Thieme

# Synthesis and Characterization of new Azecine-Derivatives as Potential Neuroleptics

Authors
**Stephanie Zergiebel[1], Christian Fleck[3], Hans-Dieter Arndt[2], Christoph Enzensperger[2], Andreas Seeling[1]**

Affiliations
1 Institute of Pharmacy, Chair of Pharmaceutical/Medicinal Chemistry, Friedrich-Schiller-University Jena, Jena, Germany
2 Institute of Organic Chemistry and Macromolecular Chemistry, Friedrich-Schiller-University Jena, Jena, Germany
3 Institute of Pharmacology and Toxicology, Friedrich-Schiller-University Jena, Jena, Germany

Key words
azecines, LE404, antipsychiotics, CAR, locomotor activity, esterase cleavage

**received** 25.01.2017
**accepted** 01.04.2017

Bibliography
**DOI** https://doi.org/10.1055/s-0043-108651
Published online: 18.5.2017
Drug Res 2017; 67: 466–475

ISSN 2194-9379

Correspondence
PD Dr. Andreas Seeling
Institute of Pharmacy
Chair of Pharmaceutical/Medicinal Chemistry
Friedrich-Schiller-University Jena
Philosophenweg 14
07743 Jena
Germany
Tel.: +49/3641/9 49814, Fax: +49/3641/9 49802
b8sean@rz.uni-jena.de

**ABSTRACT**

Dibenzo- and benzindolo-azecines represent a class of potential neuroleptics. To characterize the effectiveness at the dopamine and 5-$HT_{2A}$-receptor representative structures were synthesized and tested by radio ligand binding studies, in vivo and in vitro studies.
Neuroleptic potency and the risk of side effects of the prodrug 7-methyl-5,6,7,8,9,14-hexahydrodibenzo[d,g]azecin-3-yl isobutyrate, an ester derivative of the most promising azecine 7-methyl-5,6,7,8,9,14-hexahydrodibenzo[d,g]azecin-3-ol (LE404), was tested in vivo concerning conditioned avoidance response inhibition, locomotor activity and triggering of catalepsy vs. haloperidol as a reference. Also ester hydrolysis was examined using porcine liver esterase to thereby obtain an indication of the stability of the prodrug in vivo. An HPLC method was developed for purity control and determination of octanol/water-distribution coefficients.
It has been shown that the tested substances in their efficacy are comparable to haloperidol and risperidone, but the therapeutic index in most cases is larger. Esterification as a prodrug principle leads to significantly prolonged effectiveness.

## Introduction

Dopamine is an important neurotransmitter in the brain. It plays a crucial role in the regulation of locomotion, cognition and emotion. The result of disturbances in the dopaminergic system can be a variety of neuropsychiatric disorders, e. g., schizophrenia or Parkinson's disease [1].

Schizophrenia occurs in 1 % of the general population [2]. There are 3 different groups of symptoms: positive, negative and cognitive symptoms [3]. Hallucinations, delusions (which frequently involve persecution and/or megalomania) and severe thought disorganization are examples of positive symptoms. Negative symptoms include for instance flattening, apathy, poverty of speech, anhedonia and withdrawal from social life. Deficits in attention and memory are among the cognitive symptoms [4].

Up to now therapy of schizophrenia is still a problem, because the available drugs show a low efficacy and strong side-effects. Because aetiology of schizophrenia is unknown, it is difficult to find an appropriate target for drug discovery [5]. For drug treatment of schizophrenia a special kind of antipsychotic drug is used, called neuroleptics. These are split into 2 groups, "typical" and "atypical" drugs. The biggest difference between the 2 groups is the occurrence of extrapyramidal side effects [6]. The mechanism of action of "typical" is due to the blockade of dopamine receptors of the $D_2$ receptor subtype. The single distinguishing feature of an atypical antipsychotic is a relatively high affinity for 5-$HT_{2A}$ relative to $D_2$ receptors [7]. Newer (atypical) antipsychotics fix the positive symptoms at much less common extrapyramidal side-effects (EPSE), while having a positive impact on the negative symptoms [8].

Because the neurotransmitters dopamine and serotonin play a key role in the pathology of schizophrenia, research for a drug was promoted with the reflections to develop a molecule which contains elements of both transmitters. Synthesis of such compounds succeeded in the research group of J. Lehmann (Institute of Pharmacy, University of Jena) [9, 10]. Thus, azecines are a novel class of antidopaminergic drug candidates with preferential binding to the $D_1$ receptor subtype ($D_1$,$D_5$) [5]. Moreover, tests have shown that they bind as antagonists at the 5-$HT_{2A}$ receptor [11]. Up to now no $D_5$-selective drugs are available and the function of the $D_5$-receptor is not yet understood exactly [1]. Therefore, it is interesting to determine the affinities of azecines to the $D_5$ receptor and what effect they have. In the recent years, many structural variations were done with the aim of elucidating the structure-activity relationship (SAR). The highest receptor affinities were found in bisannelated hexahydro[d,g]azecines or N-methylated hexahydrobenzindolo[d,g] azecines [9, 12, 13].

Synthesis of dibenzo[d,g]azecines to the lead structure **3** has been done through 5 steps [10]. The derivatization was focused on various esters of short-chain fatty acids.

For receptor functionality, the substitution pattern is fundamental [11]. Various derivatives (▶ **Fig. 1**) were tested with radioligand binding studies and in vivo experiments on receptor affinity, efficiency, duration of action and side effects.

Another promising approach was found in prolongation of effectiveness by esterification of short-acting compounds containing phenolic hydroxyl groups, hereafter called ester prodrugs.

## Materials and Methods

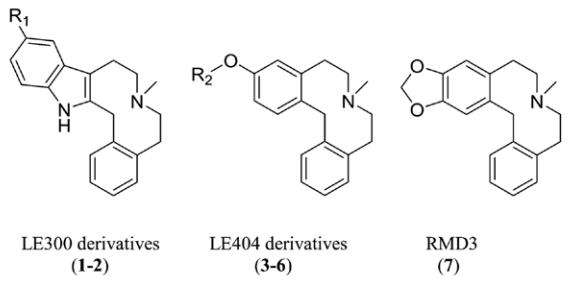
### General

All solvents and reagents were purchased from Merck (Germany), Sigma-Aldrich Chemie Ltd. (Germany), VWR International Ltd. (Germany) or Fisher Scientific Ltd. (Germany). Solvents were used in HPLC-grade and dehydrated by adding molecular sieves or collected from the solvent purification system from Inert (USA).

**3** and the ester derivatives **4**, **5** and **6** are synthesized as described (▶ **Fig. 2**). The detailed analytical data of the new compounds (**4**, **5**, **6**) are provided in the Supporting Information. The purity was determined by elemental analysis and within ± 0.2 % of the calculated value, so that a minimum purity of 95 % was ensured. The synthesis of the compound **3** has been described previously [10]. **1** [9], **2** [14], **7** [15], synthesized as free bases or better soluble salts (e. g. hydrochlorides or maleates), were sponsored by previous work at the Institute of Pharmacy, FSU Jena and conformed the requirements of > 95 % purity.

All other chemicals correspond to the purity "p.a.". The lyophilized porcine liver esterase (specification: E 3019-3,5kU/LOT# 028K7005V; Sigma-Aldrich, 91625 Schnelldorf) had an activity of 17 U/mg.

