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Cytotoxicity of β-carbolines in dopamine transporter expressing cells: Structure–activity relationships

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ABSTRACT

Some β-carbolines (BC) are natural constituents in the human brain deriving from tryptophan, tryptamine, and serotonin. In vitro and animal experiments suggest that BC-cations may cause neurodegeneration with a higher vulnerability of dopaminergic than of other neurons. Despite the possible implication of the BC-cations in the pathogenesis of Parkinson's disease (PD), the underlying mechanisms are poorly understood. The present study further explores the structural requirements for the cytotoxic effects of BCs and searches for additional compounds involved in the pathogenesis of PD. Previous studies were now extended to serotonin-derived BCs, tetrahydro-BCs, a BC-dimer, and a BC-enantiomer to reveal possible stereoselectivity.

Neutral, rather lipophilic BCs may pass the plasma membrane and the outer and inner mitochondrial membranes by diffusion whereas the cationic, more polar compounds, can be transported by the dopamine transporter (DAT). In the present study, 4 out of 17 BC-cations caused DAT-independent toxicity. This number is unexpected in view of previous findings that all BC-cations are transported by DAT. 3-Carboxylated and 6-methoxylated BCs were poor substrates. The size alone does not seem to be a limiting factor. A dimeric BC-cation was readily transported by the DAT despite its much larger structure compared to dopamine. Furthermore, (*R*)-enantiomers were preferentially transported. The neutral BCs were approximately one order of magnitude less toxic than the cationic BCs. There are considerable differences of the transport efficiency between the BCs. Potent cytotoxic tetrahydro-BCs were detected. Because precursor tetrahydro-BCs are present in the brain, the search for the occurrence of these compounds in human brain is warranted.

1. Introduction

β-Carbolines (BC) are heterocyclic molecules derived from tryptophan, tryptamine, and serotonin which occur ubiquitous in the environment and in mammals and are natural constituents in humans [1,2]. The unsubstituted, fully aromatic BC (norharman, compound **1** in Table 1), occurring naturally in human and mammalian tissues [3,4], is converted in the brain by certain *S*-adenosyl-methionine-dependent *N*-methyltransferases to the 2-methyl-β-carbolinium ion (**5**) and subsequently to the 2,9-dimethyl-β-carbolinium ion (2,9-dime-BC$^+$, **7**) [5–7]. The latter compound can be superimposed on 1-methyl-4-phenyl-pyridinium ion (MPP$^+$, **10**), which produces a Parkinson-like syndrome in humans. 2,9-Dime-BC$^+$ inhibits complex I of

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the respiratory chain in mitochondria with higher potency than MPP^+ [8]. Norharman (**1**) and harman (**2**) were identified in the human parietal association cortex (0.58 pmol/g tissue and 0.24 pmol/g tissue) and human substantia nigra (SN; 16.00 pmol/ g tissue and 1.04 pmol/g tissue). Compounds **5** and **6** were found in cortex and **5** in SN at somewhat lower concentrations than the precursor compounds. 2,9-Dime-BC^+ (**7**) and 1,2,9-trime-BC^+ (**8**) were detected in the human parietal association cortex (11 of 13 samples, 0.10 pmol/g tissue and 2 of 13 samples, 0.02 pmol/g tissue) and **7** in higher concentrations in SN (4 out of 4 samples, 0.77 pmol/g tissue, **8** in no sample) [6]. The levels of norharman and harman (**1** and **2**) were higher in CSF and plasma of medication-free PD patients than in samples of patients without a neurological disease [9,10]. Some other BCs without N^2-methylation have been detected in human and mammalian tissues. They are not mentioned here because Collins et al. hypothesized that N^2-methylation appears necessary to convert a BC into a relatively potent inhibitor of complex I of the respiratory chain and concomitantly to a neurotoxin [5].

Bilateral injection of 2,9-dime-BC^+ (**7**) into SN of rats induced a Parkinson's disease-like syndrome. The syndrome became conspicuous three weeks after application with enhanced muscle resistance and electromyographic activity recorded from the gastrocnemius and tibialis anterior muscles, a syndrome equivalent to muscle rigidity in humans. In addition, levels of dopamine and its metabolites were decreased in the striatum. Stereological counting revealed that 2,9-dime-BC^+ (**7**) had caused a significant decrease in the total number of tyrosine hydroxylase immunoreactive neurons of SN [11].

