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Original article

Dopamine/serotonin receptor ligands. Part VIII[1]: the dopamine receptor antagonist LE300 - modelled and X-ray structure plus further pharmacological characterization, including serotonin receptor binding, biogenic amine transporter testing and in vivo testings

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Abstract

-LE300, a benz[*d*]indolo[2,3-*g*]azecine with nanomolar affinities to the hD_1 receptor family, has been further pharmacologically characterized and its modelled structure was compared to its X-ray structure in order to explain NMR data, that was not in accordance to the X-ray structure. Moderate affinity at the hD_3 receptor was determined, nanomolar affinities were found at the 5-HT_{2A} and 5-HT_{2C} receptors, micromolar affinity at the 5-HT_{1A} receptor using binding assays. Functional studies indicate moderate antagonist activity at the 5-HT_{2A} site . No activity was found at dopamine, serotonin and norepinephrine transporters. These results suggested the use of LE300 for cocaine addiction treatment. High activities were found using in vivo testing: LE300 suppressed spontanous locomotor activity with an ID_{50} of 1.24 mg/kg and attenuated locomotor activity induced by 20 mg/kg cocaine with an AD_{50} of 1.50 mg/kg. It failed to substitute for the discriminative stimulus effects produced by cocaine.

Keywords: LE300; Dopamine antagonists; Serotonin antagonists; Cocaine addiction treatment; Low energy conformations; Azecines; X-ray

1. Introduction

The compound LE300 [2], which is 7-methyl-6,7,8,9,14,15-hexahydro-5*H*-benz[*d*]indolo[2,3-*g*]azecine, has been characterized both by radioligand binding assays and functional testings using cAMP and [Ca^{2+}] as a very potent antagonist for the human dopamine receptors (D_1, D_{2L}, $D_{4.4}$ and D_5) with nanomolar affinities and a 10- to 20-fold selectivity for D_1 over D_{2L} [3].

This publication deals with two topics: On the one hand, the X-ray structures of both LE300·HCl and its unprotonated form LE300 could be obtained. A remarkable difference in the chemical shifts of the respective *N*-methyl groups arises (^{13}C: δ = 44.11 ppm and 46.84 ppm / ^{1}H: 2.75 ppm and 1.99 ppm for LE300·HCl and for LE300, respectively), which cannot be explained out of the X-ray structures. In order to find out, how the structure in the lattice differs from the one in solution, molecular modelling was applied. But apart from this fairly obvious reason, LE300 served as a lead for a large number of analogs [1,4,5], binding data of which has been determined: therefore explaining and identifying the lowest energy conformations, which are reflected by the spectroscopic data, are important for further modelings of analogs, e.g. to find out, which syntheses of new compounds might be worth pursuing. Furthermore, correlating the binding affinities of analogs with the modelled structures might give some suggestions, how interaction with the receptor can be understood.

On the other hand (the second topic), intensive pharmacological evaluation of the lead LE300 was pursued. Chemically, LE300 can be regarded as a hybrid of both dopamine

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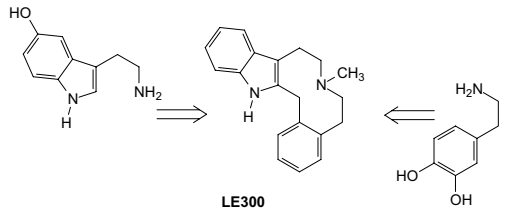

Fig. 1. LE300 as a formal structural hybrid of dopamine and serotonin without hydroxy groups.

(more precisely phenyl ethylamine) and serotonin (more precisely tryptamine) resembling parts of a molecule (see Fig. 1). Out of this reason, and because of the assumption, that a combination of affinities at several receptors, e.g. dopamine and 5-HT_{2A} might be beneficial for the avoidance of extrapyramidal side effects of neuroleptic drugs [6], LE300 is regarded as a lead structure for new compounds with neuroleptic activity. A more thorough investigation into the pharmacological profile of this lead structure was necessary, therefore, apart from completing the pharmacological profile at the dopamine receptor, i.e. determining the affinity to the D_3 receptor, measuring the affinities and agonistic/antagonistic properties at serotonin receptors is very important in order to see, if the compound in fact possesses affinities to dopamine and serotonin receptors. In the case, that no binding of LE300 to the transporter proteins of biogenic amines, which might be possible because of the obvious resemblance of structures, can be observed, another possible use of LE300 -apart from treatment of psychosis- is possible: it might also be useful for the treatment of cocaine addiction. Cocaine blocks the mono amine carriers in the nervous system. Especially the blocking of the dopamine carrier (i.e. an indirect dopamine agonism) is made responsible for the psychic addiction to cocaine. Therefore LE300′s binding profile made it interesting for investigation of an interaction with the rewarding effects of cocaine. For these reasons, in vivo testing for assessing, if LE300 is a stimulant or a depressant, were performed within the NIH Cocaine Treatment Discovery Program. Being a depressant in the locomotor activity assay is an additional indication for this compound interacting with dopamine receptors in vivo.