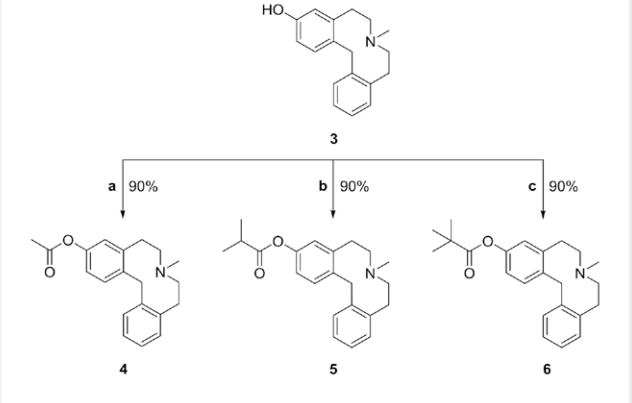
Proton $^1$H and carbon $^{13}$C-NMR spectra were recorded on a Bruker Fourier 300 spectrometer (300 MHz) or Bruker Avance 600 spectrometer (600 MHz) using DMSO-$d_6$ as solvent. Chemical shifts (δ) are given in ppm relative to residual solvent peak. References for DMSO-$d_6$ were 2.50 ppm ($^1$H) and 39.52 ppm ($^{13}$C). Electrospray ionization (ESI) was recorded on Finnigan LCQ mass spectrometer




LE300 derivatives (**1-2**) | LE404 derivatives (**3-6**) | RMD3 (**7**)

| | | |
|---|---|---|
| R1= H | 7-Methyl-6,7,8,9, 14,15-hexahydro-5*H*-indolo[3,2-*f*][3] benzazecine | **1** [9] |
| R1= 0$CH_3$ | 11-Methoxy-7-methyl-6,7,8,9,14,15-hexahydro-5*H*-indolo[3,2-f][3] benzazecine | **2** [14] |
| R2= H | 7-Methyl-5,6,7,8,9,14-hexahydrodibenzo[d,g] azecin-3-ol | **3** [13] |
| R2= (CO)$CH_3$ | 7-Methyl-5,6,7,8,9,14-hexahydrodibenz[d,g] azecin-3-yl acetate | **4** |
| R2= (CO)$CH(CH_3)_2$ | 7-Methyl-5,6,7,8,9,14-hexahydrodibenzo[d,g] azecin-3-yl isobutyrate | **5** |
| R2= (CO)$C(CH_3)_3$ | 7-Methyl-5,6,7,8,9,14-hexahydrodibenz[d,g] azecin-3-yl pivalate | **6** |
| | 7-Methyl-5,6,7,8,9,15-hexahydrobenzo[d] [1,3]benzodioxolo [5,6-g]azecine | **7** [15] |

▶ **Fig. 1** The lead compounds **1** and **3** and novel derivatives.




▶ **Fig. 2** Synthesis of **3** ester derivatives/ Reagents and conditions: **a** NaH, acetyl chloride, 0 °C→rt, $CH_2Cl_2$, 30 min; **b** NaH, isobutyryl chloride, 0 °C→rt, $CH_2Cl_2$, 30 min; **c** NaH, pivaloyl chloride, 0 °C→rt, $CH_2Cl_2$, 30 min

(spray voltage 4.5 kV, capillary temperature 200 °C). High resolution mass spectra (HRMS) were obtained from Bruker maXis impact. Elemental analyses (EA) were performed on a HEKAtech EuroVector EA3000. Infrared spectroscopy (IR) was recorded by IRAffinity-1 FTIR-spectrometer with ATR measurement head. Melting points (mp) were measured by using Büchi melting point B-540 and were uncorrected. $R_f$ values were determined by TLC, which was performed on TLC Silica gel 60 $F_{254}$ plates (Merck).

Haloperidol-hydrochloride and the internal standard substances for the determination of the octanol/water-distribution coefficients were obtained by Caesar & Loretz Ltd., 40721 Hilden, Imipramin-hydrochoride, as internal standard for the HPLC analysis, by Sigma Aldrich Chemie Ltd., 91625 Schelldorf.

HPLC gradient grade acetonitrile (HiPerSolv CHROMANORM™, VWR International B-3001 Leuven) and deionized water (TKA Gen Pure water system, Thermo Electron LED Ltd., 56412 Niederelbert) were used for preparation of mobile phases.

## General procedure for the esterification of 3 (preparation of compounds 4, 5 and 6)

1 eq. **3** was dissolved in dichloromethane (HPLC-grade) and cooled down to 0 °C in an ice bath. 1.1 eq. NaH were added slowly. After completion of gas development, 1.5 eq. of the appropriate acid chloride (acetyl chloride, isobutyryl chloride, pivaloyl chloride respectively) was added slowly and stirred 10 min at 0 °C. Then the mixture was stirred at rt for 30 min. Dichloromethane was evaporated. The residue was purified with a column chromatography on silica gel using petrol ether/acetone (70:30) to give the ester derivatives ($R_f$(**4**) = 0.36, $R_f$(**5**) = 0.38, $R_f$(**6**) = 0.41) in > 90 % yield.

## Analytical results

### 7-methyl-5,6,7,8,9,14-hexahydrodibenzo[d,g]azecin-3-yl acetate (4).

Colorless solid, mp 98 °C. $^1$H NMR (300.19 MHz, [$D_6$]DMSO): δ = 7.42-7.30 (d, 2H), 7.15-7.05 (m, 3H), 6.89-6.81 (d, 2H), 4.31-4.19 (s, 2H), 2.83-2.59 (t + t, 8H), 2.26-2.18 (s, 3H), 2.11-2.02 (s, 3H). $^{13}$C NMR (75.48 MHz, [$D_6$]DMSO): δ = 169.19, 148.65, 141.78, 140.37, 138.13, 130.82, 130.24, 129.99, 126.18, 125.9, 122.88, 119.15, 61.39, 61.10, 47.72, 36.01, 33.53, 33.5, 20.83. IR (ATR, [$cm^{-1}$]): υ = 756, 887, 926, 1011, 1196, 1366, 1458, 1489, 1751, 1975, 2160, 2808, 2909. HRMS (ESI): m/z calculated for $C_{20}H_{23}NO_2 + H^+$ [M + $H^+$]: 310.1802. Found: 310.1803. Elemental analysis (%) calculated for $C_{20}H_{23}NO_2$: C 77.64, H 7.49, N 4.53. Found: C 77.74, H 7.54, N 4.54.

### 7-methyl-5,6,7,8,9,14-hexahydrodibenzo[d,g]azecin-3-yl isobutyrate (5).

Colorless solid, mp 78 °C. $^1$H NMR (300.19 MHz, [$D_6$]DMSO): δ = 7.40-7.30 (d, 2H), 7.15-7.05 (m, 3H), 6.89-6.80 (d, 2H), 4.36-4.17 (s, 2H), 2.86-2.60 (t + t, 8H; m, 1H), 2.12-2.03 (s, 3H), 1.27-1.15 (d + d, 6H). $^{13}$C NMR (75.48 MHz, [$D_6$]DMSO): δ = 175.00, 148.71, 141.90, 140.42, 140.41, 138.11, 130.82, 130.29, 130.00, 126.17, 125.89, 122.81, 119.03, 61.36, 61.07, 47.63, 36.13, 33.64, 33.56, 33.28, 18.68, 18.68. IR (ATR, [$cm^{-1}$]): υ = 710, 756, 1065, 1119, 1142, 1458, 1489, 1744, 1975, 2153, 2793, 2978. HRMS (ESI): m/z calculated for $C_{22}H_{28}NO_2^+$ [M + $H^+$]: 338.2115. Found: 338.2117. Elemental analysis (%) calculated for $C_{22}H_{27}NO_2$: C 78.30, H 8.06, N 4.15. Found: C 78.40, H 8.09, N 4.17.

