes were rinsed twice with HBS and the cells were viewed under the microscope.

2.9.2 Confocal fluorescence microscopy

Pictures were captured using the laser scanning microscope Zeiss LSM 510 with an Axiovert 100 microscope and an oil-immersion objective Plan-Apo 63x 1.4 NA. Rhodamine was excited at a wavelength of 543 nm with the helium-neon laser and emitted light could be detected at 560-615 nm using an appropriate filter set. Differential interference contrast (DIC) pictures were made using the helium-neon laser at a wavelength of 543 nm.

2.9.2.1 Uptake of rhodamine-labelled dextran as lysosomal marker

Dextran is taken up by cells and transported into lysosomes. Therefore, dextran can be used as a lysosomal marker (Bürgi, 2001).
C6 cells cultivated on cover slips were incubated for 3 to 5 hours with 1 mg/ml tetramethylrhodamine-labelled dextran (MW: 10’000, Molecular Probes) in MEM-medium. The cell monolayer was rinsed five times with HBS and then the cells were viewed under the microscope.

2.10 MTT proliferation and cytotoxicity assay

Cells were seeded into a 96-well tissue culture plate and grown for one week. The cell monolayer of each well was rinsed twice with HBS and 100 µl HBS was added. The assay was started with the addition of 10 µl MTT stock solution (5 mg/ml in H_2O_{dd}, sterile filtered) per well. The tissue culture plate was incubated at 37°C for 2 hours. During this incubation the yellow tetrazolium MTT (3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells into a purple formazan dye (Figure 5). The reaction was stopped by the addition of 100 µl SDS 10% in 0.01 N HCl and the plate was placed on a shaker for 1 minute. The intracellular formazan was solubilised for 2 hours at 37°C and after cooling to room temperature, the absorption was measured using a micro plate reader at a wavelength of 562 nm.

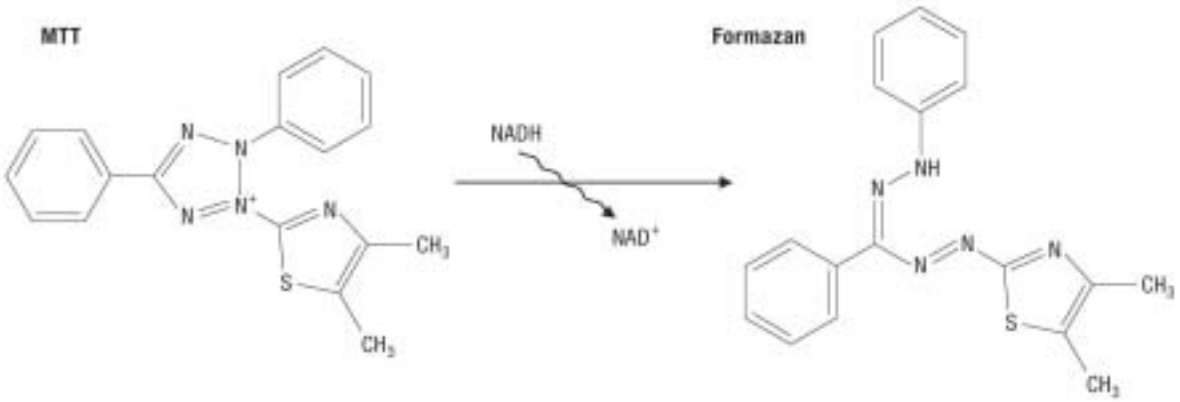

Figure 5: Reduction of MTT (3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) to a water insoluble formazan salt. Only viable, metabolically active cells are reducing MTT into the formazan dye that can be quantified spectrophotometrically.

2.11 Hypericum perforatum L. plant extracts

2.11.1 Hypericum-Lutrol extract

Hypericum extract Ze 117 was standardised to 0.2% hypericin and showed solubility problems in the cell culture medium. Therefore, a Hypericum-Lutrol extract was manufactured by Max Zeller und Söhne AG, Romanshorn that showed higher solubility than the crude Hypericum extract. A powdery extract was made of 1 part Hypericum extract Ze 117 and 2 parts Lutrol, a polyethylenglycol that is used as solubiliser for hardly soluble components. The composition of the Hypericum-Lutrol extract is shown in Table 1. This extract contained only a small amount of hyperforin.

2.11.2 Hyperforin-"free" and hyperforin-rich fractions of *Hypericum perforatum L.* extract Ze 117

Hyperforin-"free" and hyperforin-rich fractions were obtained from the laboratory of Professor M. Hamburger at the Friedrich-Schiller-Universität in Jena.
The standardised Hypericum extract Ze 117 was dissolved in a 1:1 mixture of ethanol and H_2O_{dd} resulting in an extract concentration of 20%. The filtered solution was fractionated chromatographically using a LiChrosorb RP18 column (40-63 µm, size C 440-37) with a mixture of 25% acetonitril and 75% acetonitril : H_2O_{dd} : TFA = 150 : 50 : 0.1 as mobile phase and a flow rate of 10 ml/min. The different components were detected using an UV detector (Knauer UV Detektor K-2501) and the fractions were collected in 500 ml flasks. The collected fractions were dried by using a rotary evaporator. The composition of the hyperforin-rich and hyperforin-"free" fractions are shown in Table 1. The hyperforin-"free" fraction contained only a very small amount of hyperforin which was at the detection limit.

Table 1: Analysis of the different *Hypericum perforatum L.* extracts used in this study. The analysis includes the phloroglucinol hyperforin, the naphthodianthrones hypericin and pseudohypericin, the flavonoids rutin, hyperoside, quercetin, quercitrin and biapigenin. The analysis were performed by the quality control unit of Max Zeller und Söhne AG, Romanshorn and the values are in percent of total extract. (n.d.: not determined).

	Hypericum-Lutrol extract Ze 117 (standardised to 0.2% total-hypericin)		hyperforin-rich fraction	hyperforin-"free" fraction
	analysis certificate (4.9.2001)	analysis (23.7.2004)	analysis (15.9.2003)	analysis (9.2.2004)
hyperforin	n.d.	< 0.02	1.8	0.0063
hypericin	0.037	0.036	0.033	0.0058
pseudohypericin	0.163	0.176	in trace amounts	0.055
rutin	2.8	0.93		1.75
hyperoside	n.d.	0.69	total flavonoids:	2.64
quercetin	n.d.	0.13	< 0.1	0.77
quercitrin	n.d.	0.11		0.43
biapigenin	n.d.	0.048		0.39

3 Results

3.1 Influence of chronic antidepressant exposure on membrane properties of cultured rat glioblastoma C6 cells and human skin fibroblasts

3.1.1 Phospholipid accumulation in lysosomes of rat glioblastoma C6 cells and skin fibroblasts

C6 cells and skin fibroblasts were exposed for 7 days to control medium or medium containing 10 µM DMI, 10 µM fluoxetine, 3.78 mg/ml Hypericum-Lutrol extract or 2.52 mg/ml Lutrol-control. In C6 cells, the exposure to DMI or fluoxetine led to an increase in total PL by 20%. In fibroblasts DMI induced a 60% increase, whereas the fluoxetine induced increase was only 20%. C6 cells exposed to Hypericum-Lutrol extract showed no increase in total PL, whereas an increase of 30% could be detected in fibroblasts exposed to Hypericum-Lutrol extract. Lutrol-control did not increase PL either in C6 cells or fibroblasts (results not shown).

C6 cells and fibroblasts chronically exposed to DMI, fluoxetine or Hypericum-Lutrol extract showed a changed cell morphology compared to control cells. After DMI or fluoxetine treatments, formation of cytoplasmic granules could be detected (Figure 6, DIC-pictures; Figure 7B and C). It is known that different antidepressants, mainly of the class of TCA, are lysosomotropic substances and, therefore, induce an accumulation of PL in lysosomes. This lysosomotropic action is due to inhibition of lysosomal phospholipases (Hostetler, 1984), as well as to inhibition of the PL degradation through generation of drug-PL complexes (Lüllmann et al., 1978). Previous studies in our laboratory showed that DMI induces an accumulation of PL in lysosomes due to inhibition of lysosomal phospholipases (Fauster et al., 1983; Honegger et al., 1983; Zbinden, 1997). The same could also apply to exposure to fluoxetine, a SSRI that shows similar granules.

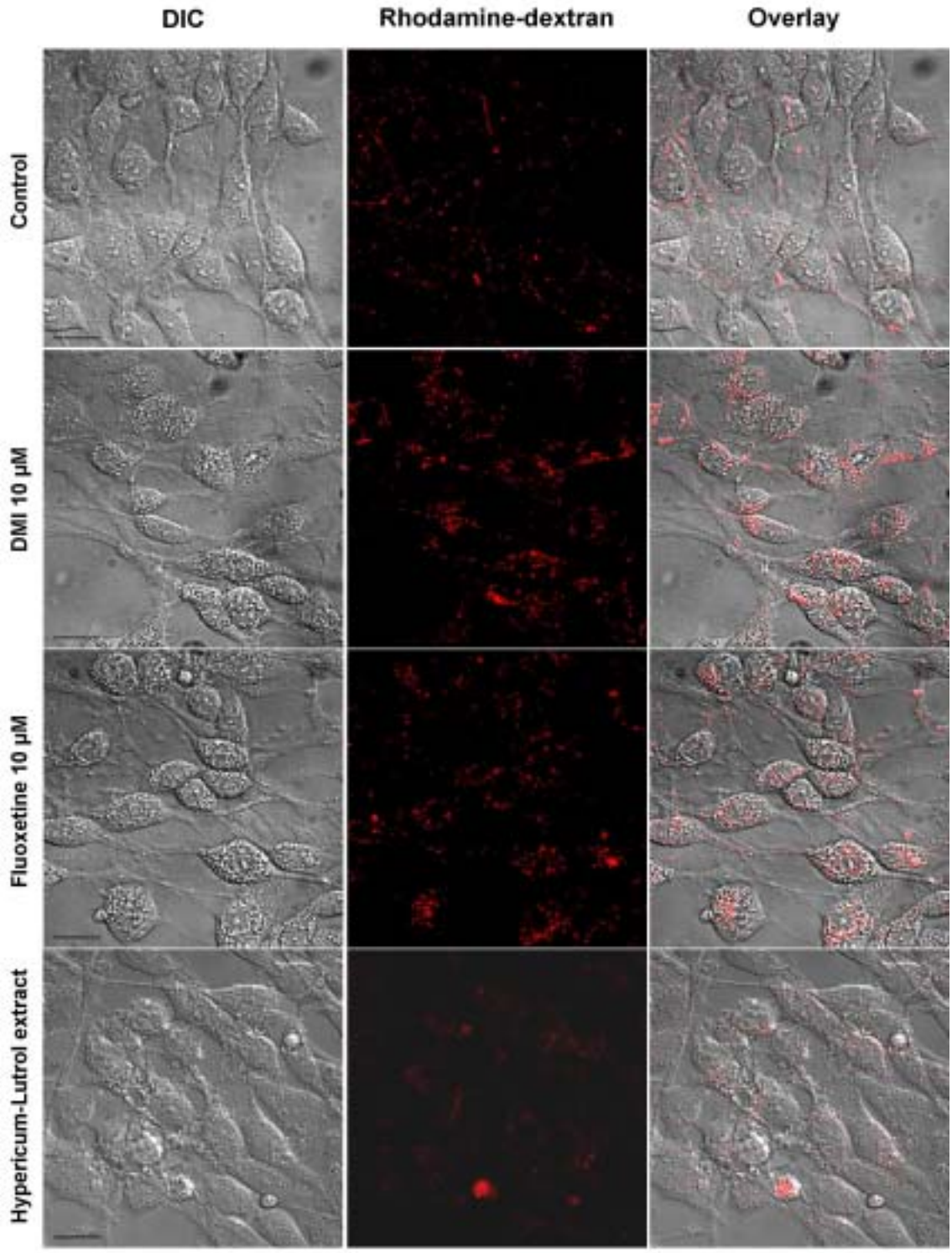

Figure 6: Morphological changes of C6 cells after chronic exposure for 7 days to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. Control C6 cells and cells chronically exposed to antidepressants were incubated for 3h with 1 mg/ml tetramethylrhodamine-labelled dextran which is used as lysosomal marker. The cells were washed 5-times with HBS and then viewed under the confocal fluorescence microscope (LSM 510). DIC: differential interference contrast; overlay: merging of DIC- and fluorescence pictures; bar = 20 µm.

To show that the granules formed are located in the lysosomal compartments of chronically exposed C6 cells, cells were incubated for 3 hours in the presence of tetramethylrhodamine-labelled dextran. Dextran is used as a lysosomal marker (Bürgi, 2001) and needs to be taken up actively by the cells. Since the C6 cells are phagocytically active, incubation for 3 hours was sufficient to label the lysosomes. As shown in Figure 6 (Overlay), the intracellular granules are clearly indicated to be within lysosomes by the rhodamine-labelled dextran.

C6 cells exposed to Hypericum-Lutrol extract showed diminished cell proliferation and no PL-accumulation compared to control cells.

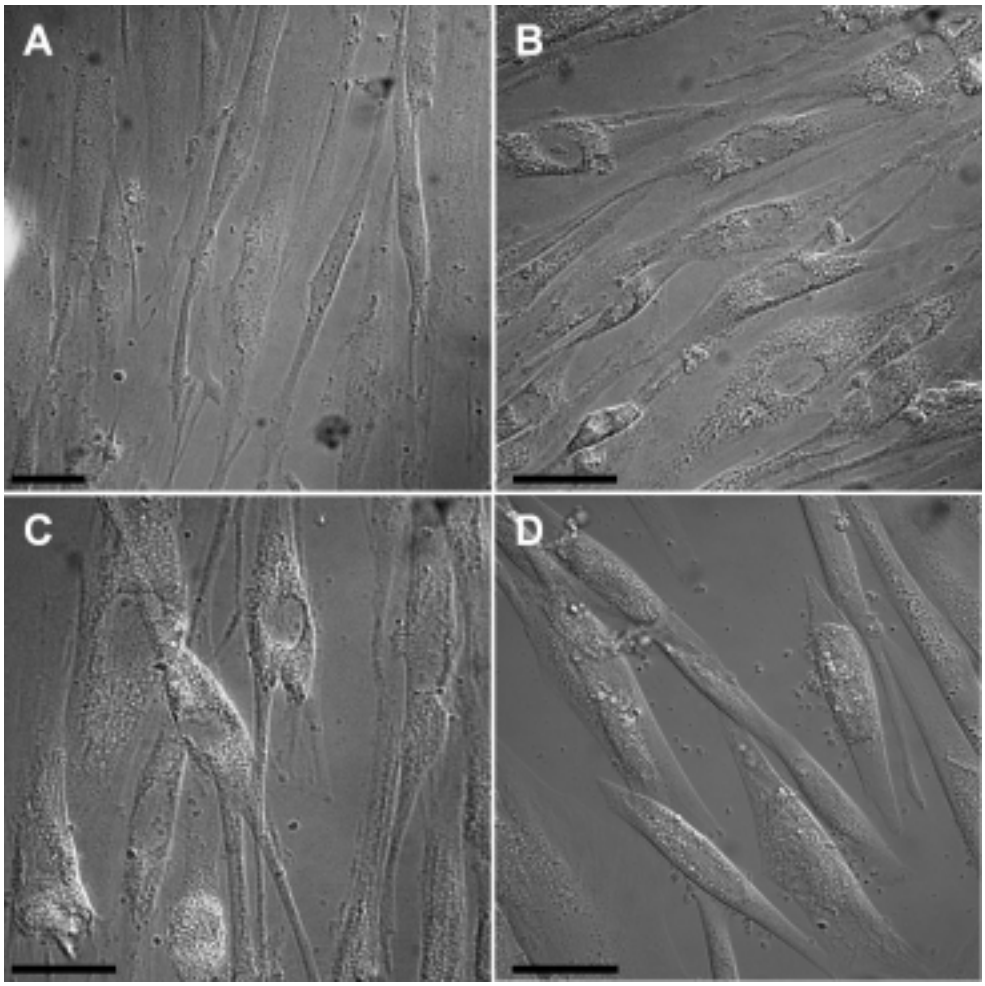

Figure 7: Cell morphology of control fibroblasts (A) and fibroblasts chronically exposed to 10 µM DMI (B), 10 µM fluoxetine (C) or 3.78 mg/ml Hypericum-Lutrol extract (D). Fibroblasts were exposed for 7 days to control-medium or medium containing 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. Differential interference contrast (DIC) images were produced using the confocal fluorescence microscope (LSM 510). Bar = 50 µm.

After chronic exposure, control fibroblasts were long and slender (Figure 7A), whereas cells exposed to DMI or fluoxetine underwent a change in shape (Figure 7B, C), became rounder and broader than the control cells. The increase in content of total PL correlated well with the appearance of granules. Fibroblasts exposed to Hypericum-Lutrol extract showed the same changes in cell morphology as cells exposed to DMI or fluoxetine, but to a lesser extent (Figure 7D).

3.1.2 Phospholipid pattern of chronically exposed rat glioblastoma C6 cells and skin fibroblasts

It is known that treatments with antidepressants lead to an increase in different PL, mainly of phosphatidylinositol (PI). PI is characterised by a fast turnover (Fauster et al., 1983) and Matsuzawa & Hostetler (1980) report that DMI inhibits phospholipase C. The latter is the major enzyme for PI degradation and, therefore, an increase in PI results after chronic DMI treatment. Since cells exposed to Hypericum-Lutrol extract showed a change in cell morphology and increased total PL content was found in fibroblasts, the PL pattern of C6 cells and fibroblasts chronically exposed to the Hypericum-Lutrol extract was studied in detail. DMI was chosen as a positive control, because its influence on the PL pattern of C6 cells (Zbinden, 1997) as well as of fibroblasts (Zühlke, 1990; Zbinden, 1997) is known.