2. Results and Discussion

2.1. Molecular Modelling

The X-ray structures of both LE300 and LE300·HCl indicate the *N*-methyl groups in an equatorial position of the azecine ring outside the anisotropic regions of the aromatic benzene and indole rings (see Figs. 2 and 3). Nuclear magnetic resonance analyses, however, indicate chemical shifts for the N-methyl carbon atom (^{13}C NMR) and the connected protons (^{1}H NMR) in a range that is not in accordance with this observation. Therefore, a molecular modelling study was carried out with the aim to investigate molecular flexibility of

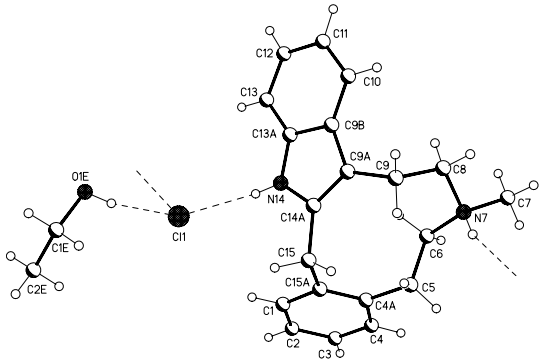

Fig. 2. X-ray structure of LE300·HCl (crystallizing with one mol of ethanol)

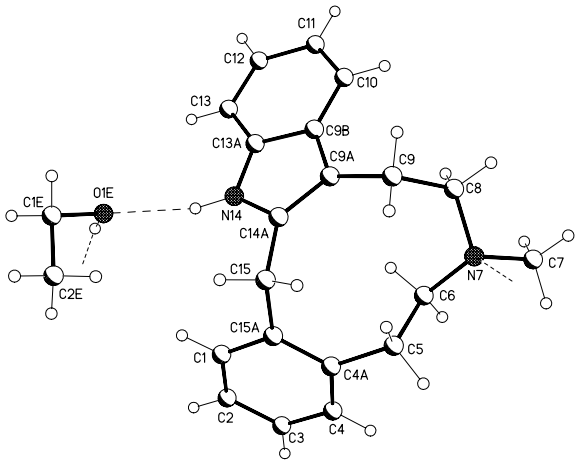

Fig. 3. X-ray structure of LE300 (crystallizing with one mol of ethanol)

these compounds in order to find an explanation for the conflictive results.

High-temperature molecular dynamic simulations (MDS) were performed for the free base and the protonated form to check the corresponding flexibility of the azecine rings. A simulated annealing protocol was used to yield an ensemble of 30 low energy conformers for each derivative. In order to enhance the significance of the results, two different force fields (cvff [7] and Amber [8,9]) were applied. To avoid artificial effects caused by the lack of shielding solvent molecules in course of the simulation, formal and atomic charges were omitted for all molecular mechanics simulations.

Animation of the trajectories verifies the chosen starting temperature of 1,500 K to be clearly sufficient to surmount all energetic barriers (maxima). Additionally, the relatively flat run of the curve indicating the annealing period from 1,500 to 0 K confirms the anticipated slow relaxation (and no splat cooling) of all sampled conformers (see Fig. 4).

Analysis of all archived conformations was accomplished on two different levels. First, the potential energy of all conformers was calculated and energetically identical conformers were clustered into common energetic families (F) starting with the lowest energy conformation to be designated as F1. Second, all structures within one family were superimposed indicating them to be identical, reflected or at least very similar. Closer examination of the latter ones

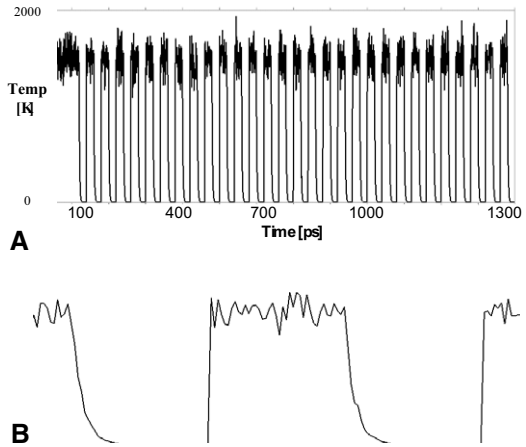

Fig. 4. Graphical analysis of the simulated annealing protocol following the entire MDS (**A**) and focussed on two individual annealing and heating periods (**B**).

adduced the geometry of the basic nitrogen atom, which is not entirely pyramidal but flattened to be responsible for the observed differences.

Analysis of the results derived from theoretical calculations indicates that independent of the applied force field or the protonation state the energetically most favourable conformation is not identical with the experimental X-ray structure. The differences may be clarified by comparing the distances between the alicyclic nitrogen atom and the aromatic ring systems (see Table 1). The force fields privilege a sandwich-like orientation of the amine group wrapped by the indole and the benzene ring (see Fig. 5). The X-ray structure, also found as a low energy conformation (F2 or F3) in this molecular mechanics study, is energetically less favourable compared to the sandwich-like conformation (ΔE 1.6 - 2.2 kcal/mol). For a fitting of both X-ray structures see Fig. 6.

Table 1
Results derived from molecular mechanics (ff) and semi-empirical calculations (AM1 [8]) for LE300 and LE300·HCl

family[a] (ff) (# of conformers)	$\Delta E_{pot.}$[b] ff	AM1	distance A[c] ff / AM1 (X-ray)	distance B[c] ff / AM1 (X-ray)
$F1_{cvff}$ (6)	0	3.0	512 / 503	418 / 402
$F2_{cvff}$ (5)	1.6	0	566 / 569 (565)	517 / 499 (507)
$F1_{Amb}$ (11)	0	0.8	518 / 516	422 / 426
$F3_{Amb}$ (2)	2.2	0	569 / 569 (565)	506 / 499 (507)
$F1_{H+/cvff}$ (7)	0	0	520 / 494	418 / 405
$F2_{H+/cvff}$ (4)	1.7	5.0	568 / 566 (550)	517 / 508 (512)
$F1_{H+/Amb}$ (8)	0	0	514 / 494	409 / 405
$F3_{H+/Amb}$ (1)	1.7	5.0	553 / 566 (550)	518 / 508 (512)

[a] energetically most favourable force field (ff) conformers derived from MDS in the cvff [5] and the Amber (Amb) force field [6,7] are grouped into family 1 (F1), the following ones in F2, and so on. For clarity, only those families comprising the X-ray conformation (i.e. F2 or F3) are compared with F1. Results obtained for the protonated derivative LE300·HCl are indicated with an H+ (e.g. $F1_{H+/cvff}$).