### 7-methyl-5,6,7,8,9,14-hexahydrodibenzo[d,g]azecin-3-yl pivalate (6).

Colorless oil, $^1$H NMR (600.15 MHz, [$D_6$]DMSO): δ = 7.37-7.30 (d, 2H), 7.13-7.08 (m, 3H), 6.85-6.80 (d, 2H), 4.31-4.25 (s, 2H), 2.75-2.63 (t + t, 8H), 2.10-2.07 (s, 3H), 1.29-1.26 (s, 9H). $^{13}$C NMR (150.91 MHz, [$D_6$]DMSO): δ = 176.36, 148.9, 141.99, 140.49, 140.43, 138.10, 130.84, 130.34, 130.02, 126.18, 125.9, 122.8, 118.99, 61.32, 61.02, 47.55, 38.48, 36.25, 33.7, 33.61, 26.79 (3x). IR (ATR, [$cm^{-1}$]): υ = 2924, 2793, 1975, 1751, 1481, 1450, 1273, 1234, 1111, 903, 756. HRMS (ESI): m/z calculated for $C_{23}H_{30}NO_2^+$ [M + H + ]: 352.2271. Found: 352.2270. Elemental analysis (%) calculated for $C_{23}H_{29}NO_2$: C 78.59, H 8.32, N 3.99. Found: C 78.76, H 8.35, N 4.12.

## Chromatographic conditions

Azecine derivatives were separated by reversed phase HPLC and quantified using imipramin-hydrochloride as internal standard.

HPLC analyses were performed isocratically using a LC-10 AS pump, an Auto Injector SIL-10A with a Diodearray Detector SPD-M10A in combination with a Communication bus module CBM-10A. Integration and calculation were done with Class LC10™ software (all parts Shimadzu Europe).

Separations were achieved using a C18 reversed-phase column (phenomenex® Gemini 5u C18 110 Å, 250 × 4.60 mm) and a precolumn (phenomenex® Security Guard Cartridge Gemini C18, 4 × 3 mm, Phenomenex Inc., 63741 Aschaffenburg, Germany). The mobile phase comprised a mixture of phosphate buffer 4 mM/acetonitrile (30:70 v/v), pH 10. Chromatography was conducted isocratically at ambient temperature at a flow rate of 0.5 ml · $min^{-1}$. Detection wave length was 220 nm.

## Chromatografic determination of octanol/water-distribution coefficients

For the determination of octanol/water-distribution coefficients a slightly modified method was used with the mobile phase methanol/water 75:25 (v/v), a flow rate of 1 ml · $min^{-1}$ and a detection wavelength of 220 nm. Thiourea was used to determine the dead time. As standard substances nicotinamide, phenacetin, procaine-hydrochloride, lidocaine-hydrochloride, tertacaine-hydrochloride and fomocaine were used. The substances were injected 3 times and the average value of the obtained retention times went into the calculation.

## Animals

For the in vivo experiments female Han-Wistar rats, aged 3–5 months, were delivered from Charles River, 97633 Sulzfeld, Germany. Body weight was between 150 and 200 g.

The rats were housed in groups of 4 animals per cage with free access to tap water and commercial food (Altromin 1320; Altromin Spezialfutter Ltd., 32791 Lage, Germany).

The housing conditions were at a temperature of 22 ± 2 °C and a dark-light cycle 12:12 h. The experiments were carried out from 8:00 a.m. till 3:00 p.m.

All animal husbandry and experimental procedures were in compliance with the current version of the German Law on the Protection of Animals and were approved by the animal protection commission of the State of Thuringia (Reg.-Nr. 02-111-11).

### Administration of substances

Before the conditioning and the experiments were started, each animal was habituated to the operator and the experimental equipment, 10 min per day over 14 days. For each test, 8 rats per dose and per time after application were used. After a washout period animals were used again.

The in vivo experiments were performed as described by Schulze et al. [11].

**4**, **5** and **6** were converted in the better soluble maleate by reaction with equimolar amounts of maleic acid. Haloperidol-hydrochloride and the maleates of the ester-derivatives (**4**, **5**, **6**) were dissolved in isotonic NaCl-solution.

Administered doses were calculated as free bases. Control groups obtained saline solution only. The intra-peritoneally applied solution was adjusted to a concentration that defines an injection volume of 1 ml per 100 g body weight. Isotonic saline solution was administered under the same conditions to the control group.

### Locomotor activity

After drug administration rats were placed in the middle of an acryl box, called open field box (50 cm × 50 cm × 50 cm, divided into 25 equal squares on the bottom). The box was manufactured by the Central Research workshops of the Hospital of the University of Jena. Horizontal and vertical movements (particularly line crossing, rearing on the edge or in the middle) were counted individually over 5 min. Grooming and defecation was also monitored, but it has not changed significantly, therefore it was not included in the evaluation. For the determination of the dose response curves (generated with Graph Pad Prism 4™.), the sum of the movements was used.

### Catalepsy

2 different tests were carried out. On the one hand the cross-leg-test and on the other hand the bar-test. In the cross-leg-test, 30; 60; 90; 120; 150; 180; 210 and 240 min after drug administration, animals were put on a desk and one of the hind limbs was placed over the forepaw and the time was measured till the rat has freed itself from this position. Afterwards the rats were exposed to the bar-test. In the process the rat was set with the front paws to a 10 cm high wooden block and the time was measured, which the rat required to put the paws down. The trial period ends after 90 s. Dose response curves and $ED_{50}$ values could be obtained by plotting the arithmetic mean of the cataleptic-times from each dosage-group (n = 8 per dose and time) against the individual logarithmic dose by Graph Pad Prism 4™.

### Pole jump

For this test a pole jump apparatus was needed, which was manufactured by the Central Research workshops of the Hospital of the University of Jena. Before starting the actual experiments, the animals needed to be trained. Previously they have a settling-time of 2 min in the box. Then a noise signal of 1 000 Hz is triggered followed by an electric shock after 4 s, which is routed through the bars of the bottom. If the rat jumps to the pole, electricity and noise signal is turned off. If it does not happen, the current flow and the sound end automatically after 20 s. During training, per day each animal had to complete 10 jumps. Only animals that were able to complete 8 out of 10 jumps within 4 s on 2 consecutive days were used for the tests, because in that case they showed stable conditioned avoidance response (CAR). The actual test consists of 20 jumps and is divided into 3 parts as described by Schulze et. al. [11].

### In vitro esterase stability

1 mg **5** and 1 ml DMSO were solubilized in 20 ml phosphate-buffer pH 7.4 (40 °C). The obtained lyophilized esterase had an enzymatic activity of 17 U/mg. The esterase stock solution (12.5 U/ml in phosphate buffer pH 7.4) should be freshly prepared. The appropriate amount of esterase stock solution was added. For incubation, the sample cup was placed in a water bath set at 40 °C. After 1; 2; 3; 4; 5; 10; 15; 20; 30; 60 and 120 min samples were taken as triplicate. At each time 0,5 ml were transferred to Eppendorff-reaction-tubes, which contain 480 µl acetonitrile (for denaturing the enzymes) and 20 µl imipramine standard solution (0,5 mg imipramine-hydrochlorid in 10 ml acetonitrile/water 50/50 (V/V)). 20 µl of these samples were injected onto the HPLC system.