C6 cells and fibroblasts were chronically exposed to control medium or medium containing 10 µM DMI, 3.78 mg/ml Hypericum-Lutrol extract, 2.52 mg/ml Lutrol-control or a combination of 10 µM DMI and 2.52 mg/ml Lutrol. The relative PL composition of the five main PL, sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE), was determined after chromatographic separation and densitometric analysis (Zühlke, 1990). The results expressed as the relative PL composition and, therefore, did not show changes in total PL content.

3.1.2.1 Cellular phospholipid pattern of chronically exposed C6 cells and skin fibroblasts

The main PL in the cells are PC and PE and these occur in the range of 45% to 50% and 30% to 35%, respectively. The amount of SPH, PS and PI was between 5% and 10% of the total PL content. The relative cellular PL composition of untreated C6 cells and untreated fibroblasts did not show big differences (Figure 8A and Figure 8B). C6 cells possessed a slightly smaller cellular PC content and a slightly higher PE content than fibroblasts.

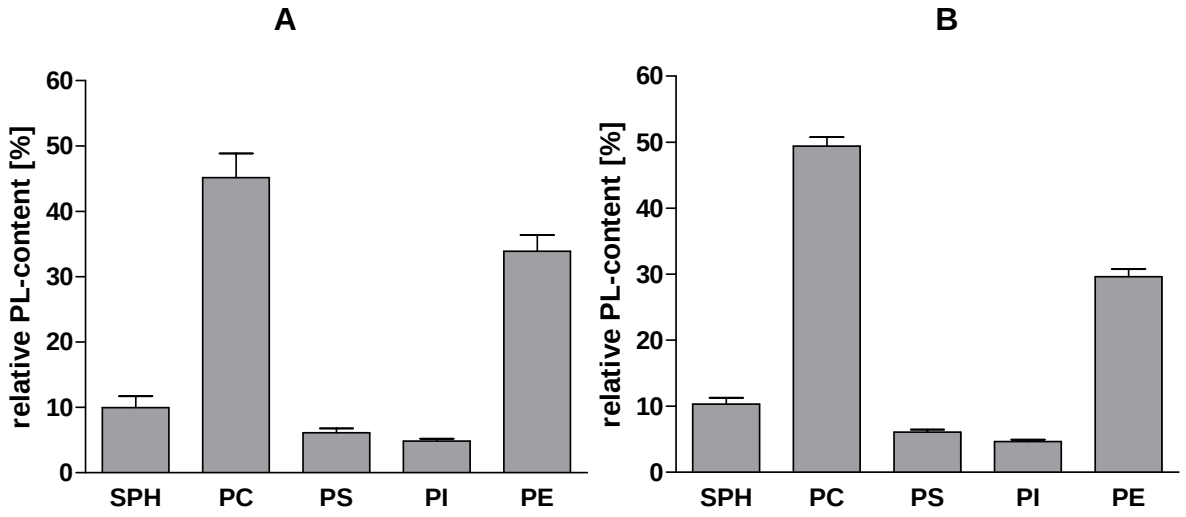

Figure 8: Relative cellular PL composition of control C6 cells (A) and control fibroblasts (B). Lipid extracts of control cells were separated by high performance thin layer chromatography (HPTLC). The relative content of each PL from the total PL-amount was determined densitometrically, using a CAMAG TLC scanner. Shown are the means ± SD of at least 3 experiments. SPH: sphingomyelin, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol, PE: phosphatidylethanolamine.

The relative PL composition of chronically exposed C6 cells and fibroblasts are shown in Figure 9. Changes in PL pattern after chronic exposure to Hypericum-Lutrol extract, DMI, combination of DMI/Lutrol or Lutrol-control were similar in both cell types. A decrease in the SPH content and an increase in the PI content after DMI treatment could be detected. The same applied to Hypericum-Lutrol extract exposed cells, whereas in C6 cells, the alterations in the SPH and PI content were not significant. The changes of the PL pattern relative to the control are shown in Figure 10.

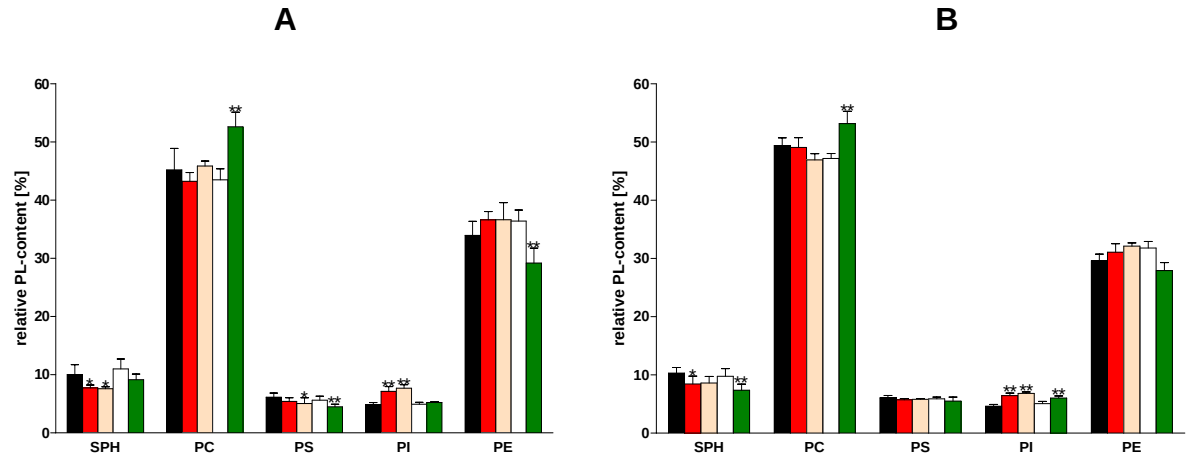

Figure 9: Relative cellular PL pattern of chronically exposed C6 cells (A) and fibroblasts (B). C6 cells and skin fibroblasts were exposed for 7 days with control medium (black bar) or medium containing 10 µM DMI (red bar), 10 µM DMI and 2.52 mg/ml Lutrol (brown bar), 2.52 mg/ml Lutrol-control (open bar) or 3.78 mg/ml Hypericum-Lutrol extract (green bar). Lipid extracts of cells were separated by HPTLC and the relative content of each PL from the total PL-amount was determined densitometrically. The bars represent the mean ± SD of at least 3 experiments. *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test). SPH: sphingomyelin, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol, PE: phosphatidylethanolamine.

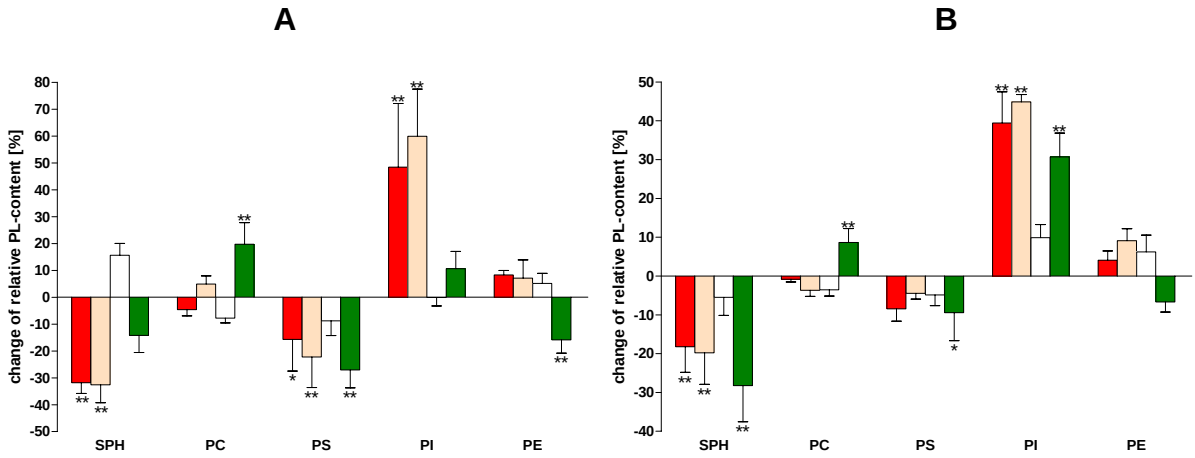

Figure 10: Changes of the relative PL amount in C6 cells (A) and fibroblasts (B) after chronic treatments. C6 cells and skin fibroblasts were exposed for 7 days with control medium or medium containing 10 µM DMI (red bar), 10 µM DMI and 2.52 mg/ml Lutrol (brown bar), 2.52 mg/ml Lutrol-control (open bar) or 3.78 mg/ml Hypericum-Lutrol extract (green bar). Lipid extracts of cells were separated by HPTLC and the relative content of each PL from the total PL-amount was determined densitometrically. Bars represent the mean ± SD of at least 3 experiments. *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test). SPH: sphingomyelin, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol, PE: phosphatidylethanolamine.

Unexpectedly, treatment with Hypericum-Lutrol extract increased the PC content significantly compared to DMI in both cell types. Additionally and in contrast to the other treatment conditions, Hypericum-Lutrol extract exposed cells showed a decrease in the relative PE content in fibroblasts and C6 cells. However, this change was significant only in C6 cells. The content in PS was reduced in both cell types after exposure to DMI, DMI/Lutrol or Hypericum-Lutrol. In C6 cells this decrease was significant for all treatment conditions, whereas in fibroblasts a significant reduction was only detected after Hypericum-Lutrol exposure.

3.1.2.2 Plasma membrane phospholipid pattern of chronically exposed C6 cells and fibroblasts

Since the PL pattern of C6 cells and fibroblasts was changed after chronic exposure to DMI or Hypericum-Lutrol extract, the PL pattern of the plasma membrane might also be altered. This would fit well with the observation that DMI inhibits various lysosomal phospholipases, thus changing the PL turnover and the PL pattern and thus inducing a disturbance of the membrane recycling.

To study the plasma membrane PL pattern, cells were exposed chronically to DMI, Lutrol-control, the combination DMI/Lutrol or Hypericum-Lutrol extract and after 7 days the cell monolayer was incubated in the presence of formaldehyde and dithiothreitol (DTT) in CMPBS. This treatment induced protrusions of the plasma membrane, resulting in the formation of plasma membrane vesicles (PMVs) (Zühlke, 1990). The vesicles formed (Figure 11) were collected by centrifugation, resuspended in Tris-buffer and the lipids were extracted with a methanol-chloroform solution after the method of Folch et al. (1957). The lipid extract was separated by thin layer chromatography and the relative PL content of the five main PL, SPH, PC, PS, PI and PE was determined densitometrically.

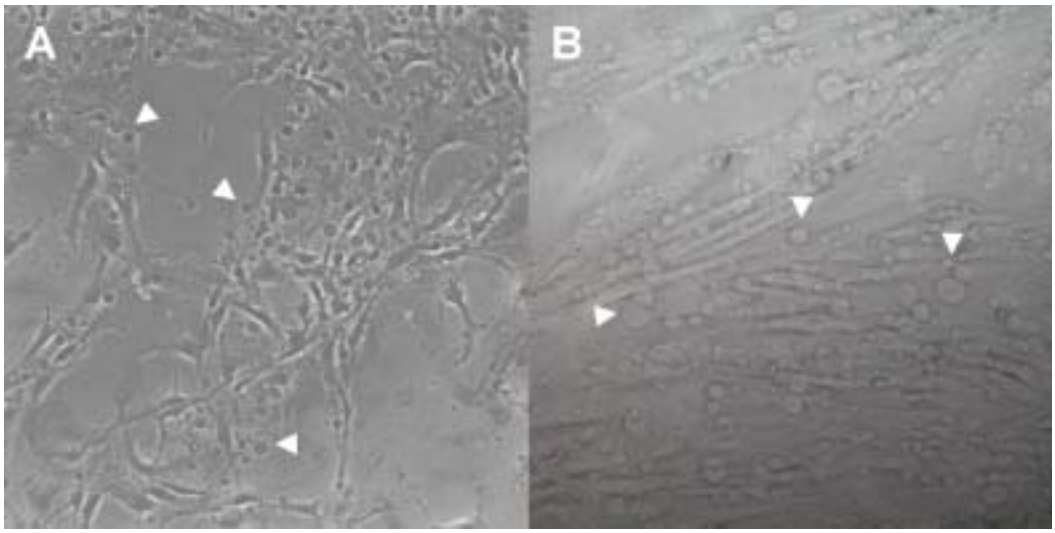

Figure 11: Formation of plasma membrane vesicles (PMVs) after treatments of C6 cells (A) and fibroblasts (B) with formaldehyde and dithiothreitol (DTT). Non-confluent cells were incubated in the presence of 25 mM formaldehyde and 2 mM DTT in CMPBS at 37°C for 90 minutes. Numerous PMVs (arrowheads) were formed from plasma membrane of intact cells. Note that PMVs derived from fibroblasts (B) appeared to be bigger than those from C6 cells (A). Photographs were taken using a phase-contrast reversal microscope at 200x magnification.

The relative PL content of whole control cells and of control PMV of C6 cells and fibroblasts are shown in Figure 12A and B. A typical increase in the SPH and PS content and a decrease in the PI and PE content was detected in PMV as already shown before in fibroblasts (Zühlke, 1990) and in C6 cells (Bürgi, 2001).

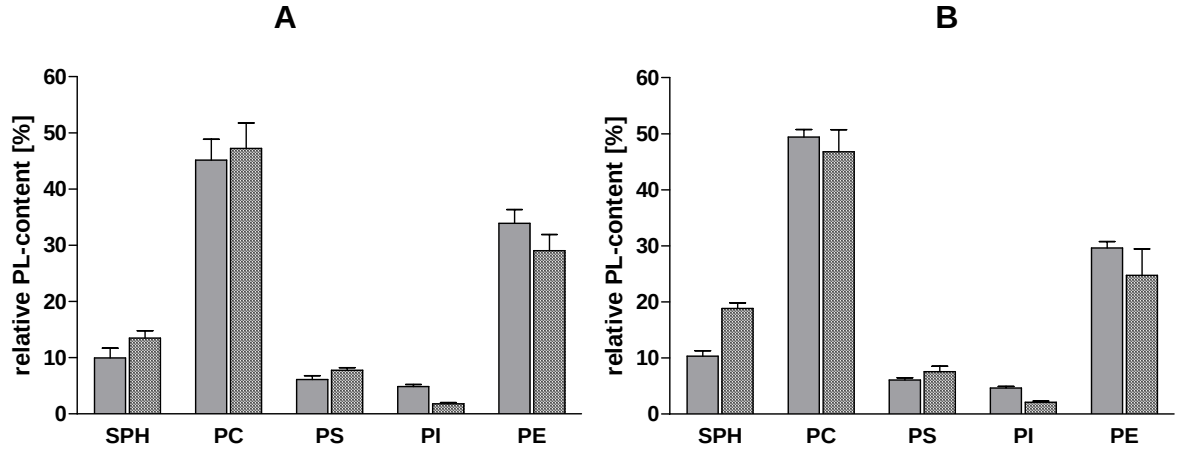

Figure 12: Relative PL pattern of whole cells and plasma membrane vesicles (PMV) of untreated C6 cells (A) and untreated fibroblasts (B). Lipid extracts of control PMV were separated by HPTLC. The relative content of each PL was determined densitometrically. Shown are the means ± SD of at least 3 experiments. SPH: sphingomyelin, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol, PE: phosphatidylethanolamine.

Figure 13 shows the relative PL composition of PMVs of chronically exposed C6 cells and fibroblasts. It is evident in both cell types that the Hypericum-Lutrol extract

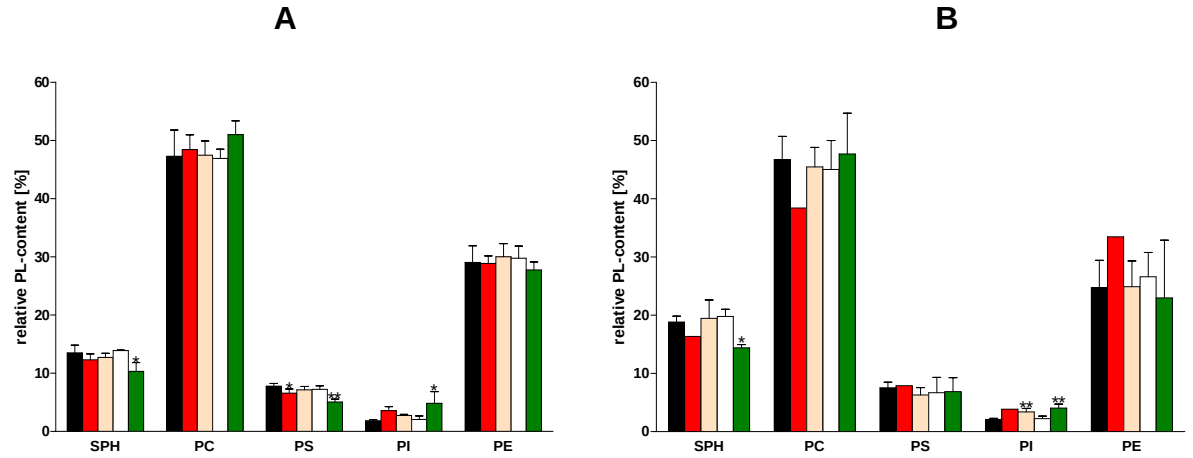

Figure 13: Relative PL pattern in PMV of chronically exposed C6 cells (A) and fibroblasts (B). C6 cells and skin fibroblasts were exposed for 7 days with control medium (black bar) or medium containing 10 µM DMI (red bar), 10 µM DMI and 2.52 mg/ml Lutrol (brown bar), 2.52 mg/ml Lutrol-control (open bar) or 3.78 mg/ml Hypericum-Lutrol extract (green bar). Lipid extracts of PMV were separated by HPTLC and the relative content of each PL from the total PL-amount was determined densitometrically. The bars represent the mean ± SD of at least 3 experiments, except for DMI treated fibroblasts only one experiment was carried out. *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test). SPH: sphingomyelin, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol, PE: phosphatidylethanolamine.