In vitro studies demonstrated cytotoxicity of β-carbolinium cations in rat pheochromocytoma (PC12) and mouse neuroblastoma 2A cells as well as primary mesencephalic cultures from mice with partial selectivity for dopaminergic neurons [12–15]. N^2-Methylated BC^+ (**5**) inhibited [^{3}H]dopamine uptake into rat striatal synaptosomes. Accumulation of 2-[^{14}C]methyl-1-methyl-7-MeO-BC^+ into synaptosomes was partially prevented by the dopamine transporter (DAT) inhibitor nomifensine [16,17]. The in vivo and in vitro findings are consistent with the notion that BCs and specifically BC cations play a role in the pathogenesis of Parkinson's disease (PD).

To study the contribution of the DAT for the increased vulnerability of dopaminergic neurons towards the cytotoxic actions of BC cations, Storch et al. [18] used human embryonic kidney cells stably transfected with cDNA of human DAT (HEK_{hDAT}). Comparison of the toxicity of N^2-methylated BC cations in the wild type HEK 293 cells (HEK_{wt}) and the HEK_{hDAT} cells and the uptake kinetics by use of the native fluorescence of the BCs revealed, that all compounds with DAT-dependent toxicity were indeed transported by the DAT. On the other hand, there was no correlation between V_{max}, calculated from the uptake kinetics of the N^2-methylated BC cations and cytotoxicity, and only a weak correlation between K_m and the ratio of the EC_{50} values determined in HEK_{hDAT} and HEK_{wt} cells. Furthermore, there was no correlation between cytotoxicity and lipophilicity [19] nor with the inhibiting potency of the respiratory chain in the mitochondria [8,20–22]. Thus, despite the important role of the BC-cations for the pathogenesis of PD and the efforts undertaken to reveal the structural requirements for cytotoxicity of the BCs in general and to explain the specific vulnerablity of dopaminergic neurons, the underlying mechanisms are poorly defined at this time.

The present study aimed at investigating further the hypothesis of Collins and colleagues that *N*-methylation is required for neurotoxicity [5] by extending their studies to various other BCs. This approach may detect additional neurotoxic BCs. Dependent on their cytotoxic potency and the probability to be formed in vivo, a search for their occurrence in humans might be warranted. Furthermore, the structural requirements for the BCs to by transported by the DAT, a prerequisite for the toxicity specifically in dopaminergic neurons, will be explored. Previous studies are now extended to the oxygenated serotonin-derived BCs, to tetrahydro-BCs, a BC-dimer, a tetrahydro-BC enantiomer to reveal possible stereoselectivity, and to other structurally related compounds like *N*-methyl-carbazole and 1-methyl-4-phenylpyridinium (MPP^+).

2. Materials and methods

2.1. Cell culture

Human embryonic kidney (HEK-293) cells were obtained from American Tissue Type Culture Collection (ATCC, reference number CLR 1573, Rockville, USA) and were grown in minimal essential medium with Earl's salt (MEM-Earle) supplemented with 2.2 g/l $NaHCO_3$, 0.518 g/l stable glutamine, 10% (v/v) fetal bovine serum (FBS) and penicillin (100 I.E./ml)/streptomycin (100 μg/ml, all from Biochrom Seromed, Berlin, Germany). Cells were maintained at 37 °C in a saturated humidity atmosphere including 5% CO_2. To assess whether HEK_{wt} cells are lacking DAT, we conducted [^{3}H]dopamine uptake kinetic experiments. There was no difference in [^{3}H]dopamine accumulation between HEK_{wt} cells incubated at 37 and 0 °C and GBR 12,909 (10 μM, 37 °C), respectively.

HEK_{hDAT} cells were prepared and characterized as previously described [18,23]. Cell culture conditions were the same as described above, except for adding 400 μg/ml geneticin (G418, Calbiochem, Schwalbach, Germany) to the medium.