### Statistical analysis

For statistical analysis Microsoft Excel®, Graph Pad Prism 4™ [GraphPad Software, Inc., Avenida de la Playa, La Jolla, CA, USA (2003)] and IBM SPSS Statistics 20 [International Business Machines Corp., New Orchard Road, Armonk, New York 10504, USA] were used.

At the locomotor activity test the sum of the horizontal and vertical movements has been registered within 5 min and plotted with respect to time after substance administration, against the applied doses, as well as the logarithm of doses. The latter formed the dose-response curves from which the $ED_{50}$ values could be calculated. The means and standard errors of reaction times of the 3 pole jump test sections (jumps: 1–5/jumps: 6–15/jumps: 16–20) were plotted against the different doses. From the number of jumps multiplied by the reaction time, the area under the curve (AUC) values were determined. By plotting this against the logarithm of doses, a dose-response curve and $ED_{50}$ values could be calculated. At the catalepsy test, the arithmetic means and standard errors of the cataleptic times were plotted against the doses. By creating dose-response curves, $ED_{50}$ values for each experimental time could be calculated.

The values of the 3 tests were examined in IBM SPSS Statistics 20 by the Kolmogorov-Smirnov test on normal distribution. Because this test was positive for all values, significances could be calculated. The significance level was set at 5 %.

## Results

### Chemistry

Acetyl chloride, isobutyryl chloride, pivaloyl chloride respectively were used for the esterification of **3**. To convert the phenol in its more reactive anion, the synthesis was accomplished in basic me-

dium. For this purpose, sodium hydride was used in dichloromethane as solvent. After purification the yields of the esters were >90%.

## Radioligand binding studies

Radioligand binding studies were carried out to test the affinity on dopamine and serotonin receptors and to check the hypothesis whether synthesized azecines will primarily attack on the $D_1$-rezeptorsubtyp. The experiments were performed as described by Kassack et al. [16]. The most promising compounds are summarized in ▶ **Table 1**. All derivatives bind in the nanomolar or subnanomolar range. Esterification of the free hydroxyl function of **3** leads to a very slight decreased affinity at the $D_1$ and $D_5$ receptor. In general, enlargement of the ester residue lowers the affinity. The methylene dioxy-derivative **7** shows $D_1$-affinity similar to the pivaloyl-derivative, but a 3 times higher $D_5$-affinity than lead compound **3**.

The azecines show a clear $D_1/D_5$-preference (▶ **Table 2**). In contrast the reference compounds haloperidol and risperidone bind preferentially to the $D_2$ receptor family. Based on the affinity of the azecines for 5-$HT_{2A}$ receptor, compared with the 5-$HT_{2A}$ receptor affinities of haloperidol and risperidone it can be postulated, according to the classification of Meltzer [7], that the azecines belong to the atypical neuroleptics.

## In vivo tests

Appropriate animal experiments were accomplished to test efficacy of substances which have emerged in the radio ligand binding studies as potentially neuroleptics. In addition, side effects such as EPSE should be detected. By correlating the results of both the therapeutic index can then be estimated for each substance. Tests were performed 30 min after administration of substances.

▶ **Table 1** Affinities of test compounds at dopamine $D_1$ and $D_5$ receptors in radio ligand binding experiments.

| substance | $K_i$ [nM] ± SEM | |
|---|---|---|
| | $D_1$ | $D_5$ |
| Risperidone [11] | 104 ± 43 | 563 |
| Haloperidol [11] | 3.4 ± 1.8 | 4.2 ± 0.3 |
| **1** [11] | 2.9 ± 0.9 | 1.2 ± 0.4 |
| **2** [14] | 0.82 ± 0.056 | 3.6 ± 0.6 |
| **7** [15] | 3.6 ± 1 | 0.5 ± 0.01 |
| **3** [11] | 0.39 ± 0.16 | 1.5 ± 0.5 |
| **4** | 1.2 | 0.7 |
| **5** | 2.9 | 1.0 |
| **6** | 3.6 | 3.5 |

## Pole jump

To investigate the efficacy of neuroleptics in relation to positive symptoms, various tests have been established [17] one of which is the inhibition of the Conditioned Avoidance Response (CAR). The Inhibition of CAR is a reliable test system for the identification of potential neuroleptics.

All tested substances inhibit the CAR at certain doses. The $ED_{50}$ values from **3**, **4**, haloperidol and risperidone are all in the range of 0.2–0.3 mg/kg (▶ **Table 3**). In case of **1**, **2**, **6** and **7** an effect can be achieved only at higher doses. $ED_{50}$ values of these substances are between 0.4 and 0.94 mg/kg. The lowest $ED_{50}$ value has been determined for **5**, with 0.13 mg/kg.

## Catalepsy

The most widely used model to study the EPSE is the triggering of catalepsy [18]. **7** has the lowest $ED_{50}$ from the azecine derivatives; it is still below the value of the effective dose for the inhibition of CAR (▶ **Table 3**). All other substances show similar $ED_{50}$ values, led by **1** and **5** with $ED_{50}$ values in the range of 5–8 mg/kg.

In addition cataleptic effects of **5** and haloperidol were tested 60; 90; 120; 150; 180; 210 and 240 min after substance administration (▶ **Fig. 3**). Up to 120 min after administration, the $ED_{50}$ values of the cross-leg tests were lower than that of the bar test. From 150 min, the values are equalizing at an $ED_{50}$-value about 1 mg/kg. At later times slightly higher $ED_{50}$ values were determined for the cross-leg test. With increasing dose, in both tests and for both substances, the times in cataleptic posture extended. The effect maximum for **5** is about 90 min. Then the cataleptic times decreased again, which results in increasing $ED_{50}$-values. This finding confirms the previous test results and shows a decrease in the effect of **5** within the experimental period. For haloperidol a steady rise effect over time is noted in both tests. The calculated $ED_{50}$ values emphasize this fact. At 120 min after application of haloperidol, the $ED_{50}$ values are very high compared with the other values. These breakaways are explained by the small sample number of tests.