[b] ΔE values in kcal/mol yielded by comparing the lowest energy conformers (set to 0) with energetically less favourable families.

[c] distance (pm) from the centroids of the indolic phenyl (**A**) and the benzene ring (**B**) to the alicyclic nitrogen atom.

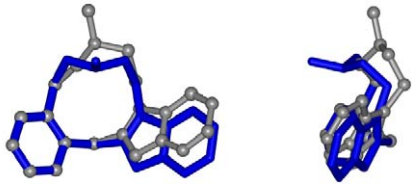

Fig. 5. Orthogonal view of the superimposed sandwich-like (dark) and X-ray analogue extended conformation (grey, in ball and sticks) of LE300 yielded by molecular mechanics calculations. Hydrogen atoms are omitted for clarity.

In the next step, one member of each family was semi-empirically geometry-optimized using the AM1 algorithm [10]. In case of the basic compound (LE300) the geometry of the minimized conformation is scarcely altered. However, one trend may be observed. The distance between the amine group and the aromatic rings is generally shortened (up to 18 pm). More relevant, however, is a comparison of the estimated energies. While the force fields prefer the sandwich-like conformation (ΔE ≈ -2 kcal/mol) the quantum mechanical calculation inverts this proportion and favours the X-ray structure conformation up to 3.0 kcal/mol (see Table 1). This may be explained by the fundamental conception of all quantum mechanical methods to optimize the electron (or charge) distribution. Thus, the atomic orbitals of the amine and the π-electrons of the aromatic rings sense each other and may be combined in an optimal fashion. This electronic interaction was intentionally prevented during the force field calculations by omitting atomic charges.

In case of the cationic LE300·HCl both calculations favour the sandwich-like conformation by 1.7 (ff) and 5.0 kcal/mol (AM1), respectively. This is evidently in contrast to the X-ray crystallographic investigation indicating for both derivatives the extended conformation. One possible explanation for this finding is the presence of ethanol (LE300 and LE300·HCl) and the chloride anion (LE300·HCl) in the crystal lattice of the unit cells forming stabilizing hydrogen bond networks. In the unit cell of LE300 the hydroxy function of ethanol is linking the indolic NH function of one molecule with the free electron pair of the basic amine of a second LE300 molecule. This polar interaction combined with hydrophobic stacking of the aromatic systems clearly favours the extended orientation of the azecine ring. A

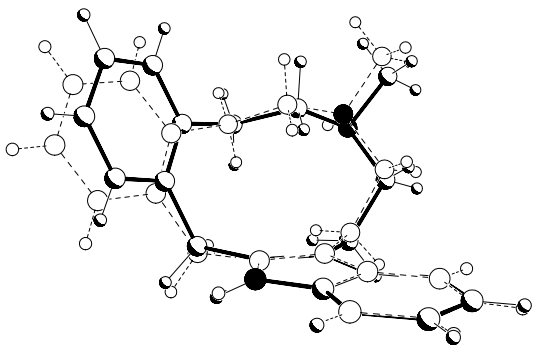

Fig. 6. Fitting of the X-ray structures of LE300 (drawn through line) and LE300·HCl (dotted line)

sandwich-like conformation would prevent the H-bond between the amine and the ethanolic OH group.

For the protonated LE300·HCl the chloride counterion displaces the oxygen atom of the alcohol and takes in its place as H-bond acceptor, for H7, H14 of the second LE300 molecule and H1e of ethanol (see Figs. 2 and 3). This allows a perfect antiparallel orientation of two indole rings and incorporation of the benzene rings into the concave azecine cycles. Once again, a sandwich-like conformation would disturb this stabilizing intermolecular linkage.

Summarizing the results of the theoretical investigations, force field calculations without consideration of charges favour optimal steric complementarity to yield compact, globular-shaped structures. Since this is a well-known property of lipophilic solute molecules in hydrophilic solvate (and vice versa), the results obtained by NMR analyses could be explained in that way. Explicit consideration of atomic charges in the course of quantum chemical calculations alters the conditions. Electron-rich centres being the aromatic rings and the basic nitrogen are pushed away, whereas opposite charged functions (π-electrons and the ammonium) approach in an optimal fashion. This explains the inverted findings obtained for the potential energy of LE300 in the force field (sandwich-like) and the semi-empiric calculation (extended). For the cationic LE300·HCl steric contributions privilege the sandwich conformation. Additional electronic features increase the tendency to form a compact sandwich form (ΔE = 5 kcal/mol).

The special conditions in the crystal lattice incorporating ethanol and partly the chloride anion (i.e. LE300·HCl) allow a perfect hydrogen bonding network if the azecine ring has an extended orientation. In the NMR experiments this ordered arrangement is disrupted by the shielding effects of the solvent molecules (DMSO). Solute-solute interactions in this dilution are very rare and the tremendous amount of solvent molecules ordered in more or less organized clusters force the solute molecules to optimize attractive and minimize repulsive inter- and intramolecular interactions. In the special case considered in this study, optimization of intramolecular interactions could explain the non-typical chemical shifts (δ = 1.99 ppm) of the *N*-methyl groups of the azecine structures. In a sandwich- (or globular-) like arrangement, the particular methyl groups are positioned in the anisotropic region of the indole and the benzene ring leading to the observed shift by approximately 0.76 ppm.