2.2. Toxicity assay

Cells were seeded at a density of 3×10^4 cells/100 μl and well in 96 well plates and grown for three days under appropriate conditions. Then, the medium was replaced by a medium containing 0, 5, 10, 50, 100, or 500 μM of the BC of interest. Results were obtained for each concentration from 16 wells. In addition, eight wells were included in each run containing 500 μM MPP^+ as a positive control. The values are the mean ± S.E.M. of 4–7 independent experiments. In cases the calculations could not be approximated to a sigmoidal function, the concentrations were increased up to 1500 μM. After 48 h of incubation, cells were washed twice with 150 μl medium per well and cell viability was determined using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay by Promega (Madison, USA). The test is composed of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and phenazine ethosulfate. The abbreviation of the test is MTS test.

2.3. β-Carboline uptake assay

The uptake of β-carbolines through the hDAT was determined using the strong autofluorescence of these compounds with minor modifications of the protocol described previously [18]. The excitation and emission wavelengths for each substance were determined by analyzing the emission spectrum for each derivative at different excitations. HEK_{hDAT} and HEK_{wt} cells were seeded into 96-well plates at a density of 2×10^5 cells/well and grown in 100 μl medium for 48 h. The medium was aspirated and cells were washed with 100 μl uptake buffer [18,23]. The samples were incubated with 100 μl uptake buffer containing different concentrations of BCs (0–500 μM) for 60 min at 37 °C. The incubation time of 60 min was used as time course experiments, performed in advance, resulted in an equilibrium concentration which was reached at about 40–50 min by all concentrations and BCs investigated. Uptake was stopped by sucking off the buffer, followed by two washes with 100 μl of ice-cold uptake buffer. Non-specific uptake was determined in parallel assays performed on ice or in the presence of 10 μM GBR 12,909 (Sigma–Aldrich, Taufkirchen, Germany). The BCs were extracted by incubating the cells with 100 μl lysis solution (0.1 M HCl, 2 mM EDTA) for 1 h on a plate shaker followed by three freeze-thaw cycles. The amount of transported compounds was determined by measuring the autofluorescence in a plate reader (Spectra Max Gemini, spectrofluorimeter, Molecular Devices, Sunnywale, CA, USA) at the appropriate excitation and emission wavelengths. The amount of the BC taken up was calculated using external standards of known concentrations of the respective BC in lysis buffer. Values were related to the amount of protein determined for each well using the bicinchoninic acid (BCA) protein assay reagent A (Pierce, Thermo Fisher Scientific, Waltham, USA) and copper(II) sulfate solution, reagent B (Sigma–Aldrich, Taufkirchen, Germany). The presented values were obtained from four to six independent experiments.

2.4. Materials

MPP^+ (**10**), GBR 12,909, the BCs **1**, **2**, and *N*-methyl-carbazole were purchased from Sigma–Aldrich, Taufkirchen, Germany. [^{3}H]dopamine was from Amersham Biosciences, Freiburg, Germany. The compounds **3**–**32** were synthesized by Yvonne Schott and Christoph Enzensperger [24]. The synthetic work included the isolation of the pure enantiomer **14** from the racemate by preparative column chromatography using Chiracel® OD column, 5 × 50, with isopropanol/*n*-hexane (10:90) as eluent. Further detailed informations on the syntheses are given in the supporting information.

Those BCs which were not readily soluble in cell culture medium without fetal calf serum (FCS), were dissolved at 37 °C by sonication and subsequently diluted in cell culture medium. In case this procedure was not sufficient, the BCs were dissolved in dimethylsulfoxide (DMSO) and further diluted by culture medium so that the final concentration of DMSO was less than 1%. Control experiments revealed that the trace of DMSO in the cell culture medium did not affect viability of HEK_{wt} and HEK_{hDAT} cells.

2.5. Statistics

V_{max}, K_m, and EC_{50} were calculated by nonlinear regression using the GraphPadPrism program (GraphPad Software, San Diego, CA, USA). Results were considered significant for *P*-values <0.05.