## Locomotor activity

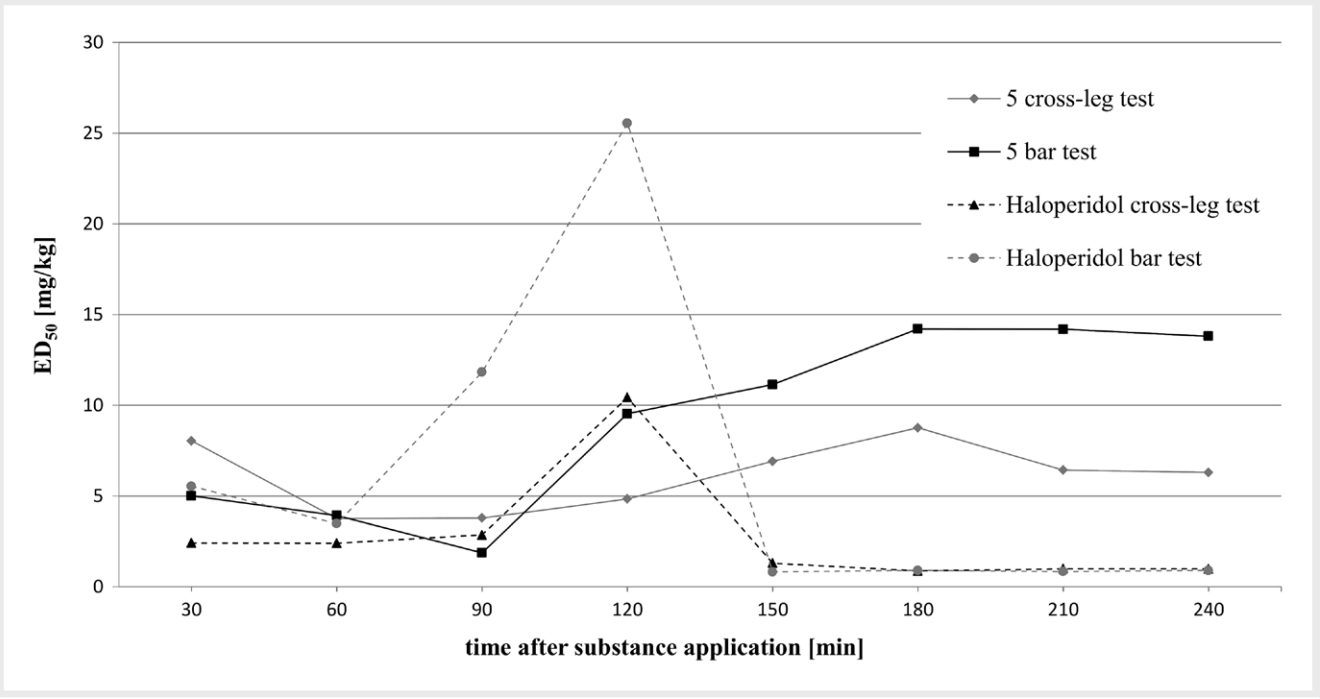
A characteristic adverse effect of neuroleptics is the restriction of locomotor activity. As possible reason for this side effect an inhibition of dopaminergic transmission in the limbic system is discussed [19]. $ED_{50}$ values, 30 min after substance application, are outlined in ▶ **Table 3**. The new compounds (except **4**) caused locomotor limitations only at higher doses compared to haloperidol and risperidone. **2** and **5** caused a significant decrease in locomotor activity even only at doses above 1 mg/kg.

▶ **Table 2** Affinities of test compounds at dopamine $D_1$-$D_5$ and serotonin 5-$HT_{2A}$ receptors in radio ligand binding experiments.

| substance | $K_i$ [nM] ± SEM | | | | | |
|---|---|---|---|---|---|---|
| | $D_1$ | $D_2$ | $D_3$ | $D_4$ | $D_5$ | 5-$HT_{2A}$ |
| Risperidone [11] | 104 ± 43 | 4.8 ± 1.3 | 13.6 ± 0.47 | 12.1 ± 5.0 | 563 | 0.18 ± 0.02 |
| Haloperidol [11] | 3.4 ± 1.8 | 0.82 ± 0.26 | 0.18 ± 0.03 | 3.5 ± 0.89 | 4.2 ± 0.3 | 103 ± 21 |
| **1** [11] | 2.9 ± 0.9 | 9.2 ± 6.9 | 40.4 ± 14.5 | 50.1 ± 12.7 | 1.2 ± 0.4 | 0.13 ± 0.01 |
| **2** [14] | 0.82 ± 0.056 | 11.9 ± 5.6 | 475 ± 48.5 | 266 ± 22 | 3.6 ± 0.6 | n.det. |
| **7** [15] | 3.6 ± 1 | 5.5 ± 1.2 | 20.6 ± 5.5 | 46 ± 9.9 | 0.5 ± 0.01 | n.det. |
| **3** [11] | 0.39 ± 0.16 | 17.5 ± 1.5 | 47.5 ± 16.3 | 7.4 ± 3.7 | 1.5 ± 0.5 | 0.67 ± 0.04 |

▶ **Table 3** $ED_{50}$-values of the in vivo tests (30 min after substance application) and therapeutic index (TI)/CAT(bar) = catalepsy bar test/CAT(CL) = catalepsy cross-leg test/CAR = conditioned avoidance response/LA = Locomotor activity.

| substance | LA $ED_{50}$ [mg/kg] | CAT(bar) $ED_{50}$ [mg/kg] | CAT(CL) $ED_{50}$ [mg/kg] | CAR $ED_{50}$ [mg/kg] | TI (CAT/CAR) | TI (LA/CAR) |
|---|---|---|---|---|---|---|
| Risperidone | 0.28 | 0.5 | 0.43 | 0.33 | 1.30–1.51 | 0.84 |
| Haloperidol | 0.21 | 5.54 | 2.42 | 0.20 | 12.1–27.7 | 1.05 |
| **1** [11] | 0.74 | 8.84 | 7.17 | 0.94 | 7.7–9.4 | 0.79 |
| **2** | 1.1 | 4.3 | 5.3 | 0.4 | 10.75–12.5 | 2.75 |
| **7** | 0.3 | 0.9 | 0.06 | 0.85 | 0.07–1.1 | 0.35 |
| **3** [11] | 0.41 | 6.54 | 6.12 | 0.32 | 13.4–20.3 | 1.28 |
| **4** | 0.1 | 2.06 | 3.26 | 0.30 | 6.9–10.9 | 0.33 |
| **5** | 1.00 | 5.01 | 8.04 | 0.13 | 38.54–61.85 | 7.69 |
| **6** | 0.67 | 5.9 | 6.1 | 0.79 | 3.0–7.6 | 0.84 |




▶ **Fig. 3** $ED_{50}$-values in catalepsy tests (bar and cross-leg test) at different times after drug administration (Haloperidol resp. **5**).

Because of the special interest in **5**, this substance and haloperidol as standard were tested for a prolonged period (data shown in ▶ **Fig. 4**). Up to 180 min **5** had 5 to 10-fold higher $ED_{50}$ values, afterwards 3–4-fold higher than haloperidol. The difference between the measuring point 1 to 3 and point 4 to 6 can be explained by an effective loss of **5** after 180 min.

## Therapeutic index

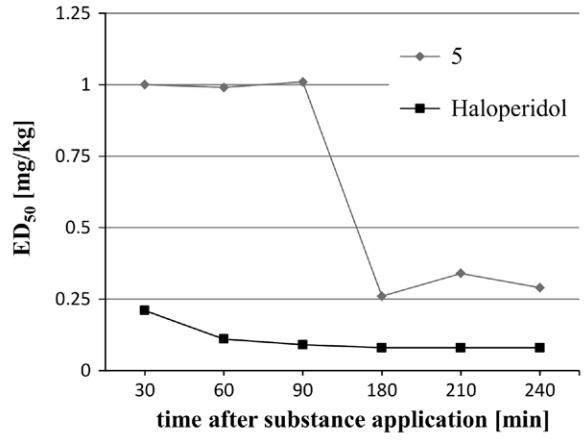
The therapeutic index (30 min after substance application) calculated from catalepsy data and pole jump data behave in parallel for the ranking of the individual substances (▶ **Table 3**). In general, the therapeutic index with respect to the triggering of the catalepsy is always greater than in relation to the restriction of locomotor activity. By far the largest therapeutic index could be calculated for **5**, with 7.69 in the restriction of locomotor activity and 38–62 in correlation with the catalepsy data. **2**, **3** and haloperidol behave in a similar manner. In relation to the restriction of locomotor activity **4**, **7** and risperidone show a therapeutic index below one.

Since **5** had by far the largest therapeutic index, further tests were carried out for 60 min after substance administration (▶ **Table 4**). Haloperidol was used as reference. Because of the low $ED_{50}$ value of haloperidol in the pole jump test, the therapeutic index (based on catalepsy and pole jump data) of the substance is greater than that of **5**. The twice as large therapeutic index of haloperidol suggests that the distance between therapeutically effective dosage and the application rate occurring at the EPSE is greater than for **5**. In correlation with the locomotor activity, the therapeutic index of **5** is 3 times higher than for haloperidol. During animal experiments, animals became significantly anxious and aggressive after haloperidol application. The over weeks domesticated animals have bitten, hissed, jumped to the investigating people and could be taken only with strong resistance from the cage. Such characteristic changes were not observed after administration of **5**.