Apart from the explanation of the concrete NMR data, we are able to model analogs, which appropriately reflect the experimental data. Correlation with the binding data can now be made possible.

2.2. Pharmacology

As already reported [3], LE 300 shows nanomolar affinties to human dopamine receptors with a 10-20-fold selectivity to the D_1 subtype family over the D_2 subtype family, including the D_3 receptor ($K_{0.5}$ = 86.9 ± 20.2 nM) (see Table 2). It also shows nanomolar affinities to 5-HT_{2A} and 5-HT_{2C} receptors using binding studies ($K_{0.5}$ = 11.9 ± 2.9 nM and $K_{0.5}$ = 36.1 ± 3.3 nM, respectively) (see Table 2), in a functional assay at the 5-HT_{2A} site it was even more potent (K_e = 3.83 ± 0.28 nM, pA_2 = 8.35 ± 0.03 nM) (see Table 3), therefore our concept to combine structural elements of both dopamine and serotonin within one molecule containing a constrained heterocyclic system to get high affinities at both receptor families turned out to be right. No relevant binding to dopamine, serotonin and norepinephrine transporters was found (see Table 4). The resulting receptogram made LE300 attractive as a potentially suitable compound for treatment of withdrawal symptoms caused by cocaine addiction.

Table 2
Binding affinity for LE300 to dopamine D_3 receptor and serotonin receptors

Receptor radioligand	$K_{0.5}$ ± SEM [nM]	Hill Slope ± SEM
5-HT_{1A} [^{3}H]8-OH-DPAT	1247.2 ± 132.5	not determined because of low affinty
5-HT_{2A} [^{3}H]Ketanserin	11.9 ± 2.9	0.81 ± 0.06
5-HT_{2C} [^{3}H]Mesulergine	36.1 ± 3.3	1.17 ± 0.11
D_3 [^{3}H]YM-09151-2	86.9 ± 20.2	0.80 ± 0.03

LE300 was tested in vivo for spontanous locomotor activity and for attenuation of of locomotor activity induced by cocaine. Fig. 7 shows average horizontal activity counts/10 min as a function of time (top graph) and dose (bottom graph), 20 min following LE300 pretreatment. The period 0-30 min was selected for analysis of dose-response data, because this is the time period in which cocaine produced maximal effects. The mean average horizontal activity counts/10 min for this 30-min period were fit to a linear function of lg dose of the descending portion of the dose effect curve (0.1 to 10 mg/kg dose range). The ID_{50} (dose producing 1/2 maximal depressant activity, where maximal depression = 0 counts/30 min) was calculated 1.24 mg/kg. A one-way analysis of variance conducted on $\log_{10}$ horizontal activity counts/10 min for the 0-30 min time period indicated

Table 3
Functional assay result for LE300 at the 5-HT_{2A} receptor

Receptor	Antagonist		
5-HT_{2A}	**K_e ± SEM [nM]**	**pA_2 ± SEM**	**Slope ± SEMh**
	86.89 ±	8.35 ± 0.03	-1.09 ± 0.03 5

Table 4
Effects of LE300 on HEK-hDAT, HEK-hSERT and HEK-hNET cells

HEK-hDAT cells	**LE300**	**Cocaine**
[^{125}I]RTI-55 Binding K_I [nM]	> 10000	472 ± 46
Hill coefficient		-1.09 ± 0.04
[^{3}H]Dopamine Uptake IC_{50} [nM]		343 ± 31
HEK-hSERT cells		
[^{125}I]RTI-55 Binding K_I [nM]	> 10000	496 ± 33
Hill coefficient		-1.03 ± 0.08
[^{3}H]Serotonin Uptake IC_{50} [nM]		344 ± 35
HEK-hNET cells		
[^{125}I]RTI-55 Binding K_I [nM]	> 10000	2710 ± 260
Hill coefficient		-0.76 ± 0.05
[^{3}H]Norepinephrine Uptake IC_{50} [nM]		275 ± 30

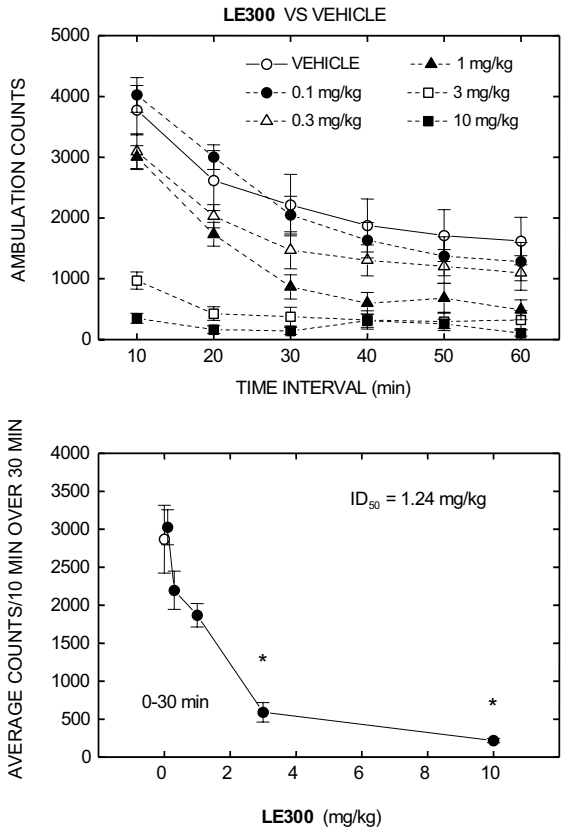

Fig. 7. Effect of LE300 on horizontal activity counts/10 min.
Upper figure: Time course of effect.
Lower figure: Dose response 0-30 min after 20 min pretreatment.
* p<0.05 Compared with 0 dose.