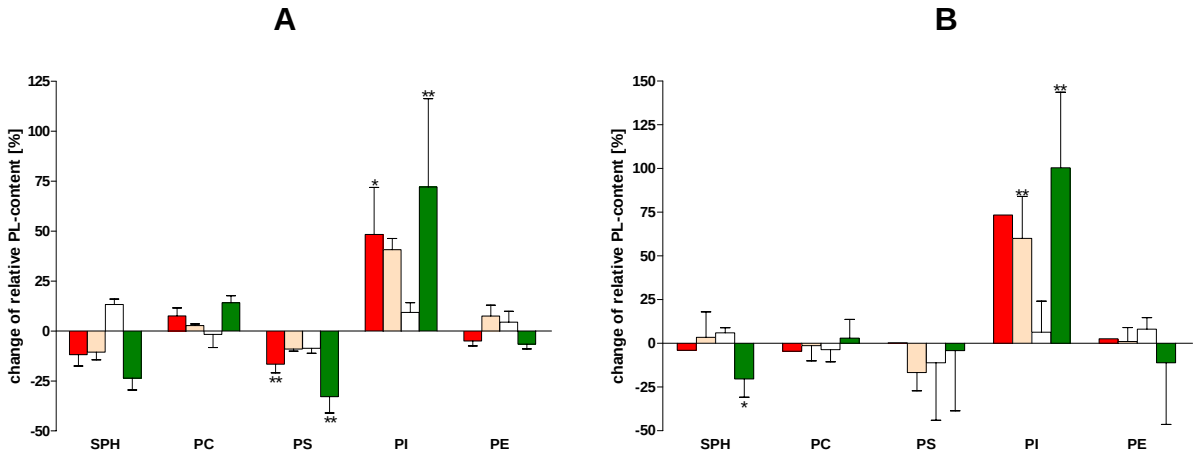

Figure 14: Changes of the relative PL content in PMV of chronically treated C6 cells (A) and fibroblasts (B). C6 cells and skin fibroblasts were exposed for 7 days with control medium or medium containing 10 µM DMI (red bar), 10 µM DMI and 2.52 mg/ml Lutrol (brown bar), 2.52 mg/ml Lutrol-control (open bar) or 3.78 mg/ml Hypericum-Lutrol extract (green bar). Lipid extracts of PMV were separated by HPTLC and the relative content of each PL from the total PL-amount was determined densitometrically. The bars represent the mean ± SD of at least 3 experiments, except for DMI treated fibroblasts, where only one experiment was carried out. *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test). SPH: sphingomyelin, PC: phosphatidylcholine, PS: phosphatidylserine, PI: phosphatidylinositol, PE: phosphatidylethanolamine.

exposure led to a decrease in the SPH content and an increase in the PI content. These changes are more apparent in Figure 14 where the PL content relative to control is shown. DMI and DMI/Lutrol increased the relative content of PI in both cell types, whereas the decrease in the content of SPH was only evident in C6 cells. In fibroblasts, only one experiment with DMI exposure was carried out, because Zühlke (1990) studied the influence of chronic DMI treatment on the PMV-PL pattern in detail and thus, the DMI effects are well known. Hypericum-Lutrol extract and DMI decreased the content of PS significantly, whereas the PC content was slightly increased in C6 cells. These effects were not observed in fibroblasts.

3.1.3 Influence of chronic treatments of C6 cells and fibroblasts on the plasma membrane fluidity

The changes of the PL pattern in plasma membrane of chronically exposed cells could also lead to a changed plasma membrane fluidity. Therefore, the fluorescence anisotropy of plasma membrane was measured using TMA-DPH as fluorescent probe. TMA-DPH preferentially incorporates into the plasma membrane surface due to its interaction with the polar head groups of PL (Prendergast et al., 1981). Only changes of the fluidity in the outer part of the plasma membrane were measured. Cell monolayers cultivated for 7 days on glass cover slips in the presence of control medium or medium containing 10 µM DMI, 10 µM fluoxetine, 2.52 mg/ml Lutrol-control or 3.78 mg/ml Hypericum-Lutrol extract were incubated at 37°C with 5 µM TMA-DPH. After excitation of TMA-DPH with polarised light, the emission intensity of the fluorophore was recorded and the fluorescence anisotropy r(G) was calculated. The anisotropy is inversely proportional to the fluidity.

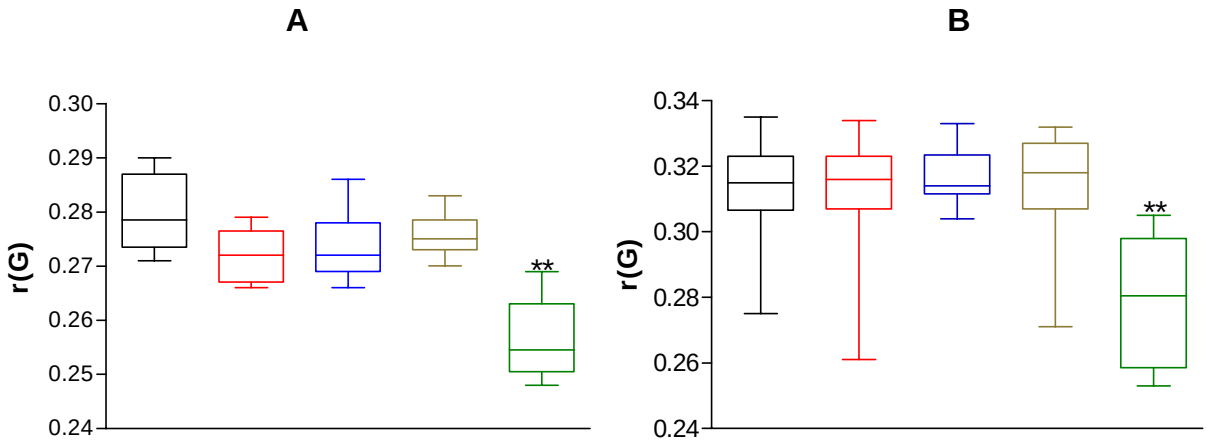

Figure 15: Fluorescence anisotropy r(G) of chronically exposed C6 cells (A) and fibroblasts (B). C6 cells and fibroblasts grown on glass cover slips were exposed for 7 days to control medium (▭) or medium containing 10 µM DMI (▭), 10 µM fluoxetine (▭), 2.52 mg/ml Lutrol-control (▭) or 3.78 mg/ml Hypericum-Lutrol extract (▭). The cell monolayers were incubated in the presence of 5 µM TMA-DPH at 37°C for 10, 15 and 20 minutes. At each time point the TMA-DPH fluorescence emission intensity was measured after excitation and the anisotropy r(G) was calculated. The line at the middle of the box that extends form the 25th to the 75th percentile is the median and the error bars extend down to the lowest value and up to the highest. **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

C6 cells showed a lower fluorescence anisotropy and, therefore, a higher membrane fluidity compared to fibroblasts (Figure 15A and B). A decreased fluorescence anisotropy was detected only in C6 cells and fibroblasts exposed chronically to the Hypericum-Lutrol extract. Consequently, this plasma membrane showed a higher fluidity than the plasma membrane of the control cells. Under all other treatment conditions, no changes in the anisotropies could be detected. However, chronic DMI or fluoxetine exposure of C6 cells showed a trend towards lower anisotropy values.

3.2 DNA and protein content of C6 cell and skin fibroblast cultures chronically exposed to antidepressant drugs

From previous experiments, there was a clear indication that chronic treatments with the Hypericum-Lutrol extract led to a reduced protein content in C6 cells as well as in fibroblasts compared to control cells. Hostanska et al. (2002) showed that an aqueous ethanolic *Hypericum perforatum* extract in a high dose expressed cytotoxic effects in different cell lines and in a lower dose cell growth inhibition. In addition, exposure to light of treated cell culture potentiated the effect on cell toxicity. Roscetti et al. (2004) also showed that *Hypericum perforatum* extracts inhibited cell growth and induced apoptosis. Hypericin (Hostanska et al., 2003) and hyperforin (Schempp et al., 2002) are the constituents of *Hypericum perforatum* extracts that are held responsible for the cytotoxic effect on cell cultures.

To find out if the reduced protein content is due to growth inhibition or cell death, the protein and DNA amounts were measured after chronic treatment. In Figure 16A and B the protein content of C6 cell and fibroblast cultures are shown. It is evident that chronic exposure of both cell types to Hypericum-Lutrol extract led to a reduced protein content compared to control cell cultures. Lutrol-control itself had no influence on the protein amount in both cell lines, whereas DMI showed a slight reduction in the protein content of chronically treated C6 cell cultures, but not in fibroblast cultures. The treatment with 10 µM DMI in combination with Lutrol showed the same reduction in the protein level in C6 cell cultures as treatment with DMI alone.

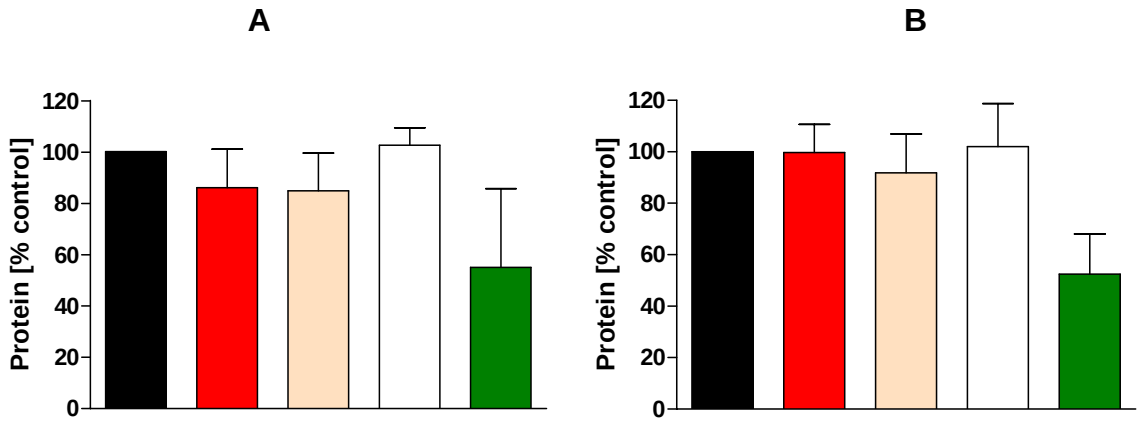

Figure 16: Protein content of chronically exposed C6 cell (A) and skin fibroblast (B) cultures. C6 cells and skin fibroblasts were exposed for seven days to control medium (black bar) or medium containing 10 µM DMI (red bar), 10 µM DMI and 2.52 mg/ml Lutrol (brown bar), 2.52 mg/ml Lutrol-control (open bar) or 3.78 mg/ml Hypericum-Lutrol extract (green bar). The protein content was measured according to the method of Lowry et al. The bars represent the mean ± SD of at least 4 experiments for C6 cells and at least 3 experiments for fibroblasts and are expressed in percent of the control value.

The amount of DNA in chronically treated C6 cell and fibroblast cultures is shown in Figure 17A and Figure 17B, respectively. Chronic treatment with Hypericum-Lutrol extract led to a pronounced reduction of the DNA content compared to control cell cultures in both cell types. Lutrol-control treated C6 cell and fibroblast cultures showed the same DNA content as control cell cultures. Exposure to 10 µM DMI led to a slight decrease in the DNA amount of C6 cell cultures, but in fibroblasts to a slight increase, whereas the treatment combination of 10 µM DMI and 2.52 mg/ml

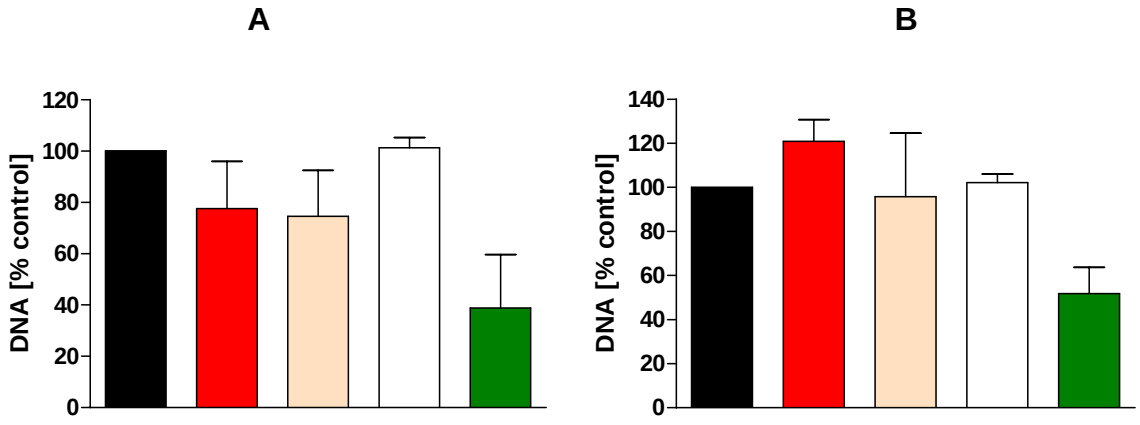

Figure 17: DNA content of chronically treated C6 cell (A) and skin fibroblast (B) cultures. C6 cells and skin fibroblasts were exposed for 7 days to control medium (black bar) or medium containing 10 µM DMI (red bar), 10 µM DMI and 2.52 mg/ml Lutrol (brown bar), 2.52 mg/ml Lutrol-control (open bar) or 3.78 mg/ml Hypericum-Lutrol extract (green bar). The DNA content was measured according to the method of R.T. Hinegardner. The bars represent the mean ± SD of at least 4 experiments for C6 cells and at least 3 experiments for fibroblasts and are expressed in percent of the control value.

Lutrol induced a decrease in the DNA content in C6 cell cultures, but had no effect on the amount of DNA in fibroblast cultures.
The DNA/protein ratio calculated from the protein and DNA content (Figure 16 and Figure 17) is shown in Figure 18. The chronic treatment with Hypericum-Lutrol extract induced a slight decrease in the mean DNA/protein ratio in C6 cells (Figure 18A), but had no influence on the ratio in fibroblasts (Figure 18B). Chronic DMI treatment of C6 cells decreased the ratio slightly, but in fibroblasts a small increase in the ratio was detected. The same applied for the combined treatment of 10 µM DMI and 2.52 mg/ml Lutrol, whereas the Lutrol-control showed no influence on the ratio.
The results shown above indicated that the treatment of C6 cells and fibroblasts to Hypericum-Lutrol extract inhibited the cell growth. To confirm these results, the metabolic activity of C6 cell cultures, assessed with the MTT-assay, and the protein content were measured before and after chronic exposure to control medium or medium containing 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. The results of these experiments are shown in Figure 19.

The increases in protein content of C6 cell cultures chronically exposed to 10 µM DMI or 10 µM fluoxetine were comparable to the one observed for control cultures after cultivation for 7 days. The metabolic activities of control cells or cells exposed to 10 µM DMI or 10 µM fluoxetine resulted in a 4-fold increase compared to cells before treatment. In contrast, chronic exposure of C6 cell cultures to 3.78 mg/ml Hypericum-Lutrol extract resulted in a small 1.6-fold increase in the protein content, compared to a 6-fold increase in control cell cultures. The metabolic activity of C6 cells exposed to Hypericum-Lutrol extract was reduced according to the reduced protein content.
These results corroborated that the reduced protein and DNA content of cell cultures after chronic exposure to the Hypericum-Lutrol was due to inhibition of the cell growth and not due to cytotoxic effects of the plant extract.