## Durations of action of 5

As has been shown in the aforementioned tests **5** is the most promising structure, whose effect was studied for up to 240 min after administration (▶ **Fig. 5**).

After 240 min throughout the test (jumps 5–20), no significant ($p \leq 0.05$) difference was visible between reaction times of the control group and the treatment group. After 60 and 150 min, all reaction times of jumps 6–15 are significantly different from the control value. Generally it can be seen that the effect decreases over time. A suppression of the natural fugue occurs only 60 min after administration.



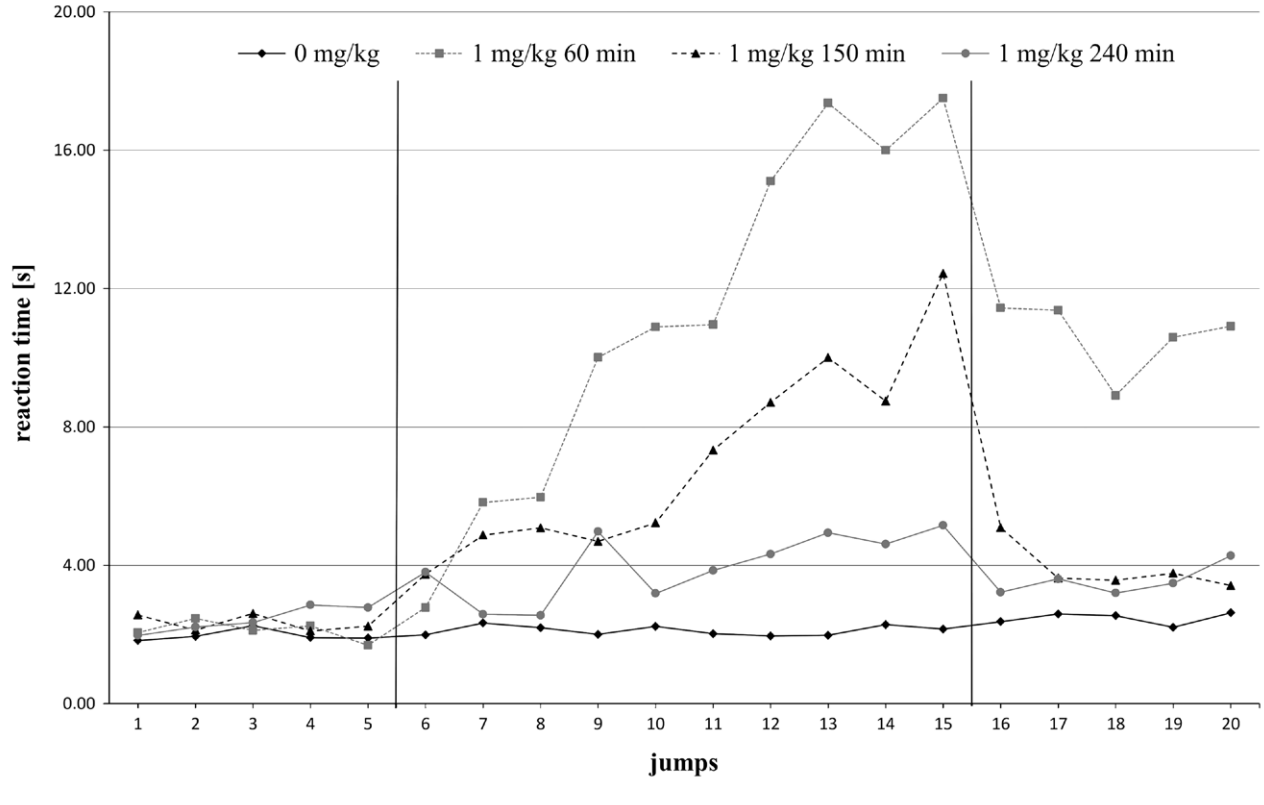

▶ **Fig. 4** $ED_{50}$-values of locomotor activity at different times after drug administration (Haloperidol resp. **5**).

## In vitro esterase stability

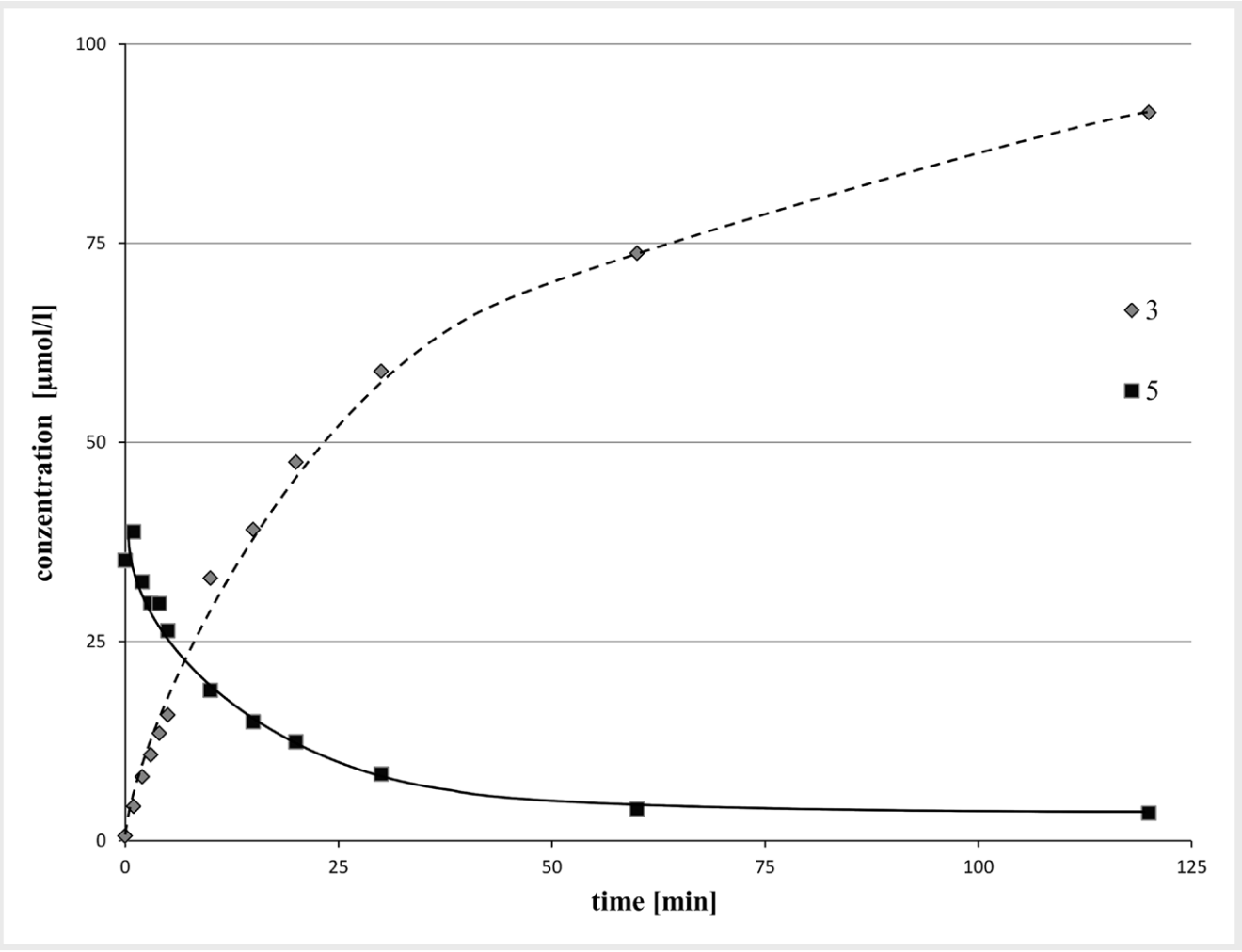
It was investigated the extent and speed of ester hydrolysis of the test compounds by porcine liver esterase. The enzymatic cleavage of **5** was examined over a period of 120 min (▶ **Fig. 6**). In consecutive experiments, the esterase activity was varied in the range between 0.125 U and 10 U. Because enzymatic ester hydrolysis follows 1st order kinetics, half-lives could be calculated. From 2.5 U, it is not possible to shorten the half-life, even when increasing the