3. Results

3.1. Effect of BCs and MPP^+ on cell viability using HEK_{wt} and HEK_{hDAT} cells

3.1.1. BCs with an unsubstituted benzene ring (aromatic tryptamine derivatives) and MPP^+

As depicted in Fig. 1, HEK_{wt} and HEK_{hDAT} cells were incubated with increasing concentrations of test substances. Due to limited space, the curves of 1-methyl-BCs (harman derivatives) were omitted from Fig. 1. Those curves are presented as Supplementary data. There were distinct differences of the cytotoxic potency between the BCs (Table 1). With respect to the non-cationic methylated BCs **2**–**4**, the cells stably transfected with hDAT were slightly less sensitive than the wild type cells. This might indicate that the membrane-inserted DAT protein interfers with the diffusion of the methylated BCs but not with the unmethylated BC **1**. The shape of the dose–response curves of the non-cationic substances was similar in both cell lines (Fig. 1). All of the aromatic BCium salts yielded by methylation in position 2 (**5**–**8**) were more toxic in HEK_{hDAT} cells than in the wild type cells. Methylation in both positions 2 and 9 increased toxicity but not selectivity for a cell type compared to the N^2-monomethylated BC^+s ($P = 0.08$ in the case of norharmanium (**5** versus **7**) and $P = 0.0093$ in the case of harmanium (**6** versus **8**), calculated for HEK_{wt}, cells; the toxicity was not statistically different in HEK_{hDAT} cells). Furthermore, a significant difference was calculated between the two cell lines (compounds **5**–**8**; Table 1).

We further investigated a molecule composed of two norharmanium molecules linked by a butan bridge bound to the respective pyridine nitrogens (**9**). We found a distinct DAT-dependent cytotoxicity ($P < 0.0001$) which was significantly stronger than that of norharman in HEK_{hDAT} cells ($P = 0.0054$). The highest DAT-dependent toxicity was observed with 1-methyl-4-phenyl-pyridinium iodide (MPP^+). This well characterized compound was investigated for comparison and served as a positive control.

9-Me-carbazole, which differs from norharman by the lack of the pyridine nitrogen was less toxic than the BC (EC_{50} in HEK_{wt} 734 ± 20.6 μM and in HEK_{hDAT} cells >1000 μM).

3.1.2. Tetrahydro-BCs: tryptophan and tryptamine derivatives

Both the noncationic S-3-carboxy-THBC (**16**) and the cationic S-2,2-dime-3-carboxy-$THBC^+$ (**17**) were not toxic in both cell populations up to a concentration of 0.5 mM. This suggests that they are not transported by the DAT. Furthermore, it seems that the carboxy-substituent hampers any diffusion because even the highest concentration (0.5 mM) of these tryptophan derivatives did not cause even a slight reduction of

cell viability in contrast to most of the BCs. Toxicity and transport by DAT was observed for the $THBC^+$ derived from tryptamine, interestingly in an enantioselective way (compare racemic 1,2,2-trimethyl-$THBC^+$ (**13**) and its *R*-(+)-enantiomer **14** (Table 1 and Fig. 1). The racemic tetrahydroharmanium (**13**) was moderately toxic but did differ significantly with regard to the type of cells. The pure *R*-(+)-enantiomer (**14**) was approximately twice as toxic as the racemate in both cell lines. It was more toxic in HEK_{hDAT} than in HEK_{wt} cells ($P < 0.0001$). This suggests that the *S*-(−)-enantiomer is relatively non-toxic. Furthermore, the finding demonstrates for the first time a stereoisomeric effect of BCs. This seems to