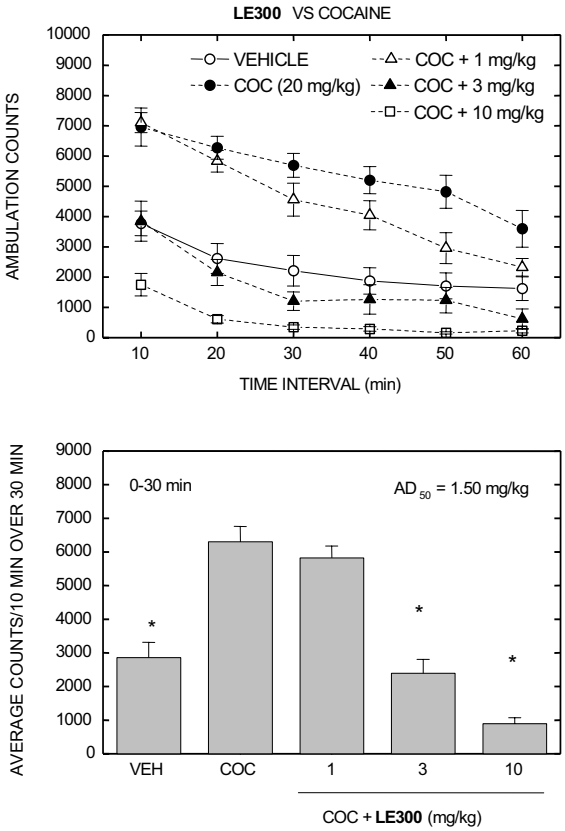

Fig. 8. Interaction between LE300 and cocaine on locomotor activity.
Upper figure: Time course of effect.
Lower figure: Dose response 0-30 min after 20 min pretreatment.
* p<0.05 Compared with cocaine alone.

a significant overall effect $F(5, 42) = 54.58$, $p<0.001$; planned comparisons (a priori contrast) against the vehicle control showed a significant difference for 3 and 10 mg/kg (all $ps<0.05$ denoted on Fig. 7 with an asterik). Fig. 8 gives the graphic representation according to cocaine interaction. Dose range was 1 to 10 mg/kg, and the AD_{50} (dose attenuating cocaine-induced stimulation by 50%) was calculated to be 1.50 mg/kg. [The ordinate value for the AD_{50} was calculated using the mean of the vehicle plus 0.9% saline (vehicle) group as the minimum value, and the mean of the vehicle plus 20 mg/kg cocaine group as the maximum value.] A one-way analysis of variance conducted on lg horizontal activity counts/10 min for the 0-30 min time period indicated a significant overall effect $F(4, 35) = 15.52$, $p<0.001$; planned comparisons (a priori contrast) against the cocaine group showed a significant difference for vehicle, 3 and 10 mg/kg (all $ps<0.05$ denoted on Fig. 8 with an asterik).

LE300 was also tested for substitution for the discriminative stimulus effects produced by cocaine. *Total session*: Within the dose range of 0.5 to 25 mg/kg, LE300 failed to substitute for the discriminative stimulus effects produced by 10 mg/kg cocaine. Response rate was decreased following 10 and 25 mg/kg, with the maximum effect (13% of vehicle control) following 25 mg/kg of LE300. A one-way, repeated measures analysis of variance conducted on response rate for the total session indicated a significant overall effect $F(6,30) = 7.35$, $p<.001$; planned comparisons (a priori contrast) against the vehicle control indicated a significant difference for the 10 and 25 mg/kg doses (all $ps<.05$ denoted on Fig. 9 with an asterisk). *First reinforcer*: The results for the first reinforcer measure were in general accordance with the total session data. Response rate was decreased to 18% of vehicle control following 25 mg/kg of LE300. A one-way, repeated measures analysis of variance conducted on response rate for the first reinforcer failed to indicate a significant overall effect $F(6,30) = 1.93$, $p = .108$; planned comparisons (a priori contrast) against the vehicle control indicated a significant difference for the 25 mg/kg dose (all $ps<.05$ denoted on Fig. 10 with an asterisk). *Other observations*: Four of six rats failed to complete the first fixed ratio when tested following 25 mg/kg of LE300. No unusual effects were observed following any dose of LE300. Because of LE300 being a potent depressant in the mouse locomotor activity assay, and because it did not generalize to cocaine in this assay, it will next be tested at the NIH in the rat drug discrimination antagonism assay.