▶ **Table 4** $ED_{50}$-values of the in vivo tests: catalepsy, pole jump and locomotor activity (60 min after substance application) and the resulting therapeutic indices.

| | 5 | Haloperidol |
|---|---|---|
| cross-leg test $ED_{50}$ [mg/kg] | 3.76 | 2.40 |
| bar test $ED_{50}$ [mg/kg] | 3.93 | 3.4 |
| locomotor activity $ED_{50}$ [mg/kg] | 0.99 | 0.11 |
| pole jump test $ED_{50}$ [mg/kg] | 0.60 | 0.20 |
| therapeutic index (CAT/CAR) | 6.27–6.55 | 12.00–17.40 |
| therapeutic index (LA/CAR) | 1.65 | 0.55 |




▶ **Fig. 5** Effect of 1 mg/kg **5** at different times after application on the CAR in the pole jump experiment. jump 1–5: before substance application/ jump 6–15: 60/150/240 min after administration of **5** without electric shock/jump 16–20: 60/150/240 min after administration of **5** with electric shock.




▶ **Fig. 6** Degradation of **5** and production of **3** by reaction with esterase solution 0,125 U.

▶ **Table 5** Retention times ($t_R$), and log $P_{O/W}$ values of the reference substances [22–26] and determined log $P_{O/W}$ values of the azecines.

| reference substance | $t_R$ (min) | log $P_{O/W}$ | test substance | $t_R$ [min] | log $P_{O/W}$ |
|---|---|---|---|---|---|
| Nicotinamide | 2.827 | −0.37 | **3** | 10.46 | 3.3 |
| Phenacetin | 3.644 | 1.58 | **4** | 11.95 | 3.9 |
| Procaine-HCl | 6.196 | 1.87 | **7** | 19.55 | 4.0 |
| Lidocaine-HCl | 6.550 | 2.26 | **5** | 26.02 | 4.3 |
| Tetracaine-HCl | 9.864 | 3.73 | **6** | 31.33 | 4.5 |
| Fomocaine | 11.611 | 3.85 | | | |

enzyme activity on 10 U. The lowest enzyme activity tested (0.125 U) results in a half-life of 31 min (▶ **Fig. 6**).

## HPLC-parameters as a basis for further biopharmaceutical studies

The limit of detection (LOD) and quantification (LOQ) were determined based on the ICH Guideline [20]. The LOD and LOQ of **3** and **5** were determined by injecting (3 times per concentration) a series of diluted solutions (5 concentrations per substance) to get a calibration curve. Furthermore solutions ($5 \cdot 10^{-6}$ mol/l) of both substances were injected 6 times and the standard deviations of the corrected peak areas were calculated, which were also included in the calculation of LOD/LOQ. The LOD for **3** was $8.68 \cdot 10^{-7}$ mol/l and $1.48 \cdot 10^{-6}$ mol/l for **5** respectively. The quantitation limit (LOQ) was $2.63 \cdot 10^{-6}$ mol/l for **3** and $4.47 \cdot 10^{-6}$ mol/l for **5**.

The resolutions of **3**, imipramine, **7**, **5** and **6** were in the range between 3.32–16.89.

## Octanol/water-distribution coefficient

According to the OECD Guideline 117 [21] a calibration curve was generated using the HPLC retention times in correlation with the corresponding log $P_{O/W}$ values. The log $P_{O/W}$ values from the refer-

ence compounds (▶ **Table 5**) were taken from the literature [22–26]. There is a linear relationship between retention time and lipophilicity of a substance and therefore its log $P_{O/W}$ value. By the approach partition coefficients for other substances can be calculated from the linear equation.

Octanol/water-distribution coefficients from the test substances are summarized in ▶ **Table 5**. In any case the derivatives are more lipophilic than the phenolic compound **3**. The pivalic acid ester has a higher lipophilicity than the isobutyric ester because of the additional methyl group. The methylene dioxy derivative **7** and the acetic acid ester **4** are classified in lipophilicity between the mother compound **3** and its esters **5** and **6**.

## Discussion

Schizophrenia is a neuro-biochemically caused transmitter imbalance in the dopaminergic system [27]. All clinically effective antipsychotic drugs selectively bind and block the $D_2$ receptor family [1]. Due to this mechanism of action adverse effects such as EPSE are unavoidable. These often therapy limiting side effects can be reduced by serotonin antagonism. A higher affinity for 5-$HT_{2A}$ relative to $D_2$ receptors characterizes a neuroleptic drug as atypical [7]. Therefore, the aim was to develop a molecule that combines the structures of both neurotransmitters. It was also strive for to reach more of a $D_1$-receptor-type selectivity. Some of these new substances revealed an antagonism at human dopamine and 5-$HT_{2A}$ receptors [11]. To assess validity of the test systems, haloperidol and risperidone were examined as reference substances in all tests. Radio-ligand-binding studies have revealed that all tested azecine-derivatives have a clear $D_1/D_5$-preference. The lead structures **1** and **3** show a strong bond at the 5-$HT_{2A}$ receptor similar to risperidone. Thus the azecines, can be classified, according to the classification of Meltzer [7], as atypical neuroleptics.

In vivo tests with selected substances were performed to underpin the radioligand binding results obtained in preliminary studies. **7** can't be applied in practice because of its narrow therapeutic index. In comparison with the other azecines it binds most strongly to the $D_2$ receptor subtype, which also explains the more pronounced side effects. The $ED_{50}$ value for the inhibition of CAR by **1** is more than 2 times higher than that of other substances. By derivatization to **2**, the effective doses were reduced, but they are still higher than those of the other azecines. **3** has the lowest binding affinity to the $D_2$ receptor of all tested substances. This is also reflected in the only slightly occurring EPSE and the associated large therapeutic index. However, **3** shows a rapid metabolism, which is most likely due to the free hydroxyl-group. Thus, a rapid loss of activity was recorded [11]. Therefore different ester prodrugs were synthesized.