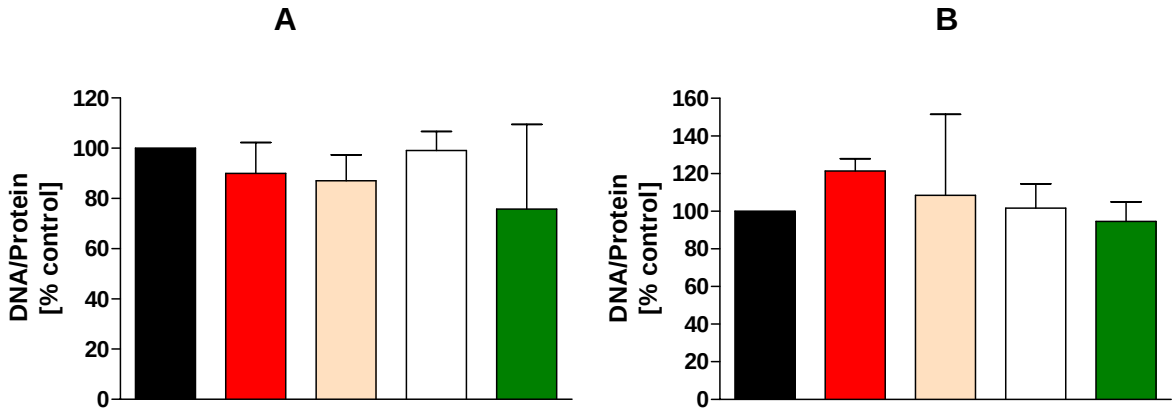

Figure 18: DNA/Protein ratio of chronically treated C6 cells (A) and skin fibroblasts (B). C6 cells and skin fibroblasts were exposed for 7 days to control medium (black bar) or medium containing 10 µM DMI (red bar), 10 µM DMI and 2.52 mg/ml Lutrol (brown bar), 2.52 mg/ml Lutrol-control (open bar) or 3.78 mg/ml Hypericum-Lutrol extract (green bar). The bars represent the mean ± SD of at least 4 experiments for C6 cells and at least 3 experiments for fibroblasts and are expressed in percent of the control value.

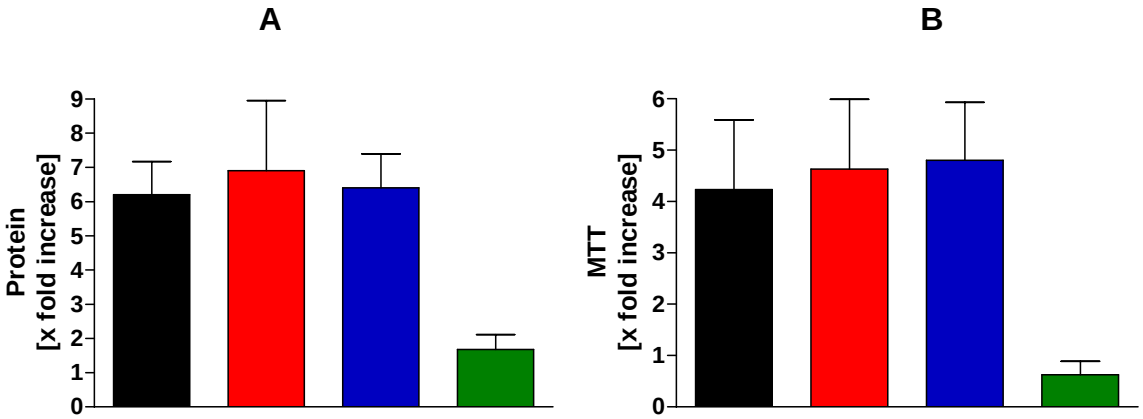

Figure 19: Effects of chronic antidepressant treatments on protein content (A) and on MTT-absorption (B) of C6 cell cultures. The protein content and MTT-absorption of C6 cells were measured before and after chronic exposure for 7 days. The cells were exposed to control medium (black bar) or medium containing 10 µM DMI (red bar), 10 µM fluoxetine (blue bar) or 3.78 mg/ml Hypericum-Lutrol extract (green bar). The bars represent the ratio of the protein content or MTT-absorption after and before the chronic treatments and show the mean ± SD of 3 experiments.

3.3 β-Adrenoceptor binding of C6 cells chronically exposed to antidepressant drugs of three different classes

Chronic treatments of cell cultures with most antidepressants lead to down-regulation of β-adrenoceptors. This down-regulation is dose- and time-dependent as shown in fibroblasts (Honegger et al., 1986) as well as in different region of rat brain (Stanford et al., 1983) and rat cerebral cortex (Beer et al., 1987; Argenti & D'Mello, 1994).
In this part, it is shown that chronic exposure of C6 cells to antidepressants of different classes down-regulate the number of cell surface β-adrenoceptors. For this purpose the TCA DMI, the SSRI fluoxetine and a plant extract of *Hypericum perforatum L.* (St. John's wort) were used. Rat glioblastoma C6 cells were treated with the respective drugs, or the medium alone as control, for 6 consecutive days, with medium changes every second day and a last medium change 24 hours before the radioligand binding experiment.

3.3.1 Dose-dependent down-regulation of the cell surface β-adrenoceptor number in C6 cells

3.3.1.1 Down-regulation of the cell surface β-adrenoceptor number with desipramine

C6 cells were cultivated for 6 days in the presence of different DMI concentrations or absence of the TCA. The surface β-adrenoceptor number of DMI treated and untreated control C6 cells was measured, using the hydrophilic tritium-labelled β-adrenoceptor antagonist ^{3}H-CGP-12177.

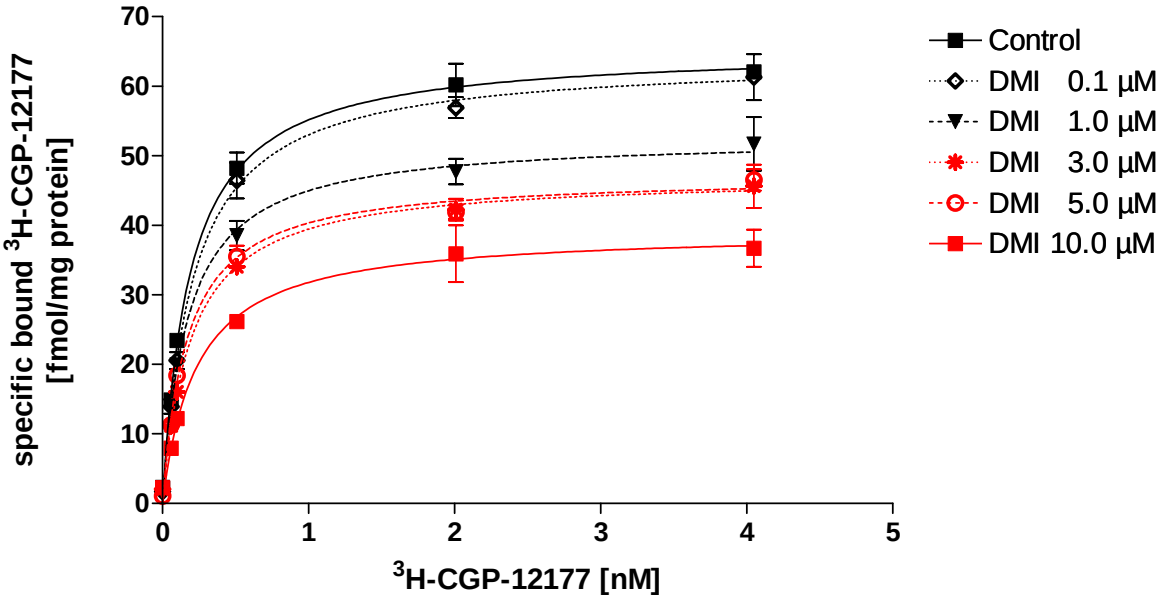

Figure 20: Number of β-adrenoceptors of chronically DMI treated and control C6 cells. Cell suspensions were incubated with increasing concentrations of radioactive labelled β-adrenoceptor antagonist ^{3}H-CGP-12177 in a shaking water bath for 60 minutes at 37°C. The unspecific binding was determined using the β-adrenoceptor antagonist timolol in a concentration of 1 µM. The specific binding was determined as the difference between total and unspecific binding of the radioligand. Each data point represents the mean ± SD of triplicates of one representative binding experiment.

The binding of ^{3}H-CGP-12177 to β-adrenoceptors showed a saturation effect and reached a plateau between 2 nM and 4 nM as depicted in Figure 20. DMI concentration of at least 1 µM is required to down-regulate the cell surface β-adrenoceptor number of chronically treated C6 cells. This concentration led to a loss of 20% of cell surface receptors compared to control cells. Chronic treatment with 10 µM DMI reduced the surface β-adrenoceptors number by 40%. DMI treatments had no influence on the affinity (K_D) of the β-adrenoceptor for the radioligand (Table 2).

Table 2: Radioligand binding parameter. The B_{max} and K_D values were analysed from the saturation binding curves of Figure 20, using the nonlinear regression curve fit option for one site binding of GraphPad Prism for Windows (GraphPad Software, San Diego California USA) which is based on the following equation: $Y=B_{max}*X/(K_d+X)$. B_{max} and K_D are expressed in fmol/mg protein and B_{max}-values in % are normalised to the control B_{max} value.

	B_{max} [fmol/mg Protein]	B_{max} [%]	K_D [nM]
Control	65.4 ± 1.11	100.0 ± 1.69	0.19 ± 0.014
DMI 0.1 µM	64.0 ± 1.03	97.8 ± 1.57	0.21 ± 0.014
DMI 1.0 µM	52.7 ± 1.20	80.6 ± 1.84	0.17 ± 0.017
DMI 3.0 µM	47.1 ± 0.98	72.1 ± 1.50	0.20 ± 0.018
DMI 5.0 µM	47.2 ± 1.11	72.1 ± 1.70	0.17 ± 0.018
DMI 10.0 µM	39.2 ± 1.19	60.0 ± 1.81	0.24 ± 0.030

A competition binding experiment was carried out to test the possibility of an interference of accumulated DMI in chronically treated cells with the radioligand ^{3}H-CGP-12177 on the binding of the β-adrenoceptor. C6 cells were incubated with increasing concentrations of DMI in the presence of a constant concentration of 4 nM ^{3}H-CGP-12177 at 37°C in a shaking water bath. Figure 21 depicts the competition of

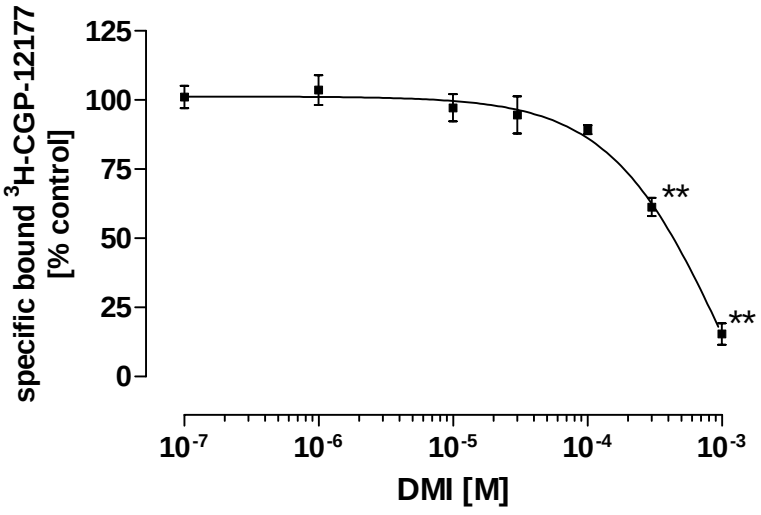

Figure 21: Competition radioligand binding experiment on surface β-adrenoceptors of intact C6 cells with increasing concentrations of DMI in presence of 4 nM ^{3}H-CGP-12177. The specific binding of ^{3}H-CGP-12177 was determined using 1 µM timolol and this control binding value was set to 100.0% (± 14.3%). The ^{3}H-CGP-12177 binding measured after competition with different concentrations of DMI was expressed as a percentage of the control value. Shown are means ± SD of three independent measurements of one representative experiment. **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

DMI with ^{3}H-CGP-12177 at the β-adrenoceptor binding site. Only DMI concentrations higher than 300 µM showed a significant inhibition of ^{3}H-CGP-12177 binding. Such high DMI concentrations are not likely after chronic treatment with DMI.
For subsequent chronic DMI treatments a concentration of 10 µM DMI was chosen. This concentration is likely to be reached in human brain *in vivo*, after chronic DMI treatment. *In vitro*, in cell cultures, chronic exposure to 10 µM DMI resulted in a reproducible and pronounced β-adrenoceptor down-regulation. DMI concentrations higher than 50 µM were not chronically tolerated by C6 cells and led to cell death.

3.3.1.2 Down-regulation of the cell surface β-adrenoceptor number with fluoxetine

C6 cells were exposed for 6 days to different fluoxetine concentrations or to control medium. The number of surface β-adrenoceptors of fluoxetine treated and control C6 cells were measured, using the radioligand ^{3}H-CGP-12177.

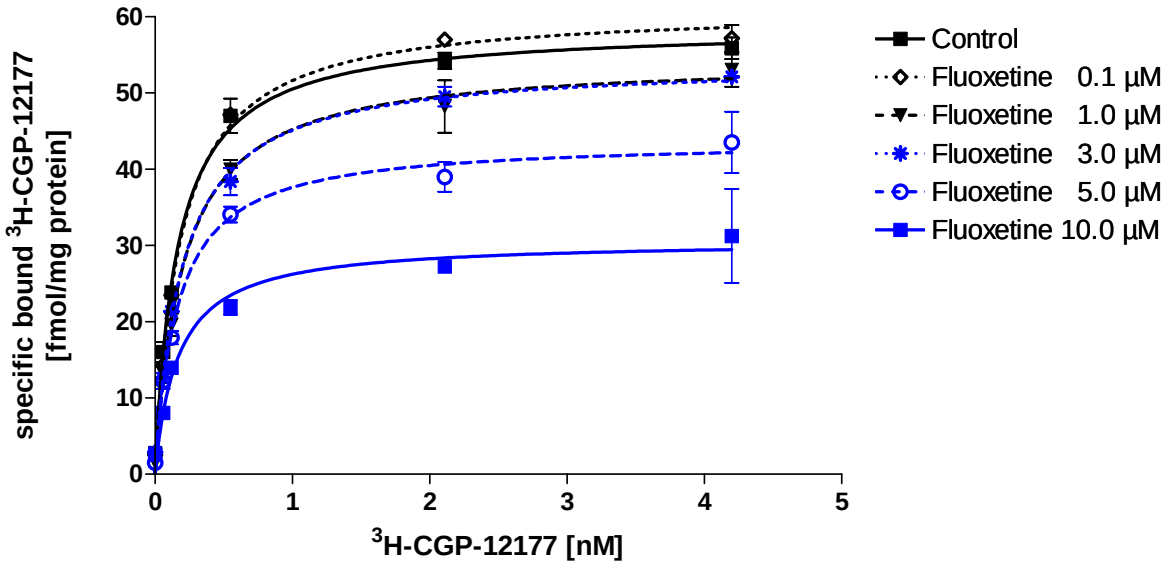

Figure 22: Number of β-adrenoceptor of chronically fluoxetine treated and control C6 cells. Cell suspensions were incubated with increasing concentration of radioligand. Unspecific binding was determined using 1 µM timolol. The specific binding was the difference between total and unspecific binding of ^{3}H-CGP-12177. Shown are the means ± SD of triplicates of one typical binding experiment.

As shown in Figure 22, a minimal fluoxetine concentration of 5 µM was required to down-regulate the cell surface β-adrenoceptor number of chronically treated C6 cells. This concentration led to a loss of 25% of cell surface receptors compared to control cells. Chronic treatment with 10 µM fluoxetine diminished the surface β-adrenoceptor number by 50% compared to control. Chronic treatment of C6 cells with fluoxetine did not influence the binding affinity (K_D) of the β-adrenoceptor for the radioligand ^{3}H-CGP-12177 (Table 3).

Table 3: B_{max} and K_D values of chronically fluoxetine treated or control C6 cells. B_{max} and K_D were calculated from the saturation binding curves of Figure 22, using the nonlinear regression curve fit option for one site binding of GraphPad Prism and are expressed in fmol/mg Protein. B_{max} [%] is expressed as the percentage of the control B_{max} value.

	B_{max} [fmol/mg Protein]	B_{max} [%]	K_D [nM]
Control	58.64 ± 1.41	100.0 ± 2.40	0.16 ± 0.017
Fluoxetine 0.1 µM	61.15 ± 1.44	104.3 ± 2.45	0.19 ± 0.019
Fluoxetine 1.0 µM	54.37 ± 1.12	92.7 ± 1.91	0.20 ± 0.018
Fluoxetine 3.0 µM	54.01 ± 1.44	92.1 ± 2.46	0.20 ± 0.023
Fluoxetine 5.0 µM	43.82 ± 1.11	74.7 ± 1.90	0.17 ± 0.019
Fluoxetine 10.0 µM	30.62 ± 1.59	52.2 ± 2.71	0.17 ± 0.039

The decrease in the number of β-adrenoceptors with increasing concentrations of fluoxetine might be due to competition of the radioligand with cellular accumulated fluoxetine for the binding site at the receptor. To test such a possibility, a competition radioligand experiment was carried out. Control C6 cells were incubated at 37°C during one hour in a shaking water bath in the presence of increasing concentrations of fluoxetine from 0.1 µM to 1 mM and a constant radioligand concentration of 4 nM ^{3}H-CGP-12177.