LE300 seems to be an interesting lead structure for neuroleptic drugs, but our new results strongly suggest LE300 also as a lead for cocaine addiction treatment. Several structural modifications both at the aromatic ring systems and the heterocyclic ring system have been made for further improvement and are currently under pharmacological investi-

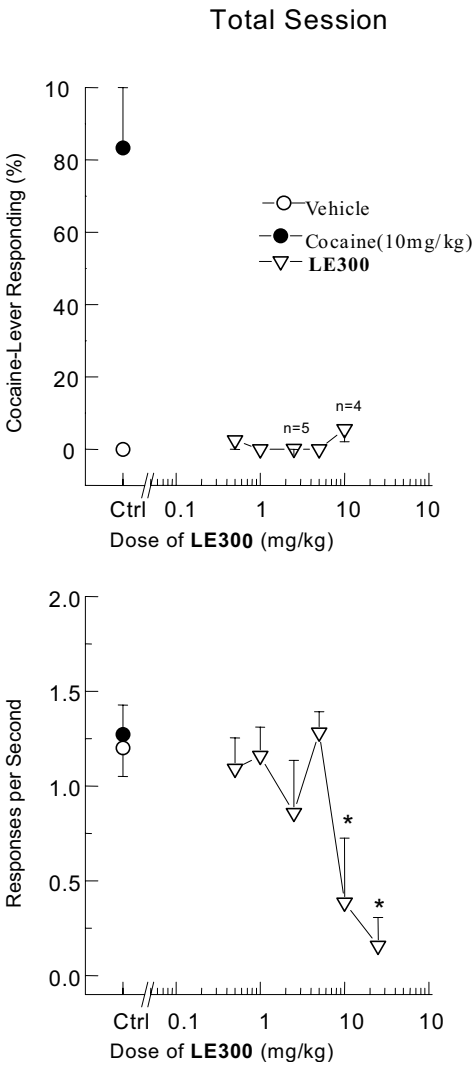

Fig. 9. *Total Session*: The upper panel shows the mean (±SEM) percentage of responses emitted on the cocaine-appropriate lever during the total session as a function of dose, for doses with three or more rats completing the first fixed ratio. The lower panel shows the mean response rate (±SEM) as a function of dose for all subjects tested. The sample size is equal to six at all data points except where noted. To the left of the axis break, control (Ctrl) data are shown for the vehicle (2% methylcellulose) and for the training dose of cocaine (10 mg/kg). Data for the substitution study of LE300 for the training dose of cocaine are shown to the right of the axis break.
*p<0.05 compared with vehicle control.

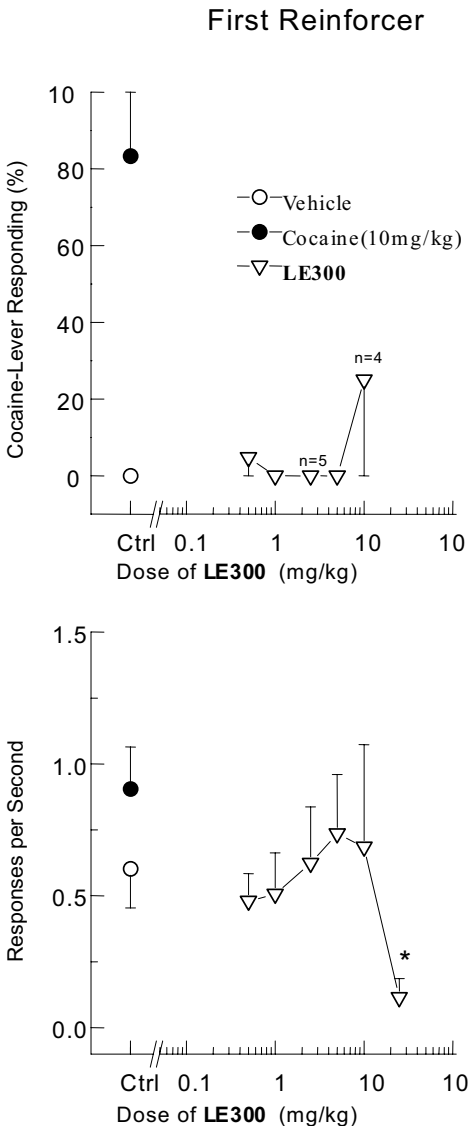

Fig. 10. *First Reinforcer*: The upper panel shows the mean (±SEM) percentage of responses emitted on the cocaine-appropriate lever for the first reinforcer as a function of dose, for doses with three or more rats completing the first fixed ratio. The lower panel shows the mean response rate (±SEM) as a function of dose for all subjects tested. The sample size is equal to six at all data points except where noted. To the left of the axis break, control (Ctrl) data are shown for the vehicle (2% methylcellulose) and for the training dose of cocaine (10 mg/kg). Data for the substitution study of LE300 for the training dose of cocaine are shown to the right of the axis break.
*p<0.05 compared with vehicle control.

gation. Because of its promising properties in the Cocaine Treatment Discovery Program (CTDP), further in vivo testings (rat drug discrimination antagonism assay) are currently performed.

3. Experimental protocols

3.1. Molecular Modelling

A theoretical study was performed in order to find a reasonable explanation for the obvious discrepancy of the chemical shifts of the *N*-methyl groups of LE300 and LE300·HCl compared to the X-ray crystallographic data. High-temperature molecular dynamics simulations (MDS) were carried out with the Discover module of the MSI software package [7] to generate a multitude of different starting conformers for a further, more detailed investigation. Using two different force fields (cvff [5] and Amber [8,9]), a simulated annealing protocol was applied for the free base LE300 and the protonated form (LE300·HCl). MDS were initialized at a temperature of 1500 K for a period of 100 picoseconds (ps). The sampling procedure was started at 1500 K for 20 ps followed by a freezing period of 20 ps cooling the temperature slowly down to 0 K. The yielded structure was archived.

This protocol was repeated 30 times for each derivative (in total 1.2 ns) to gain 30 low energy conformations. Subsequently geometry-optimization was accomplished in the corresponding force field until a convergence criterion of 0.01 kcal/Å was reached. Superposition of all relaxed conformers over the six carbon atoms of the benzene ring provided clusters of common conformational families. The lowest energy conformers of the energetically most favourable families were subjected to charge-considering semi-empirical AM 1 [10] minimizations with the AMPAC/MOPAC module of Insight II [7].