With increasing size of the ester moiety the affinity for the individual receptors is reduced, according to the radio-ligand binding studies. In vivo, it has been shown that **5** has by far the largest therapeutic index and the $ED_{50}$ value for the CAR inhibition is only 1/3 of the **3** value. It has now raised the question whether this effect is based on **5** itself or on **3** resulting from ester cleavage. For this purpose, the rate of the ester hydrolysis was examined by reaction with commercial porcine liver esterases. The lowest tested enzyme activity (0.125 U) resulted in a half-life of 31 min. At this time, always the first test was carried out in the animal experiments. In 3 animal models of efficacy and side effect characterization a maximum effect could be seen in the range of 90 to 150 min. In test solutions with an enzyme activity of 0.125 U **5** decreased to 9.8 % within 120 min, whereas **3** increased, so the observed effect on the rats is based solely on **3**. Thus **5** can be regarded as a prodrug of **3**. Complex ester groups like pivaloylester reduce the effectiveness. Moreover it should be noted, that small, sterically not demanding esters show a too rapid hydrolysis and are therefore not eligible for a prolonged effect. On the basis of the previous results the isobutyric ester **5** shows the longest effectiveness. In order to establish structure-activity relationships and to achieve an even stronger protraction additional derivatives have to be synthesized and tested. A possible approach may be amino acids esterification for example with valine. Investigations had shown that synthesis of the prodrug valaciclovir (valin esterification of aciclovir) results in significant higher plasma levels as aciclovir [28]. This esterification is possible by dicyclohexyl carbodiimide (DCC) coupling [29].

## Conflict of interest

The authors declare no competing financial interest.

## References


[1] Emilien G, Maloteaux JM, Geurts M et al. Dopamine receptors-physiological understanding to therapeutic intervention potential. Pharmacol Ther 1999; 84: 133–156

[2] Bonnot O, Dumas N. Schizophrenic disorders in adolescence. Rev Prat 2014; 64: 499–504

[3] Carpenter WT Jr, Buchanan RW. Schizophrenia. N Engl J Med 1994; 681–690

[4] Abi-Dargham A, Laruelle M. Mechanisms of action of second generation antipsychotic drugs in schizophrenia: Insights from brain imaging studies. Eur J Psychiatry 2005; 20: 15–27

[5] Roth BL, Sheffler DJ, Kroeze WK. Magic shotguns versus magic bullets: selectively non-selective drugs for mood disorders and schizophrenia. Nat Rev Drug Discov 2004; 3: 353–359

[6] Leucht S, Pitschel-Walz G, Abraham D et al. Efficacy and extrapyramidal side-effects of the new antipsychotics olanzapine, quetiapine, risperidone, and sertindole compared to conventional antipsychotics and placebo. A meta-analysis of randomized controlled trials. Schizophr Res 1999; 35: 51–68

[7] Meltzer HY, Matsubara S, Lee JC. Classification of typical and atypical antipsychotic drugs on the basis of dopamine D-1, D-2 and serotonin2 pKi values. J Pharmacol Exp Ther 1989; 251: 238–246

[8] Kapur S, Seeman P. Does fast dissociation from the dopamine d(2) receptor explain the action of atypical antipsychotics?: A new hypothesis. Am J Psychiatry 2001; 158: 360–369

[9] Witt T, Hock FJ, Lehmann J. 7-Methyl-6,7,8,9,14,15-hexahydro-5H-benz[d]indolo[2,3-g]azecine: A new heterocyclic system and a new lead compound for dopamine receptor antagonists. J Med Chem 2000; 43: 2079–2081

[10] Hoefgen B, Decker M, Mohr P et al. Dopamine/Serotonin Receptor Ligands. 10:1 SAR studies on azecine-type dopamine receptor ligands by functional screening at human cloned D1, D2L, and D5 receptors with a microplate reader based calcium assay lead to a novel potent D1/D5 selective antagonist. J Med Chem 2006; 49: 760–769

[11] Schulze M, Siol O, Robaa D et al. Molecular combination of the dopamine and serotonin scaffolds yield in novel antipsychotic drug candidates – characterization by in vivo experiments. Drug Res 2012; 62: 252–260

[12] Kassack MU, Hofgen B, Decker M et al. Pharmacological characterization of the benz[d]indolo[2,3-g]azecine LE300, a novel type of a nanomolar dopamine receptor antagonist. Naunyn Schmiedeberg's Arch Pharmacol 2002; 366: 543–550

[13] Mohr P, Decker M, Enzensperger C et al. Dopamine/serotonin receptor ligands. 12(1): SAR studies on hexahydro-dibenz[d,g]azecines lead to 4-chloro-7-methyl-5,6,7,8,9,14-hexahydrodibenz[d,g]azecin-3-ol, the first picomolar D5-selective dopamine-receptor antagonist. J Med Chem 2006; 49: 2110–2116

[14] Enzensperger C, Kilian S, Ackermann M et al. Dopamine/serotonin receptor ligands. Part 15: Oxygenation of the benz-indolo-azecine LE 300 leads to novel subnanomolar dopamine D1/D5 antagonists. Bioorg Med Chem Lett 2007; 17: 1399–1402

[15] Robaa D, Eldin AbulAzm S, Lehmann J et al. A novel non-phenolic dibenzazecine derivative with nanomolar affinities for dopamine receptors. Chem Biodivers 2011; 8: 431–439

[16] Kassack MU, Hofgen B, Lehmann J et al. Functional screening of G protein-coupled receptors by measuring intracellular calcium with a fluorescence microplate reader. J Biomol Screen 2002; 7: 233–246

[17] Arnt J. Pharmacological specificity of conditioned avoidance response inhibition in rats: inhibition by neuroleptics and correlation to dopamine receptor blockade. Acta Pharmacol Toxicol (Copenh.) 1982; 51: 321–329

[18] de Oliveira L, Cunha AO, Mortari MR et al. Cataleptic activity of the denatured venom of the social wasp Agelaia vicina (Hymenoptera, Vespidae) in Rattus norvegicus (Rodentia, Muridae). Prog Neuropsychopharmacol Biol Psychiatry 2006; 30: 198–203

[19] Ahlenius S, Hillegaart V. Involvement of extraphyramidal motor mechanisms in the suppression of locomotor-activity by antipsychotic-drugs - a comparison between the effects produced by presynaptic and postsynaptic inhibition of dopaminergic neurotransmission. Pharmacol Biochem Behav 1986; 24: 1409–1415

[20] ICH Guideline, I., Topic Q 2 B/validation of analytical procedures: Methodology. 1996; http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html 20.08.2016

[21] OECD Guideline 117, OECD Guideline for testing of chemicals. 1989; http://www.oecd.org/chemicalsafety/risk-assessment/1948169.pdf 10.09.2016

[22] Hansch CM, Muir RM, Fujita T et al. The correlation of biological activity of plant growth regulators and chloromycetin derivatives with Hammett constants and partition coefficients. J Am Chem Soc 1963; 2817–2824

[23] Brandstrom A. The use of distribution coefficients in predicting lipid concentrations of drugs. Acta. Pharm. Suec 1964; 1: 159–164

[24] Hansch C, Anderson SM. The effect of intramolecular hydrophobic bonding on partition coefficients. J Org Chem 1967; 2583–2586

[25] Hansch C, Leo A. Pomona College Medicinal Chemistry Project, Log P Database, 1987. Claremont, CA 91711.

[26] http://www.drugbank.ca/drugs/DB00721 22.03.2011

[27] Deutsche Gesellschaft für Psychiatrie, Psychiotherapie und Nervenheilkunde (DGPPN). Behandlungsleitlinie Schizophrenie, S3 Praxisleitlinien in Psychiatrie und Psychotherapie. Vol. 1. 2006, Steinkopff Darmstadt.

[28] MacDougall C, Guglielmo BJ. Pharmacokinetics of valaciclovir. J Antimicrob Chemother 2004; 53: 899–901

[29] Dhokchawle BV, Tauro SJ, Bhandari AB. Ester prodrugs of ketoprofen: Synthesis, hydrolysis kinetics and pharmacological evaluation. Drug Res 2016; 66: 46–50