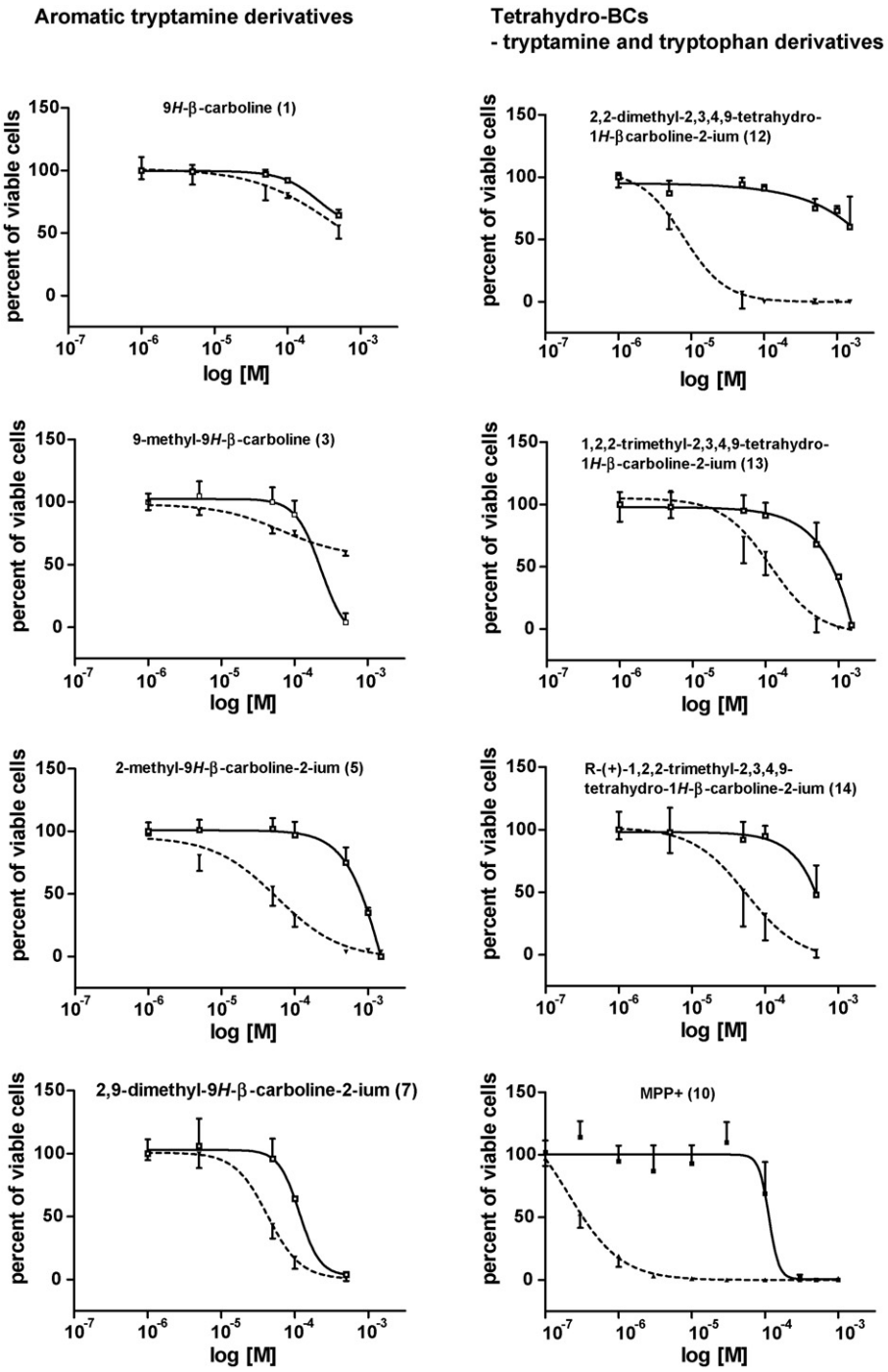


Fig. 1 – Dose effect relationship of increasing concentrations of BCs (range: 0–500 μM, in some cases up to 1500 μM) on cell viability using the MTS-assay. HEK_{hDAT} (closed triangles, dotted lines) and HEK_{wt} (open squares, solid lines) cells were exposed to the BCs for 48 h. Then, the percentage of vital cells was determined. The first column comprises aromatic tryptamine derivatives, the second column tetrahydro-BCs derived from tryptamine and tryptophan and MPP+, column 3 are serotonin derivatives, and column 4 are methoxy-BCs. The values are the mean and SEM from four to six independent experiments. For reasons of limited space, graphs of 1-methyl-BC derivatives are presented as Supplementary data.