All computations were carried out on SGI Indigo^2 R10000 and SGI Indy R4600 workstations.

3.2. Pharmacology

5-HT$_{1A}$(Binding): HA_7 (human receptor) are grown to confluence in DMEM containing 10% fetal bovine serum (FBS), 0.05% penicillin-streptomycin, and 400 μg/mL of G418. The cells are scraped from 100 x 20 mm plates and centrifuged at 500 x *g* for 5 minutes. The pellet is homogenized in 50 mM Tris-HCl (pH = 7.7), with a polytron, centrifuged at 27,000 x *g*, and resuspended at 10 mg protein/mL in the same buffer. The homogenate is the stored at –70°C in 1 mL aliquots. The thawed cells are washed once and resuspended at 10 mg protein/80mL in 25 mM Tris-HCl containing 100 μM ascorbic acid and 10 μM nialamide at pH = 7.4. The assay is performed in triplicate in a 96-well plate. 100 μL of test compound or buffer and 0.8 mL of cell homogenate (0.1 mg protein/well) are added to 100 μL of [^{3}H]8-OH-DPAT (0.5 nM final concentration). Nonspecific binding is determined with 1.0 μM dihydroergotamine. The plates are incubated at 25°C for 60 min and then filtered. The incubation is terminated by rapid filtration through glass fiber filter plates on a Tomtec cell harvester. The filters are washed four times with ice-cold 50 mM Tris-HCl (pH = 7.7), dried overnight, and bagged with 10 mL scintillation cocktail before counting for two minutes on a Wallac Betaplate 1205 liquid scintillation counter.

5-HT$_{2A}$(Binding): NIH-3T3-GF_6 cells (rat receptor) are grown as described for the HA_7 cells. On the day of the experiment the cells are thawed, resuspended in 50 mM Tris-HCl (pH = 7.7), and centriguged at 27,000 x *g* for 12 minutes. The pellet is then resuspended at 1 mg protein/80 mL in 25 mM Tris-HCl (pH = 7.7), and 0.8 mL of cell homogenate (0.01 mg/protein/well) is added to wells containing 100 μL of the test drug or buffer and 100 μL of [^{3}H]ketanserin (0.4 nM final concentration). Nonspecific binding is determined with 1.0 μM ketanserin.

5-HT$_{2C}$(Binding): NIH-3T3-P∅ cells (rat receptor) are grown as described for the HA_7 cells. The final pellet is resuspended at 3 mg protein/80 mL in 50 mM Tris-HCl (pH = 7.7), 4 mM $CaCl_2$, 10 μM pargyline, and 0.1% ascorbic acid. Wells containing 100 μL of the test drug or buffer, 100 μL of [^{3}H]mesulergine (0.4 nM final concentration), and 0.8 mL of cell homogenate (0.03 mg protein/well) are incubated at 25°C for 60 minutes. Nonspecific binding is determined with 10 μM of mesulergine.

D_3: CHOp-cells (human receptors) are grown to confluence in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS), 0.05% penicillin-streptomycin, and 600 μg/mL of G418, according to the procedures described for HA_7 cells. The final pellet is resuspended at 1 mg protein/80 mL in 50 mM Tris,containing 120 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 4 mM $MgCl_2$, and 1 mM EDTA (pH = 7.4). Wells containing 100 μL of the test drug or buffer, 100 μL of [^{3}H]YM-09151-2 (0.21 nM final concentration), and 0.8 mL of cell homogenate (0.01 mg protein/well) are incubated at 25°C for 60 minutes. Nonspecific binding is determined with 1 μM chlorpromazine.

5-HT$_{2A}$ (*Receptor assay on rat aorta spiral*): male albino Wistar rats (200-300 g body weight) are sacrificed and their aortas quickly removed, cleaned, and cut into a spiral. The spiral is mounted in an 8 mL water-jacketed tissue bath containing Krebs-hydrogencarbonate solution (118 mM NaCl, 2.5 mM $CaCl_2$, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 11.5 mM glucose). The spiral is incubated with oxygenated (5% CO_2 in oxygen) Krebs solution at 37°C under 2.0 g tension for 30 minutes in the presence of 500 μM pargyline and 10 μM benextramine tetrahydrochloride [11]. Excess unreacted pargyline and benetrexamine are removed from the bath by flushing the system several times with Krebs solution. For standardization purposes, the spiral is cumulatively contracted with increasing concentrations of 5-HT in the presence of 0.1 mM ascorbic acid. The 5-HT-induced contractions are recorded using an isometric transducer (Metrigram) coupled to a Gould multichannel polygraph. Compounds, that do not produce a contraction on their own are tested for 5-HT_{2A} antagonist activity. The test compound is incubated with the spiral for 60 minutes, and the 5-HT standard curve is repeated in the presence of the drug. Antagonist activities are calculated for each tissue from full concentration-response curves obtained before and after addition of a single antagonist concentration. At least three different concentrations are used, and only one antagonist concentration is tested on each tissue. pA_2 values are determined from Schild plots using a statistical analysis programme developed by B. Eynon (SRI International) [12].