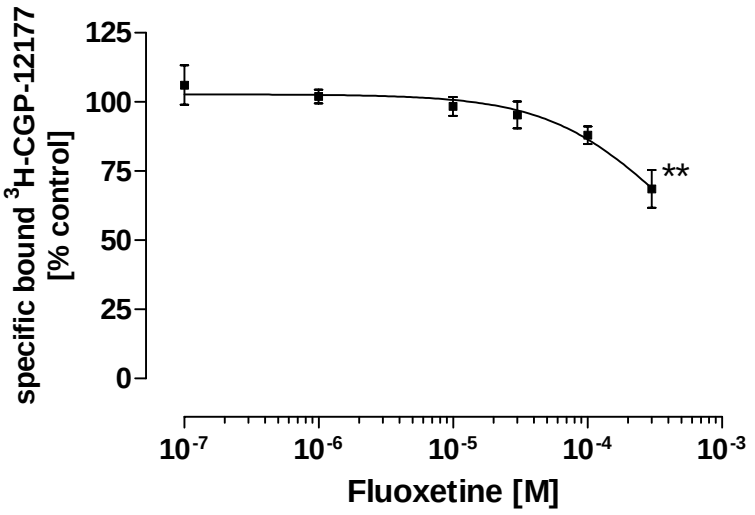

Figure 23: Competition radioligand binding experiment on surface β-adrenoceptors of intact C6 cells with increasing concentrations of fluoxetine in the presence of 4 nM ^{3}H-CGP-12177. The specific binding of ^{3}H-CGP-12177 was determined using 1 µM timolol and this control binding value was set to 100.0% (± 1.0%). The ^{3}H-CGP-12177 binding measured after competition with different concentrations of fluoxetine was expressed as the percentage of the control value. Shown are the means ± SD of three independent measurements of one individual experiment. **: $p < 0.01$ vs. control (GraphPad Prism one-way ANOVA, Tukey-Kramer post-test).

Significant competition of fluoxetine with ^{3}H-CGP-12177 for binding at β-adrenoceptors was only detected at a concentration of 300 µM as shown in Figure 23. Fluoxetine in a concentration of 1 mM led to a specific binding of -6.9 ± 0.65% compared to control (not shown). This is probably due to the fact that fluoxetine in very high concentrations competes not only for receptor binding sites, but also for unspecific binding sites. Such high fluoxetine concentrations are not likely reached after chronic exposure.

For further experiments a concentration of 10 µM fluoxetine was chosen for three reasons. First, this concentration led to a maximal β-adrenoceptor down-regulation of chronically exposed C6 cells that was reproducible in every experiment. Second, concentrations higher than 20 µM were not tolerated by the cells and led to cell death, similar to higher DMI concentrations. And third, brain concentrations of 1 to 10 µM fluoxetine were detected using fluorine magnetic resonance spectroscopy in humans after chronic treatment (Strauss & Dager, 2001; Strauss et al., 2002).

3.3.1.3 Down-regulation of the cell surface β-adrenoceptor number with Hypericum-Lutrol extract

C6 cells were exposed for 6 days to either medium alone or medium containing 0.38, 0.76, 1.51, 2.27 or 3.78 mg/ml Hypericum-Lutrol extract, corresponding to 0.5, 1, 2, 3 or 5 µM hypericin and to 47, 94, 188, 282 or 470 nM hyperforin, or 2.52 mg/ml Lutrol. The surface β-adrenoceptor number of the C6 cells treated with Hypericum-Lutrol extract, Lutrol or control medium were measured using ^{3}H-CGP-12177 as a radioligand.

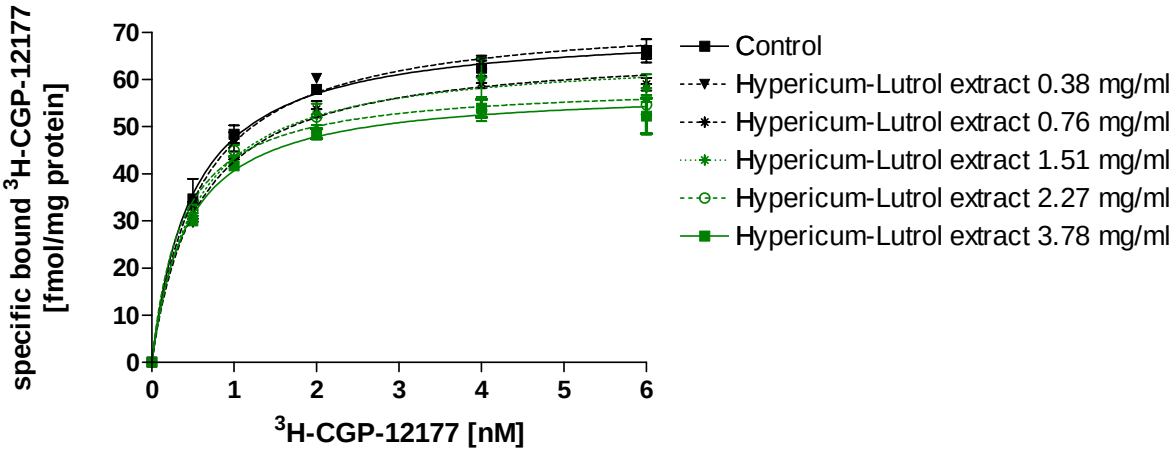

Figure 24: Hypericum-Lutrol extract concentration dependent down-regulation of β-adrenoceptor number of chronically exposed C6 cells. C6 cells were exposed for 6 days to 0.38, 0.76, 1.51, 2.27 or 3.78 mg/ml Hypericum-Lutrol extract equal to 0.5, 1, 2, 3 or 5 µM hypericin. Cell suspensions were incubated with increasing concentration of the tritium-labelled β-adrenoceptor antagonist ^{3}H-CGP-12177 in a shaking water bath for 60 minutes at 37°C. Unspecific binding was determined using 1 µM timolol and specific binding was determined as the difference between total and unspecific binding. Data represent means ± SD of triplicates of one typical experiment.

Figure 24 shows that a Hypericum-Lutrol extract concentration equal to or higher than 2.27 mg/ml, corresponding to 3 µM hypericin was required to down-regulate the number of cell surface β-adrenoceptor of chronically treated C6 cells. Chronic exposure to 2.27 mg/ml Hypericum-Lutrol extract led to a loss of 17% of cell surface receptors compared to control cells. Chronic treatment with a Hypericum-Lutrol extract concentration equal to 5 µM hypericin led to a reduction of surface β-adrenoceptor number by ~20% compared to control cells. Lutrol did not influence

the β-adrenoceptor number of chronically exposed C6 cells (data not shown). Chronic exposure of C6 cells to Hypericum-Lutrol extract showed no influence on the binding affinity of the β-adrenoceptor to the radioligand (Table 4).

Table 4: Binding parameters of C6 cells chronically exposed to Hypericum-Lutrol extract. The saturation binding curves form Figure 24 were used to calculated B_{max} and K_D values by means of the nonlinear curve fit of GraphPad Prism.

	B_{max} [fmol/mg Protein]	B_{max} [%]	K_D [nM]
Control	71.17 ± 0.94	100.0 ± 1.32	0.50 ± 0.029
Hypericum-Lutrol extract 0.38 mg/ml	73.76 ± 1.99	103.6 ± 2.80	0.58 ± 0.065
Hypericum-Lutrol extract 0.76 mg/ml	66.67 ± 1.66	93.7 ± 2.33	0.57 ± 0.059
Hypericum-Lutrol extract 1.51 mg/ml	65.63 ± 1.70	92.2 ± 2.39	0.52 ± 0.058
Hypericum-Lutrol extract 2.27 mg/ml	59.21 ± 1.62	83.2 ± 2.28	0.37 ± 0.052
Hypericum-Lutrol extract 3.78 mg/ml	58.12 ± 1.45	81.7 ± 2.04	0.43 ± 0.051

A competition radioligand binding experiment was carried out to determine the influence of increasing concentrations of Hypericum-Lutrol extract on the binding properties of ^{3}H-CGP-12177. Control C6 cells were incubated at 37°C during one hour in a shaking water bath in the presence of a constant concentration of 4 nM ^{3}H-CGP-12177 and 7.57 µg/ml to 37.8 mg/ml Hypericum-Lutrol extract, corresponding to 0.01 µM to 50 µM hypericin (Table 5).

Table 5: Hypericum-Lutrol extract concentrations used for the competition radioligand binding experiment. The concentrations are expressed as mg/ml Hypericum-Lutrol extract and the corresponding hypericin and hyperforin concentration. (*: not included for the competition radioligand binding).

Hypericum-Lutrol extract	Hypericin	Hyperforin
7.57 µg/ml	0.01 µM	1 nM
75.7 µg/ml	0.1 µM	9 nM
227.0 µg/ml	0.3 µM	28 nM
756.6 µg/ml	1.0 µM	94 nM
2.27 mg/ml	3.0 µM	282 nM
3.78 mg/ml*	5.0 µM	470 nM
7.57 mg/ml	10.0 µM	940 nM
22.7 mg/ml	30.0 µM	2819 nM
37.8 mg/ml	50.0 µM	4699 nM

Hypericum-Lutrol extract concentrations equal or higher than 7.57 mg/ml, corresponding to 10 µM hypericin, were needed to obtain a significant competition on the β-adrenoceptor as shown in Figure 25.

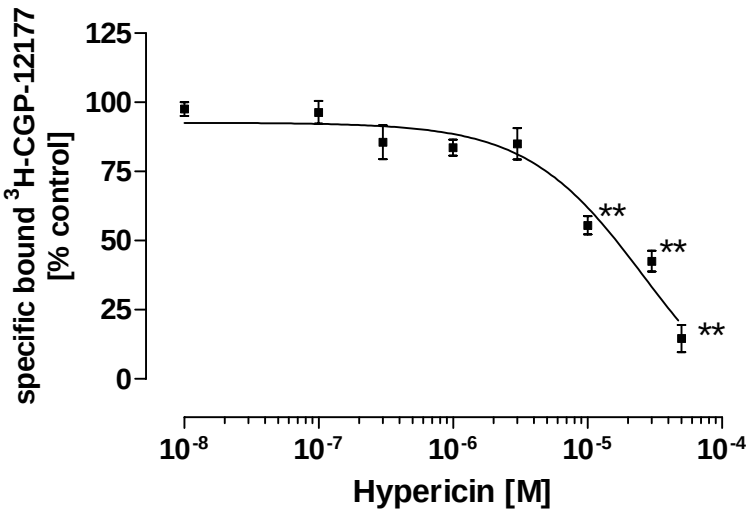

Figure 25: Competition radioligand binding experiment on surface β-adrenoceptors of intact C6 cells with increasing concentrations of Hypericum-Lutrol extract in presence of 4 nM ^{3}H-CGP-12177. The specific binding of ^{3}H-CGP-12177 was determined in the presence of 1 µM timolol and this control binding value was set to 100.0% (± 2.9%). The ^{3}H-CGP-12177 binding measured after competition with different Hypericum-Lutrol extract concentrations, expressed as hypericin concentrations, was expressed as the percentage of the control value. Shown are the means ± SD of three independent measurements of one typical experiment. **: $p < 0.01$ vs. control (GraphPad Prism one-way ANOVA, Tukey-Kramer post-test).

For subsequent experiments a concentration of 3.78 mg/ml Hypericum-Lutrol extract corresponding to 1.26 mg/ml Hypericum extract or 5 µM hypericin was chosen. Higher Hypericum-Lutrol extract concentrations were not tolerated by the C6 cells for chronic exposure and resulted in cell death.

3.3.2 Down-regulation of the cell surface β-adrenoceptor number: A comparison between C6 cells chronically exposed to Hypericum-Lutrol extract, fluoxetine or DMI

As shown in the previous chapters, chronic treatments of rat glioblastoma C6 cells with antidepressants from three different classes led to a dose dependent down-regulation of cell surface β-adrenoceptors. To confirm these results and to rule out possible variations in the isoproterenol induced cAMP responses, C6 cells were treated in the same experiments with DMI, fluoxetine or Hypericum-Lutrol extract and the number of surface β-adrenoceptors and isoproterenol stimulated cAMP (see cyclic AMP chapter) were measured. C6 cells were chronically exposed to 10 µM DMI, 10 µM fluoxetine or to 3.78 mg/ml Hypericum-Lutrol extract equal to 1.26 mg/ml Hypericum extract, 5 µM hypericin or 470 nM hyperforin.

The radioligand binding study was performed using the β-adrenoceptor antagonist ^{3}H-CGP-12177 at three different concentrations (2, 3 and 4 nM) at which the binding was saturated and reached a plateau. The determination of the unspecific binding was performed in the presence of 1 µM timolol, a specific β-adrenoceptor antagonist.

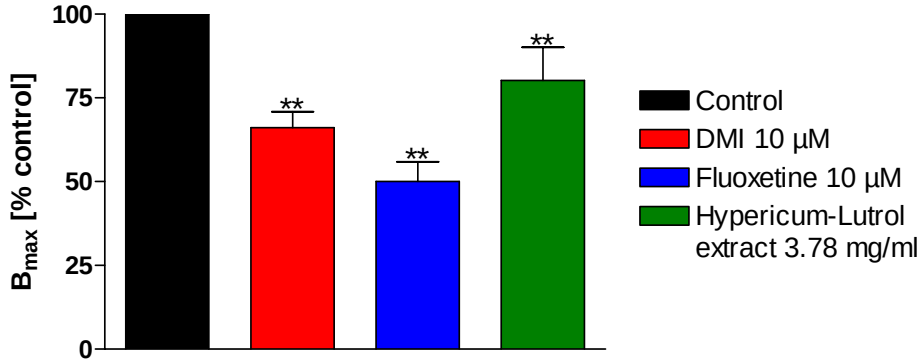

Figure 26: Number of surface β-adrenoceptors (B_{max}) of rat glioblastoma C6 cells chronically exposed to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. Cultures were exposed for 6 days with 4 changes of medium. Results shown represent the mean ± SD of 7 (fluoxetine) to 8 independent experiments. B_{max} 100% is equal to the number of β-adrenoceptors of untreated control cells (49.9 ± 9.1 fmol/mg protein). The β-adrenoceptor number was determined with the radioactive labelled β-adrenoceptor antagonist ^{3}H-CGP-12177 using 1 µM timolol for determining unspecific binding. Specific binding was determined as the difference between total and unspecific binding. **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

Chronic treatments with the antidepressants from three different classes led to significant down-regulation of the surface β-adrenoceptors of C6 cells as shown in Figure 26. The respective B_{max} values were expressed as percent of the B_{max} value of untreated C6 cells and they are listed in Table 6. Surprisingly, chronic exposure of C6 cells to fluoxetine, a selective reuptake inhibitor, showed the most pronounced reduction in the number of surface β-adrenoceptors, followed by cells exposed to DMI, a TCA.

Table 6: B_{max} values of C6 cells treated chronically with DMI, fluoxetine or Hypericum-Lutrol extract. The values are expressed as a percentage of the B_{max} value of control cells.

	DMI 10 µM	Fluoxetine 10 µM	Hypericum-Lutrol extract 3.78 mg/ml
$B_{max} \pm$ SD [% control]	66.1 ± 4.8 (n=8)	50.0 ± 6.0 (n=7)	80.2 ± 9.9 (n=8)

3.3.3 Down-regulation of the cell surface β-adrenoceptor number in C6 cells chronically exposed to a hyperforin-"free" or a hyperforin-rich fraction of the Hypericum-Lutrol extract

The capacities of a hyperforin-rich and a hyperforin-"free" fraction to induce β-adrenoceptor down-regulation were assessed. C6 cells were exposed for 6 days to media containing 10 µM DMI, 500 µg/ml hyperforin-"free" fraction or 50 µg/ml hyperforin-rich fraction or to control medium. The 500 µg/ml hyperforin-"free" fraction contained 304 nM hypericin and 59 nM hyperforin. The 50 µg/ml hyperforin-rich fraction contained 33 nM hypericin and as much as 1677 nM hyperforin. The radioligand binding was performed as already described.

The concentration of the hyperforin-rich fraction used for the chronic exposure of C6 cells could not be increased to the level used for the hyperforin-"free" fraction, because of cytotoxic effects. The exposure to 500 µg/ml of the hyperforin-"free" extract fraction led to a significant loss of 20% of surface β-adrenoceptors (Figure 27A), whereas after chronic exposure to the tolerable 50 µg/ml of the hyperforin-rich extract fraction only a slight, non significant, decrease of 7% (Figure 27B) was observed. Chronic exposure of C6 cells to the hyperforin-"free" fraction in a

concentration of 250 µg/ml did not reduce the β-adrenoceptor number significantly (results not shown). The down-regulation of surface β-adrenoceptors by 10 µM DMI

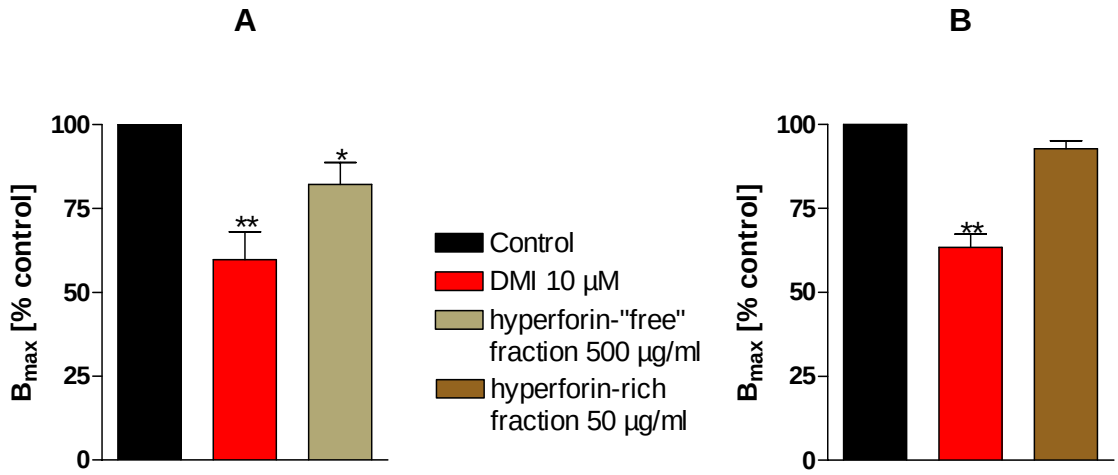

Figure 27: Effects on the number of β-adrenoceptors of rat glioblastoma C6 cells chronically exposed to the hyperforin-"free" fraction (A) or the hyperforin-rich fraction (B). Cultures were exposed for 6 days with 4 changes of medium. Results shown represent the mean ± SD of 2 (B) to 4 (A) independent experiments. Due to the cytotoxic effect of the hyperforin-rich fraction on the C6 cells, its concentration could not be increased to that used for the hyperforin-"free" fraction. The β-adrenoceptor number was determined with the tritium-labelled β-adrenoceptor antagonist ^{3}H-CGP-12177 using 1 µM timolol for determining unspecific binding. *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

was rather constant, as shown in Figure 27A and Figure 27B (59.8% compared to 63.4%), and was in line with that observed earlier in this chapter (66.1% in Figure 26). The values of the radioligand binding of Figure 27 are shown in Table 7.