Biogenic amine transporter binding: Unknowns are weighed and a 10 mM stock solution in DMSO is prepared out of them. Subsequent dilutions are made in assay buffer, achieving a final concentration of 0.1%. Pipetting is conducted using a Biomek 2000 robotic work station. HEK293 cells expressing hDAT, hSERT or hNET inserts are grown to 80% confluence on 150 mm diameter tissue culture dishes and serve as the tissue source. Cell membranes ar prepared as follows. Medium is poured off the plate, and the plate is washed with 10 mL of Ca- and Mg-free PBS. Lysis buffer (10 mL; 2 mM HEPES with 1 mM EDTA) is added. After 10 min, cells are scrapped from plates, poured into centrifuge tubes, and centrifuged 30,000 x *g* for 20 min. The supernatant fluid is removed, and the pellet is resuspended in 12-32 mL of

0.32 M sucrose using a Polytron at setting 7 for 10s. The suspension volume depends on the density of binding sites within a cell line and is chosen to reflect binding of 10% or less of the total radioactivity. For the assays, each assay tube contains 50 µL of membrane preparation (about 10-15 mg of protein), 25 µL of unknown or buffer (Krebs-HEPES, pH = 7.4; 122 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 µM pargyline, 100 µM tropolone, 0.2% glucose and 0.02% ascorbic acid, buffered with 25 mM HEPES), 25 µL of [^{125}I]RTI-55 (40-80 pM final concentration) and additional buffer sufficient to bring up the final volume to 250 µL. Membranes are preincubated with unknowns for 10 minutes prior to the addition of the [^{125}I]RTI-55. The assay tubes are incubated at 25°C for 90 minues. Binding is terminated by filtration over GF/C filters using a Tomtec 96-well cell harvester. Filters are washed for six seconds with ice-cold saline. Scintillation cocktail is added to each square and radioactivity remaining on the filter is determined using a Wallac γ- or β-plate reader. Specific binding is defined as the difference in binding observed in the presence and absence of 5 µM mazindol (HEK-hDAT and HEK-hNET) or 5 µM imipramine (HEK-hSERT). Three independent competition experiments are conducted with duplicate determinations. GraphPAD Prism is used to analyze the ensuing data, with IC_{50} values converted to K_I values using the Cheng-Prusoff equation.

Locomotor activity: A dose response study of LE300-induced locomotor depression was conducted using 40 Digiscan locomotor activity testing chambers (40.5 x 40.5 x 30.5 cm) housed in sets of two, within sound-attenuating chambers. A panel of infrared-beams (16 cm) and corresponding photodetectors were located in the horizontal direction along the sides of each activity chamber. A 7.5-W incandescent light above each chamber provided dim illumination. Fans provided an 80-dB ambient noise level within the chamber. Separate groups of 8 non-habituated male Swiss-Webster mice (Hsd:ND4, aged 2-3 months) were injected via the intraperitoneal (IP) route with either vehicle activity testing. Just prior to placement in the apparatus, all mice received a saline injection. In all studies, horizontal activity (interruption of photocell beams) was measured for 1 h within 10-min periods. Testing was conducted with one mouse per activity chamber. The cocaine interaction study was also conducted using the above described chambers. Twenty minutes following IP vehicle or LE300 injections (1, 3 or 10 mg/kg), groups of 8 non-habituated mice were injected with either 0.9% saline or 20 mg/kg cocaine IP and placed in the Digiscan apparatus for a 1 h session.

Test of substitution for the discriminative stimulus effects of cocaine: LE300 was tested for its ability to substitute for the discriminative stimulus effects of cocaine (10 mg/kg) in rats. Testing began December 18, 2002 and ended May 19, 2003. Experiments were conducted according to the standard operating protocol for CTDP (Cocaine Treatment Discovery Program by the NIH) drug discrimination testing in rats (SOP, March 28, 1997). Six male Sprague-Dawley rats were trained to discriminate cocaine (10 mg/kg) from saline using a two-lever choice methodology. Food was available as a reinforcer under a fixed ratio 10 schedule when responding occurred on the injection appropriate lever. All tests occurred in standard, commercially available chambers (Coulbourn Instruments), using 45 mg food pellets (Bioserve) as reinforcers. Training sessions occurred in a double alternating fashion, and tests were conducted between pairs of identical training sessions (i.e., between either two saline or two cocaine training sessions). Tests occurred only if, in the two preceding training sessions, subjects met the criteria of emitting 85% of responses on the injection correct lever for both the first reinforcer (first fixed ratio) and the total session. Test sessions lasted for twenty minutes, or until twenty reinforcers had been obtained. Doses of the test compound for which fewer than three rats completed the first fixed ratio were not considered in the characterization of discriminative stimulus effects. Intraperitoneal injections (1 ml/kg) of LE300, or its vehicle (2% methylcellulose), occurred 30 minutes prior to the start of the test session. A starting dose for LE300 of 0.5 mg/kg was determined based upon data supplied by the project officer, and a dose range of 0.5 to 25 mg/kg was examined. This range included doses that were inactive to those that had biological activity as evidenced by a decrease in response rate to 13% of vehicle control.

3.3. X-ray analysis

Preparation of crystals: Crystals of LE300 can be directly obtained by recrystallising LE300 [2] from anhydrous ethanol.

LE300·HCl is prepared by dissolving 100mg (0.35 mmol) of LE300 in 130 mL of anhydrous diethyl ether and bubbling gasous HCl through this solution. After a short while a turbidity is formed and the reaction is discontinued. Letting this solution stand for a further 40 minutes completes precipitation. The solid is filtered and dried. To obtain crystals, the solid is dissolved in anhydrous ethanol, which is allowed to evaporate slowly at 4°C.

Crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC-199342 (LE300) and CCDC-198889 (LE300·HCl).

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