Table 7: B_{max} values of C6 cells chronically exposed to DMI, hyperforin-"free" fraction or hyperforin-rich fraction. The values are expressed as percent of the B_{max} value of untreated control cells as shown in Figure 27.

	DMI 10 µM	hyperforin-"free" fraction 500 µg/ml	hyperforin-rich fraction 50 µg/ml
A	59.8 ± 8.2 (n=4)	82.2 ± 6.5 (n=4)	
B	63.4 ± 4.0 (n=2)		92.8 ± 2.4 (n=2)

3.4 Chronic treatments of rat glioblastoma C6 cells with antidepressants from three different classes and their influences on cyclic AMP responses

As shown in the previous chapter, chronic treatments with antidepressants led to a down-regulation of surface β-adrenoceptors in C6 cells. We wanted to know if these changes in the number of β-adrenoceptor might have functional consequences in the signal transduction pathway of these cells. Accordingly, the stimulated cAMP was determined with three stimuli that are interfering with different steps in the signalling pathway of the β-adrenoceptor. First, isoproterenol, a β-adrenoceptor agonist, was used to determine the receptor mediated cAMP stimulation. Second, the G protein induced cAMP formation was measured by using fluorides, like NaF that activates the α_s-subunit by interacting with the nucleotide binding site of α_s and mimicking GTP. Third, the AC stimulated cAMP was quantified by forskolin, a direct activator of the AC. It is known that in cell cultures, the cAMP signal is dependent on the cell density. The isoproterenol stimulated cAMP responses diminish as the protein content and cell density per culture increase as shown in fibroblasts (Manganiello & Breslow, 1974; Styger, 1994) as well as in C6 cells (Zbinden, 1997). Chronic exposure of C6 cells and of fibroblasts to Hypericum-Lutrol extract resulted in diminished protein content relative to control cultures, as described in chapter 3.2, the cell density of the seeded cells was increased 5-fold compared to control cells in order to obtain a comparable cell density after chronic exposure. The cAMP content was measured in C6 cells chronically exposed to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. The Hypericum-Lutrol extract contained 5 µM hypericin and 470 nM hyperforin and corresponded to 1.26 mg/ml Hypericum extract. The cell monolayer was washed twice with 2 ml HBS prewarmed to 37°C and the cells were pre-incubated with 1 mM IBMX in HBS for 15 minutes at 37°C to inhibit the phosphodiesterases responsible for cAMP degradation. The stimulants isoproterenol, forskolin or NaF or solvent were added and the cells were incubated at 37°C for further 10 minutes. The reaction was stopped by the removal of the incubation solution and the cell monolayer was washed twice with 2 ml HBS. For the extraction of the cAMP, the cells were broken up in the presence of 3 ml 0.1 N HCl by freezing

for at least 3 hours at –20°C. Aliquots of the thawed HCl solution were frozen and lyophilised over night. The cAMP content was determined by a competitive protein-binding assay described in the method section. The stimulated cAMP content was calculated by subtracting basal cAMP content (solvent stimulation) from the total cAMP content (stimulation with isoproterenol, forskolin or NaF).

3.4.1 Isoproterenol stimulation of chronically treated C6 cells

C6 cells were exposed chronically to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. The cells were stimulated for 10 minutes in the presence of isoproterenol to determine the β-adrenoceptor mediated cAMP response. Chronically exposed C6 cells were stimulated with 10 nM or 10 µM isoproterenol. The concentration of 10 nM isoproterenol led to a submaximal stimulation of β-adrenoceptors and therefore, to a submaximal cAMP response; whereas with 10 µM isoproterenol a maximal stimulation of the β-adrenoceptors is achieved which is leading to a maximal cAMP response. These concentrations were chosen because Brütsch (2003) found in C6 cell cultures chronically exposed to fluoxetine no inhibition of the cAMP response stimulated with 10 nM isoproterenol, but a ~20% reduction after stimulation with 10 µM isoproterenol.

Chronic exposure of C6 cells to the TCA DMI or the SSRI fluoxetine inhibited the isoproterenol-stimulated cAMP at both isoproterenol concentrations (Figure 28). The inhibition was more pronounced with the submaximal concentration of 10 nM isoproterenol except for Hypericum-Lutrol extract exposed cells where a reduction of ~20% was measured after cAMP stimulation with both isoproterenol concentrations.

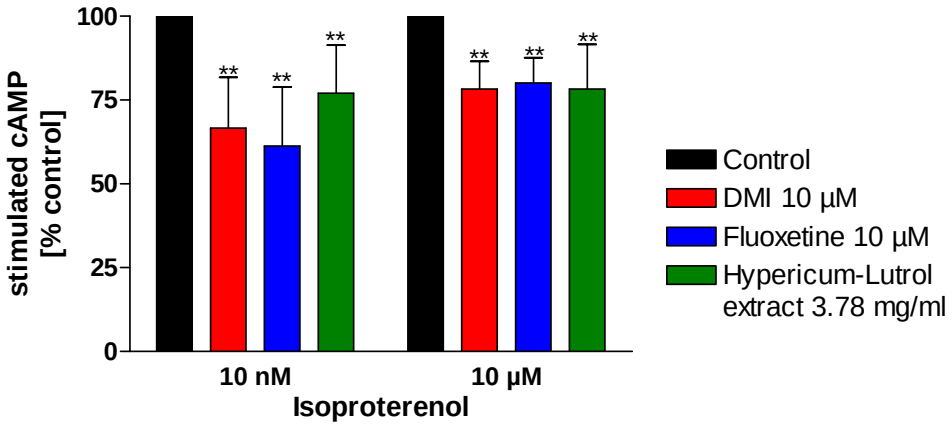

Figure 28: Effects of chronic exposure of C6 cells to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract on β-adrenoceptor mediated stimulation of the AC by 10 nM or 10 µM isoproterenol. Cultures were exposed for 6 days with 4 medium changes. Data represent means ± SD of 10 (Hypericum-Lutrol extract) to 12 individual experiments. 100% stimulated cAMP is equal to the stimulated cAMP amount of untreated control cells by 10 nM or 10 µM isoproterenol (667.5 ± 443.6 pmol/mg protein and 2653.4 ± 472.2 pmol/mg protein, respectively). **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

C6 cells chronically exposed to DMI or fluoxetine showed the same reduction of ~20% in the cAMP accumulation after stimulation by 10 µM isoproterenol (Table 8).

Table 8: Isoproterenol-stimulated cAMP values of C6 cells chronically exposed to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. The stimulated cAMP values are those of Figure 28 and are expressed in percent of the control value.

	stimulated cAMP ± SD [% control]	
	Isoproterenol	
	10 nM	10 µM
DMI 10 µM	66.6 ± 15.2 (n=12)	78.3 ± 8.2 (n=12)
Fluoxetine 10 µM	61.3 ± 17.6 (n=12)	80.1 ± 7.5 (n=12)
Hypericum-Lutrol extract 3.78 mg/ml	77.0 ± 14.4 (n=10)	78.3 ± 13.3 (n=10)

3.4.1.1 Chronic effects of DMI, fluoxetine or Hypericum-Lutrol extract on the isoproterenol concentration response curve in C6 cells

The cAMP concentrations were measured in C6 cells chronically exposed to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract stimulated by increasing concentrations of isoproterenol from 0.1 nM to 100 µM.

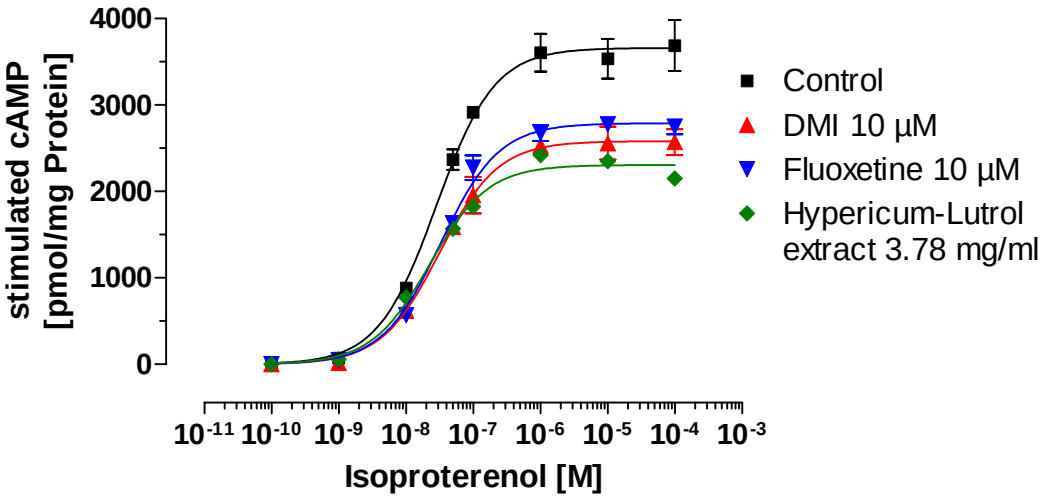

Figure 29: Effects of chronic exposure of C6 cells to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract on the β-adrenoceptor mediated stimulation of the AC by increasing concentrations of isoproterenol. The results of one experiment are shown. Cultures were exposed for 6 days with 4 medium changes. Each data point represents the mean ± SD of triplicates. The curve was fitted using GraphPad Prism's built in equation for sigmoidal dose response: y=bottom + (top-bottom)/(1+10^(logEC_{50}-x)), where x = logarithm of concentration and y = stimulated cAMP in pmol/mg protein.

All treatments resulted in clear effects on isoproterenol concentration dependent cAMP signal (Figure 29). Up to a concentration of 10 nM, isoproterenol showed the same effectiveness in stimulating cAMP with all treatments. Furthermore, stimulation with 1 µM isoproterenol for 10 minutes induced a maximal cAMP response that was not further increased by 10 or 100 µM isoproterenol.

The maximal stimulated cAMP in DMI or fluoxetine exposed C6 cells was inhibited by ~30% compared to control and was in the range measured in the previous chapter. Exposure of C6 cells to Hypericum-Lutrol extract showed the most pronounced effect on the cAMP accumulation with a reduction of ~35% compared to control (Table 9). This result might reflect the fact that the cAMP accumulations in C6 cells chronically

exposed to Hypericum-Lutrol extract were subjected to a high standard deviation as shown in Table 8.

Table 9: Parameters of the concentration-effect curve. The maximal stimulated cAMP response in pmol/mg protein and in percent of control and the EC_{50} of the treatments were calculated from the data shown in Figure 29 using GraphPad Prism software. Shown are the means ± SD of 3 data points of one individual experiment. The EC_{50} value is expressed in nM and the 95% confidence intervals (C.I.) are shown in brackets.

	max. stimulated cAMP		EC_{50} (95% C.I.)
	[pmol/mg protein]	[%] of control	[nM]
Control	3660 ± 50.2	100.0 ± 8.0	28.1 (24.0 - 32.9)
DMI 10 µM	2582 ± 35.2	69.7 ± 4.1	31.6 (28.9 - 34.5)
Fluoxetine 10 µM	2789 ± 33.7	74.5 ± 2.4	32.5 (25.4 - 41.6)
Hypericum-Lutrol extract 3.78 mg/ml	2307 ± 33.2	63.8 ± 3.2	22.4 (18.7 - 26.8)

3.4.2 NaF stimulated cyclic AMP of chronically exposed C6 cells

C6 cells were exposed chronically to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. The cells were stimulated for 10 minutes in the presence of 20 mM NaF that stimulates the AC indirectly by activating the α_s-subunit of G proteins.

Figure 30 shows that chronic exposure of C6 cells to DMI, fluoxetine or Hypericum-Lutrol extract led to diminished cAMP responses of ~20% (Table 10), compared to control cells.

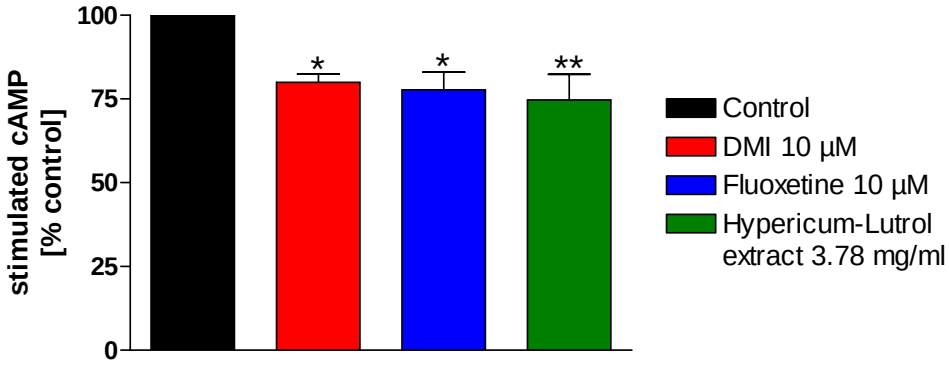

Figure 30: Effects of chronic exposure of C6 cells to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract on the G protein mediated stimulation of the AC by 20 mM NaF. Cultures were exposed for 6 days with 4 medium changes. Results shown represent the mean ± SD of 4 (fluoxetine) to 5 individual experiments. 100% stimulated cAMP is equal to the stimulated cAMP amount of untreated control cells by 20 mM NaF (29.8 ± 14.8 pmol/mg). *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

Table 10: NaF stimulated cAMP values of chronically exposed C6 cells to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. The stimulated cAMP values are those of Figure 30 and are expressed in percent of the control value.

	stimulated cAMP ± SD [% control] NaF 20 mM
DMI 10 µM	80.0 ± 5.5 (n=5)
Fluoxetine 10 µM	77.7 ± 10.7 (n=4)
Hypericum-Lutrol extract 3.78 mg/ml	74.7 ± 17.2 (n=6)

The stimulation of C6 cells with 20 mM NaF resulted in a cAMP signal of 29.8 ± 14.8 pmol/mg protein that was only twice the basal cAMP value. Compared to the forskolin stimulation that resulted in 100-fold cAMP stimulation over basal level, the NaF induced signal was very small. Thus, when interpreting the cAMP results obtained with NaF stimulation, this point has to be considered.

3.4.3 Forskolin stimulation of chronically exposed C6 cells

C6 cells were exposed chronically to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. The cells were stimulated for 10 minutes in the presence of 100 µM forskolin which activates the AC directly.

As shown in Figure 31, chronic exposure to the TCA DMI or the SSRI fluoxetine inhibited the forskolin stimulated cAMP by ~20% (Table 11), whereas exposure of C6 cells to Hypericum-Lutrol extract had no influence on the direct activation of the AC.

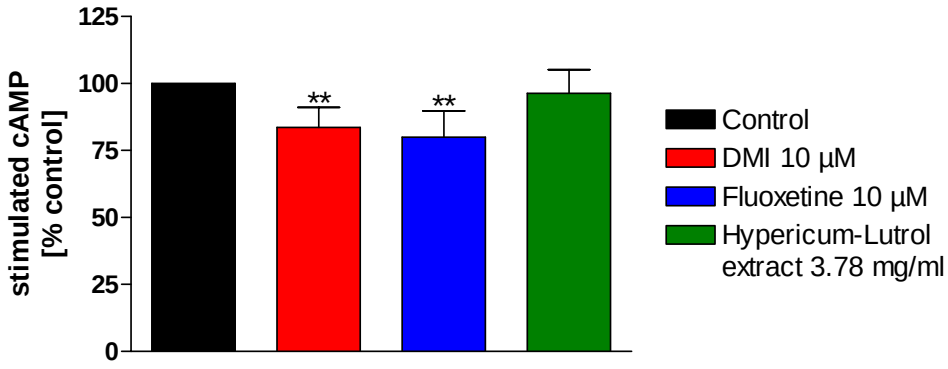

Figure 31: Effects of chronic exposure of C6 cells to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract on the direct stimulation of the AC by 100 µM forskolin. Cultures were exposed for 6 days with 4 medium changes. Data represent means ± SD of 9 (Hypericum-Lutrol extract) to 11 individual experiments. 100% stimulated cAMP is equal to the stimulated cAMP amount of untreated control cells by 100 µM forskolin (2989.0 ± 1302.4 pmol/mg protein). **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

Table 11: Forskolin stimulated cAMP values of chronically exposed C6 cells to 10 µM DMI, 10 µM fluoxetine or 3.78 mg/ml Hypericum-Lutrol extract. The stimulated cAMP values originate from Figure 31 and are expressed in percent of the control value.

	stimulated cAMP ± SD [% control]
	Forskolin 100 µM
DMI 10 µM	83.6 ± 7.5 (n=11)
Fluoxetine 10 µM	79.9 ± 9.9 (n=11)
Hypericum-Lutrol extract 3.78 mg/ml	96.3 ± 8.8 (n=9)

3.5 Cyclic AMP-response after chronic treatments of C6 cells with fractions of the Hypericum extract Ze 117 with low or high hyperforin content

The influences of the hyperforin-rich and hyperforin-"free" fraction of the Hypericum extract were tested on the β-adrenergic signal transduction pathway. C6 cells were exposed chronically and the isoproterenol and forskolin stimulated cAMP content were measured.

3.5.1 Isoproterenol induced cyclic AMP response

In a first step the isoproterenol induced cAMP accumulations in C6 cells chronically exposed to the hyperforin-rich or hyperforin-"free" fraction were determined.
The hyperforin-rich fraction was used in concentrations of 1 µg/ml to 50 µg/ml, whereas the concentrations of the hyperforin-"free" fraction ranged from 10 µg/ml to 600 µg/ml. The amount of the hyperforin-rich fractions could not be raised to the levels of the hyperforin-"free" fraction, because the cells chronically exposed to such high concentrations of the hyperforin-rich fraction showed cytotoxic effects that led to cell death.
The isoproterenol stimulated cAMP accumulation of C6 cells exposed to hyperforin-rich or hyperforin-"free" fractions showed clear dose dependencies (Figure 32A and B). The respective values from Figure 32 are listed in Table 12.

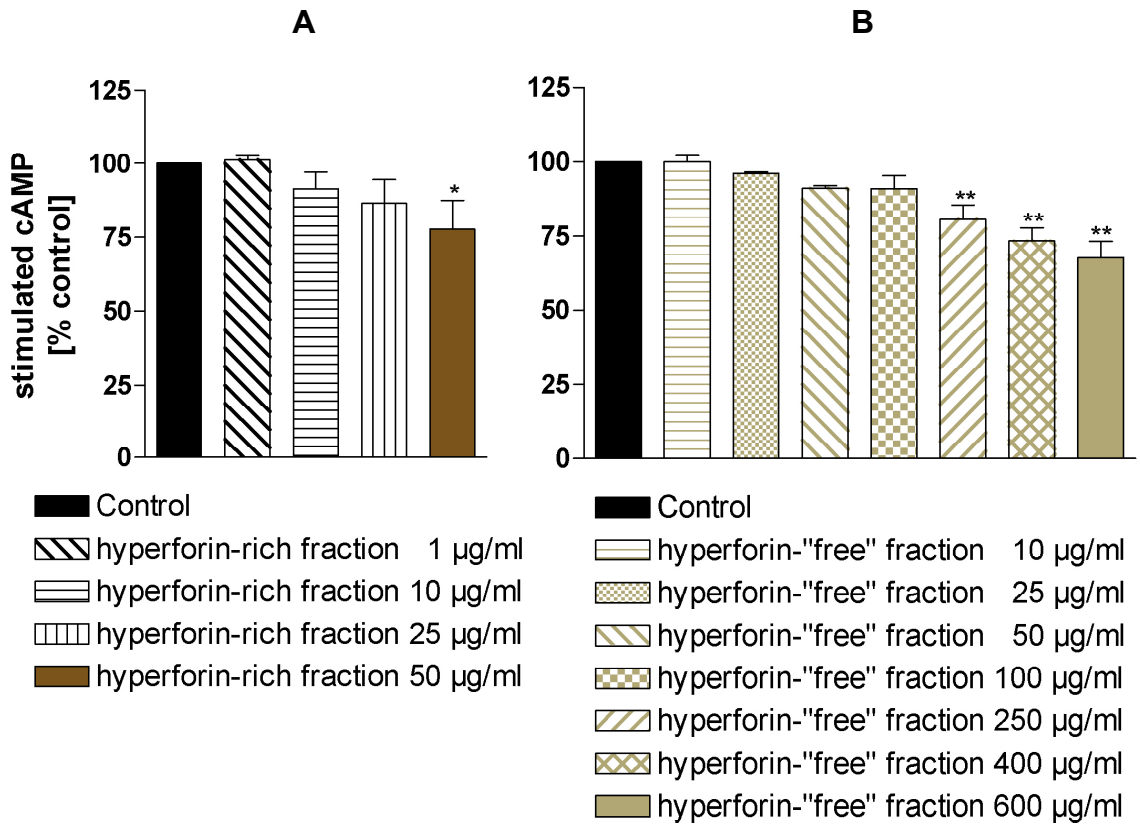

Figure 32: Effects of chronic exposure of C6 cells to increasing concentrations of the hyperforin-rich fraction (A) or hyperforin-"free" fraction (B) on the β-adrenoceptor mediated stimulation of the AC by 10 µM isoproterenol. Cultures were exposed for 6 days with 4 medium changes. Data show the means ± SD of 3 to 6 individual experiments. 100% stimulated cAMP is equal to the stimulated cAMP concentration of untreated control cells by 10 µM isoproterenol (hyperforin-"free" fraction: 2680.1 ± 253.4 pmol/mg; hyperforin-rich fraction: 2316.6 ± 468.7 pmol/mg). *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

Hyperforin-rich fraction resulted a significant reduction of ~20% of the cAMP accumulation at the highest concentration of 50 µg/ml. The cAMP accumulation after chronic exposure to hyperforin-"free" fraction was significantly reduced with concentrations equal or higher to 250 µg/ml. The highest tolerable concentration of 600 µg/ml reduced the stimulated cAMP by ~30% compared to control. The reductions of 20 to 30% of the accumulated cAMP after chronic exposure to the hyperforin-rich or hyperforin-"free" fraction were comparable to the effect after chronic exposure to the 3.78 mg/ml of the Hypericum-Lutrol extract as described in chapter 3.4.1.

Table 12: cAMP levels stimulated by 10 µM isoproterenol of C6 cells chronically exposed to increasing concentrations of the hyperforin-"free" or hyperforin-rich fractions. The values of the accumulated cAMP are those of Figure 32 and are expressed in percent of the stimulated cAMP concentration of control cells. *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

concentrations of the fractions [µg/ml]	hyperforin-rich fraction			hyperforin-"free" fraction		
	hyperforin [nM]	hypericin [nM]	stimulated cAMP ± SD [% control] Isoproterenol 10 µM	hyperforin [nM]	hypericin [nM]	stimulated cAMP ± SD [% control] Isoproterenol 10 µM
1	34	1	101.3 ± 1.5 (n=3)			
10	335	7	91.4 ± 5.6 (n=3)	1	11	100.0 ± 2.2 (n=3)
25	838	16	86.4 ± 8.2 (n=3)	3	28	96.2 ± 0.5 (n=3)
50	1677	33	77.9 ± 9.5 (n=3) *	6	57	91.2 ± 0.9 (n=3)
100				12	113	91.1 ± 4.4 (n=6)
250				29	283	80.7 ± 4.9 (n=6) **
400				47	453	73.4 ± 4.4 (n=6) **
600				70	680	67.7 ± 5.5 (n=6) **

3.5.2 Comparison of the cyclic AMP accumulation in C6 cells after chronic exposure to hyperforin-"free" fraction, DMI, fluoxetine or Hypericum-Lutrol extract

The comparison of the hyperforin-"free" fraction with the full Hypericum plant extract and the synthetic antidepressant drugs should provide evidence if hyperforin is in fact crucial for the inhibition of the cAMP accumulation and probably an antidepressant effect or if this substance could be neglected. Accordingly, C6 cells were exposed to the hyperforin-"free" fraction, Hypericum-Lutrol extract, DMI or fluoxetine in the same experiment. The effects of these treatments on the stimulated cAMP responses induced by isoproterenol and forskolin were determined.

3.5.2.1 Stimulation with isoproterenol

C6 cells chronically exposed to 10 µM DMI, 10 µM fluoxetine, 3.78 mg/ml Hypericum-Lutrol extract or 600 µg/ml hyperforin-"free" fraction were stimulated with 10 nM or 10 µM isoproterenol and the stimulated cAMP was measured. As shown in Figure 33 all treatments led to a significant reduction of the stimulated cAMP level with both isoproterenol stimuli. The stimulated cAMP response induced by 10 nM isoproterenol showed a reduction of ~30% compared to control. The level of the accumulated cAMP stimulated by 10 µM isoproterenol was reduced by ~20% in

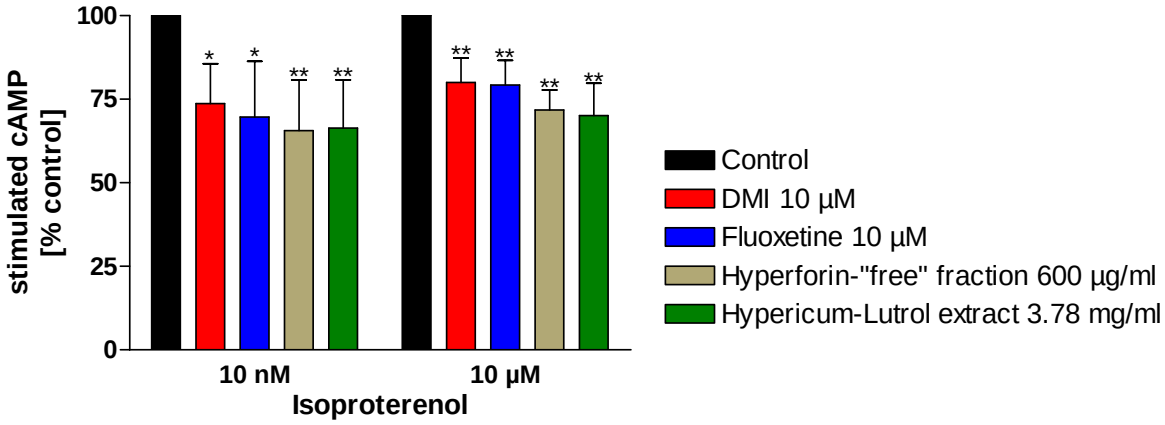

Figure 33: Effects of chronic exposure of C6 cells to 10 µM DMI, 10 µM fluoxetine, 3.78 mg/ml Hypericum-Lutrol extract or 600 µg/ml hyperforin-"free" fraction on the β-adrenoceptor mediated stimulation of the AC by 10 nM or 10 µM isoproterenol. Cultures were exposed for 6 days with 4 medium changes. Results shown represent the means ± SD of 4 (Hypericum-Lutrol extract) to 5 individual experiments. 100% stimulated cAMP is equal to the stimulated cAMP amount of untreated control cells by 10 nM or 10 µM isoproterenol (329.0 ± 166.4 pmol/mg or 2465.6 ± 417.8 pmol/mg). *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

the case of DMI or fluoxetine and in the case of the plant extracts by ~30% (Table 13). The differences in the means of the stimulated cAMP between the synthetic antidepressants and the extracts of *Hypericum perforatum*, however, were not significant.

Table 13: Isoproterenol induced cAMP levels of C6 cells chronically exposed to DMI, fluoxetine, hyperforin-"free" fraction or to Hypericum-Lutrol extract. The values of the cAMP level are expressed in percent of the stimulated cAMP concentration of control cells stimulated by 10 nM or 10 µM isoproterenol.

	stimulated cAMP ± SD [% control]	
	Isoproterenol	
	10 nM	10 µM
DMI 10 µM	73.7 ± 11.9 (n=5)	80.0 ± 7.4 (n=5)
Fluoxetine 10 µM	69.7 ± 16.7 (n=5)	79.3 ± 7.3 (n=5)
Hyperforin-"free" fraction 600 µg/ml (corresp. to 680 nM hypericin, 70 nM hyperforin)	65.6 ± 15.2 (n=5)	71.8 ± 6.0 (n=5)
Hypericum-Lutrol extract 3.78 mg/ml (corresp. to 5 µM hypericin, 470 nM hyperforin)	66.4 ± 14.4 (n=4)	70.1 ± 9.7 (n=4)

3.5.2.2 Stimulation with forskolin

The effects on the forskolin induced cAMP of chronically exposed C6 cells to 600 µg/ml hyperforin-"free" fraction, 3.78 mg/ml Hypericum-Lutrol extract, 10 µM DMI or 10 µM fluoxetine were determined. Forskolin activates the AC directly.

Chronic exposure of C6 cells to DMI or fluoxetine led to a decrease in the stimulated cAMP level compared to control (Figure 34). Surprisingly, treatment with the hyperforin-"free" extract led to a comparable reduction of the stimulated cAMP level, whereas chronic exposure to the Hypericum-Lutrol extract showed no inhibition of the cAMP level in C6 cells, as already shown in chapter 3.4.3.

Chronic exposure of C6 cells to fluoxetine or the hyperforin-"free" fraction led to a ~20% reduction in the stimulated cAMP (Table 14). DMI exposure led only to a non-significant reduction of ~15%, compared to control. This may depend on the limited number of experiments that were performed.

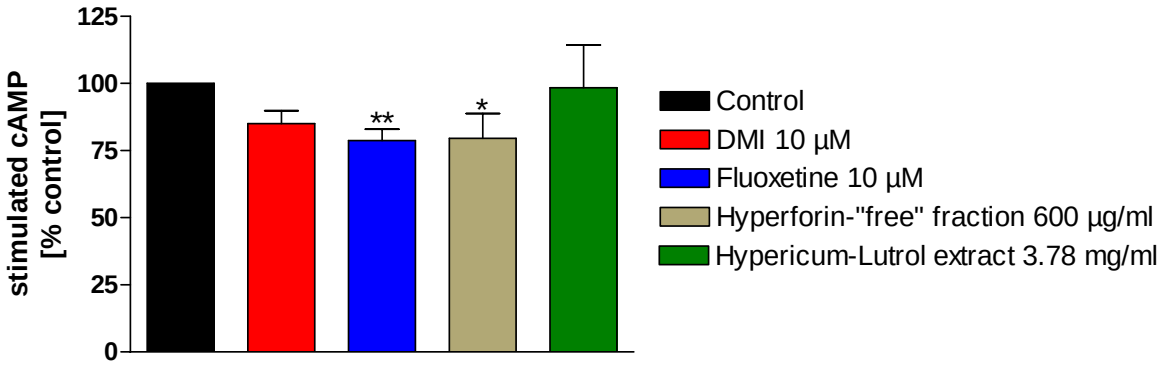

Figure 34: Effects of chronic exposure of C6 cells to 10 µM DMI, 10 µM fluoxetine, 3.78 mg/ml Hypericum-Lutrol extract or 600 µg/ml hyperforin-"free" fraction on the direct stimulation of the AC by 100 µM forskolin. Cultures were exposed for 6 days with 4 medium changes. Data represent the means ± SD of 4 individual experiments. 100% stimulated cAMP is equal to the stimulated cAMP amount of untreated control cells by 100 µM forskolin (3117.9 ± 243.3 pmol/mg). *: $p < 0.05$; **: $p < 0.01$ vs. control (one-way ANOVA, Tukey-Kramer post-test).

Table 14: Forskolin induced cAMP levels of C6 cells chronically exposed to DMI, fluoxetine, hyperforin-"free" fraction or to Hypericum-Lutrol extract. The values of the cAMP level are expressed in percent of the stimulated cAMP concentration of control cells stimulated by 100 µM forskolin.

	stimulated cAMP ± SD [% control]
	Forskolin 100 µM
DMI 10 µM	85.1 ± 4.7 (n=4)
Fluoxetine 10 µM	78.7 ± 4.3 (n=4)
Hyperforin-"free" fraction 600 µg/ml	79.5 ± 9.3 (n=4)
Hypericum-Lutrol extract 3.78 mg/ml	98.4 ± 15.9 (n=4)

4 Discussion

Despite more than 50 years of research efforts on antidepressant drugs the mode of action is not well understood. The immediate inhibition of neurotransmitter reuptake from the synaptic cleft does not directly account for the amelioration of depressive symptoms in humans. It is thought that secondary, adaptive effects following the inhibition of neurotransmitter reuptake are responsible for the clinical improvement of depression that occurs only after prolonged medication of 2 to 3 weeks. The most consistent observation after chronic antidepressant treatment is the down-regulation of the β-adrenoceptor number in the brain of laboratory animals, but also in cell culture systems. It has been shown that in the CNS only the number of the β_1-adrenoceptor subtype is reduced, whereas the number of the β_2-adrenoceptor subtype remains unchanged (Riva & Creese, 1989). Consequently, down-regulation of the β_1-adrenoceptor is thought to be a marker for antidepressant effectiveness. How this reduction is achieved and which underlying mechanisms are responsible, are not yet defined. C6 cells express 4 times more β_1- than β_2-adrenoceptors (Zhong & Minneman, 1993) and it has been shown that chronic, but not acute exposure to antidepressant drugs reduces the β-adrenoceptor number (Fishman & Finberg, 1987). In addition, C6 cells show a functional β-adrenoceptor dependent signalling system with G_s proteins tightly coupled to the receptor (Rasenick & Kaplan, 1986). Thus, this cell type is a good model system to study the number of β-adrenoceptors, G protein and AC activity.

Most of the antidepressant drugs belong to the group of cationic amphiphilic drugs (CADs). All members of this group are able to induce phospholipid (PL) accumulation in cells. This phospholipidosis can be observed in cell cultures, in animals and also, but rarely, in humans (Reasor & Kacew, 2001). Due to their amphiphilic properties, CADs accumulate in acidic cellular compartments, e.g. lysosomes. Accumulation of PL has been observed after chronic exposure to antidepressants in fibroblasts (Zühlke, 1990) and C6 cells (Bürgi, 2001). Chronic exposure of cells to DMI influences the cell membrane properties resulting in a changed membrane PL composition and altered membrane fluidity. These changes may affect the cellular distribution of cell membrane proteins like receptors and further proteins involved in

the second messenger signalling system. Accordingly, membrane transport from the cell surface to intracellular compartments may be affected and thus contribute to the observed down-regulation of the β-adrenoceptor number.

In the present work, possible influences on the β-adrenergic-sensitive signal pathway of C6 cells and fibroblasts after chronic exposure to antidepressants were investigated. The antidepressant drugs tested included a plant extract of *Hypericum perforatum L.*, the tricyclic DMI and the SSRI fluoxetine. In addition, the role of hyperforin, a controversial constituent of the Hypericum extract, was studied by using hyperforin-"free" and hyperforin-rich fractions of the Hypericum extract.

After chronic exposure of C6 cells and fibroblasts to the Hypericum extract or DMI, membrane properties were changed. Both drugs induced changes in the PL composition of cellular and isolated plasma membrane with a pronounced increase in the relative amounts of phosphatidylinositol. The plasma membrane fluidity of both cell types was increased after treatment with Hypericum extract.

Chronic exposure of C6 cells to antidepressants influenced the cell surface β-adrenoceptor number and the second messenger response. It was found that DMI, fluoxetine and the extract of *Hypericum perforatum* down-regulated the β-adrenoceptor number dose-dependently. Moreover, the β-adrenoceptor dependent second messenger signal was comparably reduced at every treatment condition.

The surface β-adrenoceptor number after chronic exposure of C6 cells to the hyperforin-"free" fraction was reduced to the same extent as observed for the Hypericum extract. The hyperforin-rich fraction did not show a significant receptor down-regulation. Interestingly, both fractions reduced the β-adrenoceptor dependent second messenger accumulation as much as did the whole hydroalcoholic Hypericum extract.

In contrast to DMI and fluoxetine, chronic exposure of C6 cells and fibroblasts to the Hypericum extract showed a distinct influence on cell growth and viability. The plant extract reduced the protein- and DNA-content of C6 cell cultures, leaving the cellular protein/DNA ratio unchanged.

4.1 Influence of chronic antidepressant exposure on cell growth and viability

Chronic exposure of C6 cells to DMI or fluoxetine did not influence cell growth. Lutrol, used as solubiliser for the Hypericum extract, was included as a control for the plant extract and showed no change in the cell growth property. Exposure of cell cultures to the Hypericum-Lutrol extract inhibited cell proliferation drastically, as reflected by reduced protein- and DNA-content. The cellular ratio between protein- and DNA-content did not change, indicating that the average cellular protein content was comparable to that of control cells and of cells exposed to DMI, fluoxetine or Lutrol.

Different mechanisms may contribute to cell growth inhibition induced by the Hypericum-Lutrol extract. Cytotoxic effects have been shown for hypericin (Schempp et al., 2002) and hyperforin (Hostanska et al., 2003). Hyperforin induces cell death by activating caspases that are key regulatory proteins in the initiation of apoptosis. It can not be excluded that also other constituents of the Hypericum extract may have the potential to display cytotoxic effects. Blocking of the cell cycle and the consequent inhibition of cell division and proliferation by constituents of the Hypericum extract may represent another mechanism for inhibition of cell growth. It is possible that all of these mechanisms could contribute to the observed cell growth inhibition of the Hypericum extract. On the other hand, inhibition of cell proliferation and cell toxicity occur in concentrations that are most probably not reached *in vivo.*

4.2 Phospholipid composition and membrane properties of C6 cells and human skin fibroblasts chronically exposed to antidepressants

The total amounts of PL were increased after chronic exposure of fibroblasts and C6 cells to DMI or fluoxetine. In contrast to the effects in fibroblasts, C6 cells exposed to Hypericum-Lutrol extract did not augment the total PL amount. The morphology of the cells containing increased amounts of PL was changed. It was characterised by vacuolisation and broadening of the cells. Vacuoles enclosing drug and/or drug-PL complexes contained the fluorescently labelled lysosomal marker dextran. This indicated that the vacuoles might be lysosomes and that these were enlarged by chronic antidepressant treatments. The accumulation of PL was probably due to CAD-properties of DMI, fluoxetine and certain constituents of the Hypericum-Lutrol extract. The difference in PL accumulation between C6 cells and fibroblasts after chronic exposure to Hypericum-Lutrol extract might be a consequence either of less potent phospholipidosis inducing properties of the relevant constituents of the plant extract or of cell type specific properties. Fibroblasts show a marked tendency to accumulate and retain non-degradable substances in lysosomes, whereas C6 cells preferably exocytose lysosomal content. Consequently, these cell-specific properties may contribute to the difference between the C6 cells and the fibroblasts with regard to the PL accumulation after chronic exposure to Hypericum-Lutrol extract.

a) Membrane phospholipid pattern

Chronic treatments of C6 cells and fibroblasts with Hypericum-Lutrol extract or DMI showed complex changes in the PL pattern of the cellular membrane. These alterations could not be extrapolated to the changes detected in isolated plasma membrane. Lutrol exposure did not show any significant change of the PL content in the two cell types.

An increase in the amount of phosphatidylinositol (PI) was the most pronounced change in the cellular and plasma membrane PL composition. It has been shown that

DMI preferentially forms complexes with acidic PL like PI (Lüllmann & Wehling, 1979) which can accumulate in lysosomes. The increased content of PI may not only be due to lysosomal storage, but also due to an increased content of PI in the cell membrane bilayers. The elevated amount of PI may indicate that the content of phosphorylated PI is also increased. PI and its phosphorylated forms play a crucial role in endocytosis and transport of vesicles and thus also for the proteins localised in the plasma membrane. As a consequence, these effects may result in a down-regulation of the number of cell surface β-adrenoceptors after chronic exposure to antidepressant drugs.

Additionally, the content of SPH was decreased after chronic exposure to Hypericum-Lutrol extract or DMI. An obvious difference regarding the PL composition between the Hypericum-Lutrol extract and the DMI exposed fibroblasts and C6 cells reflects the contrasting regulation of the PC and the PE content. Hypericum-Lutrol extract exposure led to an increase in the content of PC, whereas the PE content was decreased. DMI treatment increased the PE content and decreased the PC content. PC can be formed out of PE by sequential methylation of PE by methyltransferases. This is a minor pathway in mammalian cells, but it may be altered after chronic exposure to antidepressants. Consequently, the activity of methyltransferases may be increased after chronic exposure to Hypericum-Lutrol extract, whereas chronic treatment with DMI may decrease the activity of methyltransferases. The changes in content of PC, PE and SPH were less obvious in plasma membrane than in cellular membrane, however. Since β-adrenoceptors are receptors with seven transmembrane spanning regions and located preferentially in the plasma membrane, it is not likely that the minor changes in the bulk plasma membrane content of PC, PE and SPH might explain the observed alterations of the β-adrenoceptor signalling system.

b) Membrane fluidity

Chronic exposure of C6 cells and fibroblasts to DMI, fluoxetine or Lutrol did not change the plasma membrane fluidity, whereas exposure to Hypericum-Lutrol extract led to a reduction in the fluorescence anisotropy. This indicated that treatment with Hypericum-Lutrol extract increased the plasma membrane fluidity. It is possible that this increase in the membrane fluidity is partially mediated by hyperforin, since it has been shown by Eckert et al. (2004) that chronic treatment of mice with hyperforin increases brain membrane fluidity significantly. It can not be excluded that other undefined substances of the Hypericum-Lutrol extract may have contributed to the enhanced membrane fluidity. Despite the change in cell membrane fluidity after Hypericum-Lutrol extract exposure, it is not likely that the β-adrenoceptor signalling system was influenced, because DMI and fluoxetine showed comparable effects on the β-adrenoceptor number and cAMP accumulation, without altering the membrane fluidity.

4.3 Influence of chronic exposure with antidepressants on the β-adrenoceptor number and the second messenger system of C6 cells

a) β-Adrenoceptor number

Chronic exposure of C6 cells to the Hypericum-Lutrol extract, DMI or fluoxetine led to a dose-dependent down-regulation of the cell surface β-adrenoceptor number. Interestingly, exposure of C6 cells to the SSRI fluoxetine caused the most pronounced reduction.

The down-regulation of the β-adrenoceptor number is probably not due to an inhibition of the receptor expression, since it has been shown that the mRNA level of the β-adrenoceptor was not changed after chronic exposure of C6 cells to DMI

(Kientsch, 1998). An enhanced endocytosis of the β-adrenoceptor may explain the observed receptor down-regulation and which results in a decreased surface receptor number. On the other hand, the total number of β-adrenoceptors in cell homogenates is also reduced (Fishman & Finberg, 1987). Results from our laboratory indicate that the number of cell surface β-adrenoceptors and of total β-adrenoceptors are reduced, albeit to a different degree, after chronic exposure of C6 cells to DMI. It is possible that the receptors are degraded more efficiently in response to DMI exposure or that the receptors are partially localised inside compartments in which quantification is not possible. Another reason for the β-adrenoceptor down-regulation may be the predominant increase in the PI content of cellular and plasma membranes. This may lead to a changed endocytosis and transport of vesicles and, consequently, also of proteins, e.g. receptors, localised in the plasma membrane. An antidepressant-independent increase in the PI content in cell membrane should result in a down-regulation of β-adrenoceptors. In preliminary experiments, however, in which the feeding of C6 cells with PI-liposomes induced an increase in the cellular PI content, no consistent decrease in the cell surface β-adrenoceptor number could be measured. Consequently, the down-regulation of the β-adrenoceptor number may not be a direct result of changes in the bulk plasma membrane properties. It can not be excluded that after chronic antidepressant exposure only small regions of cell membrane surrounding β-adrenoceptors were changed. Such changes in small, limited areas of the cell membrane might not be measurable in bulk membranes by determinations of the fluidity and the PL composition.

b) Cyclic AMP response

The β-adrenoceptor mediated cAMP responses of C6 cells chronically exposed to DMI, fluoxetine or Hypericum-Lutrol extract were reduced by 20% compared to control cells. This attenuated cAMP accumulation was accompanied by a decrease in the number of cell surface β-adrenoceptors. The inhibition of the cAMP accumulation induced by the Hypericum-Lutrol extract correlated with the extent of the receptor

down-regulation. In the case of DMI and fluoxetine, the receptor down-regulation was the most pronounced with 40% and 50% reductions, respectively. It is possible that the Hypericum-Lutrol extract down-regulated functional β-adrenoceptors only, whereas the synthetic antidepressants, in addition to functional receptors, reduced also the number of non-functional, spare receptors.

Different antidepressants reduced the cAMP accumulation of chronically exposed cells acting on different levels of the β-adrenoceptor dependent second messenger cascade. The β-adrenoceptor dependent isoproterenol-mediated and the G protein dependent NaF-mediated cAMP accumulations in cells exposed to Hypericum-Lutrol extract, DMI or fluoxetine showed comparable relative values. In contrast to the synthetic antidepressant drugs, the plant extract did not inhibit the AC activity. It is possible that the Hypericum-Lutrol extract reduces the intrinsic G protein activity, the coupling efficiency between G proteins and AC, or changes the cellular G protein localisation pattern. Each of these mechanisms, alone or in combination, may result in a reduced G protein responsiveness. The direct inhibition of the AC activity by DMI and fluoxetine indicates that the down-regulation of the number of β-adrenoceptors may not be necessary for a reduction in the cAMP accumulation and for an antidepressant effect. Since DMI and fluoxetine acutely inhibited the AC activity by 20% and 15% (data not shown), respectively, an immediate antidepressant effect would be expected. Nevertheless, in patients chronic treatment with antidepressant drugs is required to improve depression. Possibly, the concentrations of an antidepressant drug sufficient to inhibit the AC activity in the brain is reached only after chronic accumulation.

The effects of chronic exposure of Hypericum-Lutrol extract, DMI or fluoxetine on the β-adrenoceptor number and accumulation of cAMP are summarised in Table 15.

Table 15: Effects of chronic exposure of C6 cells to DMI, fluoxetine or Hypericum-Lutrol extract on the β-adrenoceptor number and cAMP accumulation. (⇓: reduction; ⇔: no change).

	β-adrenoceptor number	cAMP accumulation induced by		
		Isoproterenol	NaF	Forskolin
DMI	⇓	⇓	⇓	⇓
Fluoxetine	⇓	⇓	⇓	⇓
Hypericum-Lutrol extract	⇓	⇓	⇓	⇔

4.4 Role of hyperforin for the effectiveness of the Hypericum-Lutrol extract on the β-adrenoceptor number and the cyclic AMP accumulation

The influence of hyperforin, a constituent of the Hypericum extract, on the β-adrenoceptor number and cAMP accumulation was investigated. For this purpose, hyperforin-"free" and hyperforin-rich fractions of the Hypericum extract were compared to the hydroalcoholic Hypericum-Lutrol extract.

Chronic exposure of C6 cells to 3.78 mg/ml Hypericum-Lutrol extract or 500 µg/ml hyperforin-"free" fraction reduced the β-adrenoceptor number to a comparable extent. In contrast, the 50 µg/ml hyperforin-rich fraction did not show a significant reduction of the receptor number, but it exerted severe toxic effects on cell growth and viability. Consequently, higher concentrations of the hyperforin-rich fraction were not tolerated by the cells and led to cell growth inhibition and cell death. Cytotoxic effects of hyperforin have been shown by Hostanska et al. (2003), who related it to the activation of key regulatory proteins of apoptosis. Furthermore, the hyperforin-rich fraction was found to be enriched in lipophilic compounds. It is possible that, in addition to hyperforin, these lipophilic compounds were responsible for the distinct cytotoxic effect by disrupting the cellular membrane organisation. The influence of the hyperforin-"free" fraction on β-adrenoceptor number was at least as pronounced as the one of the Hypericum-Lutrol extract. This indicated that hyperforin was not an indispensable prerequisite for down-regulating the β-adrenoceptor number. It has been shown that, in contrast to a Hypericum extract, hyperforin as pure substance did not down-regulate the β-adrenoceptor number in chronically exposed C6 cells (Kientsch et al., 2001). Results from our laboratory showed that the presence of hyperforin in extracts of the Hypericum plant increased the potency in down-regulating β-adrenoceptors. This indicates that hyperforin may act as a co-effector beside other constituents of the Hypericum extract.

The cAMP accumulations after chronic exposure of C6 cells to 3.78 mg/ml Hypericum-Lutrol extract, 600 µg/ml hyperforin-"free" fraction and 50 µg/ml hyperforin-rich fraction were determined. The accumulation of the second messenger

cAMP was reduced to comparable levels after treatments with the two fractions and the full plant extract. The hyperforin-rich fraction was the most potent of the extracts tested and the hyperforin-"free" fraction was more potent than the Hypericum-Lutrol extract. This indicates that hyperforin may enhance the activity of the extract, but that it is not crucial for the reduction of the cAMP accumulation. Further, the hyperforin-"free" fraction inhibited the AC activity, like DMI or fluoxetine. Possibly, constituents of the Hypericum-Lutrol extract were enriched in the hyperforin-"free" fraction, leading to the inhibition of the AC activity. Consequently, chronic exposure of cells to higher concentrations of the Hypericum-Lutrol extract would also result in an inhibition of the AC activity, but such an effect would not be measurable because of the cytotoxic effect of the plant extract at such high concentrations.

4.5 Conclusions

The clinically effective plant extract of *Hypericum perforatum L*. displayed effects similar to those of the SSRI antidepressant fluoxetine and the TCA DMI in our cell culture system. These effects were observed on the cellular level as well as on the level of the β-adrenoceptor-sensitive signal pathway.

The effects of DMI, fluoxetine and Hypericum-Lutrol extract on membrane properties, PL accumulation and PL pattern of chronically exposed fibroblasts and C6 cells were inconsistent. Thus, these effects are not likely to contribute to β-adrenoceptor down-regulation and alterations in the cAMP accumulation.

Chronic exposure of C6 cells to DMI, fluoxetine or Hypericum-Lutrol extract resulted in a down-regulation of the number of β-adrenoceptors and in a reduction of the cAMP accumulation. These changes were probably not a consequence of changes in the properties of cellular and plasma bulk membranes. The possibility remains that chronic exposure of cells to antidepressants changes the properties of small regions of the plasma membrane and that these alterations might not be detectable by measuring the properties of bulk membranes. Modification of such micro-domains could influence the β-adrenoceptor signal pathway (receptor number and/or post-receptor cascade).

The role which hyperforin plays for the effectiveness of Hypericum extracts was investigated by using hyperforin-"free" and hyperforin-rich fractions of the Hypericum extract. From our results, it can be concluded that hyperforin may enhance the effectiveness of extracts by interacting with other constituents of the Hypericum extract. On the other hand, hyperforin is not essential for inducing β-adrenoceptor down-regulation and for reducing the cAMP accumulation *in vitro*. Consequently, *in vivo*, in addition to a lack of drug interactions, a hyperforin-free Hypericum extract may be as effective and even safer as a hyperforin-containing extract for treating mild to moderate depression.

5 References

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Curriculum vitae

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