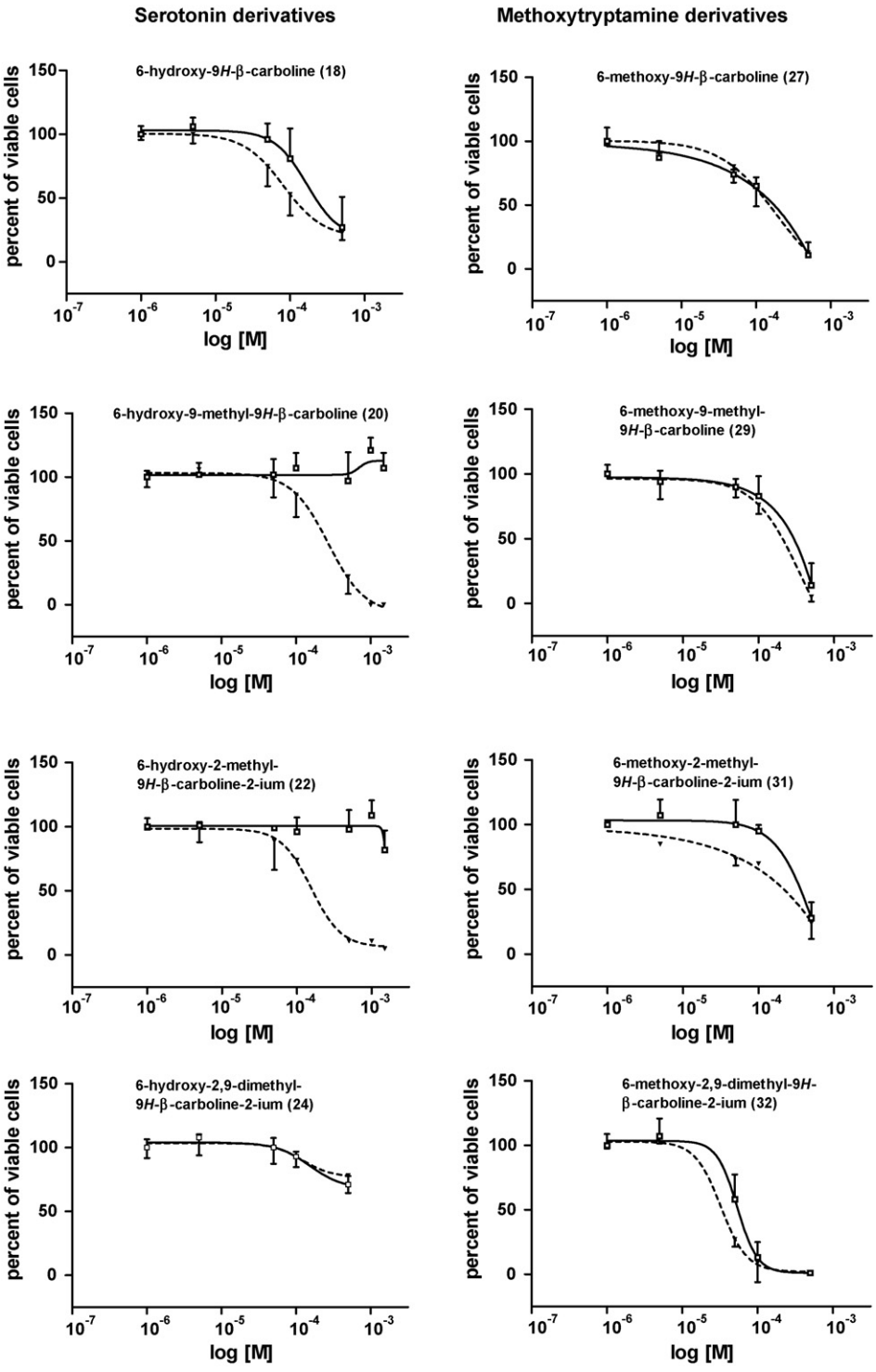


Fig. 1. (Continued).

be remarkable not only for the DAT-mediated transport but possibly also for the toxic effect which is presumably mediated by inhibition of complex 1 of the respiratory chain in the mitochondria (see Section 1).

As observed with the aromatic tryptamine derivatives, a cationic property represents the prerequisite for a DAT-dependent transport of BCs (compare **11** and **12–15**).

3.1.3. BCs with a hydroxy group in position 6 (serotonin derivatives) and harmolium

Generally, the 6-hydroxylation increases the DAT dependent toxicity of the noncationic BCs (compare **18–21** to **1–4**). The formation of inner salts by transprotonation from the acidic phenolic hydroxy group to the basic nitrogen may contribute to this property as depicted in Fig. 2 because the cationic

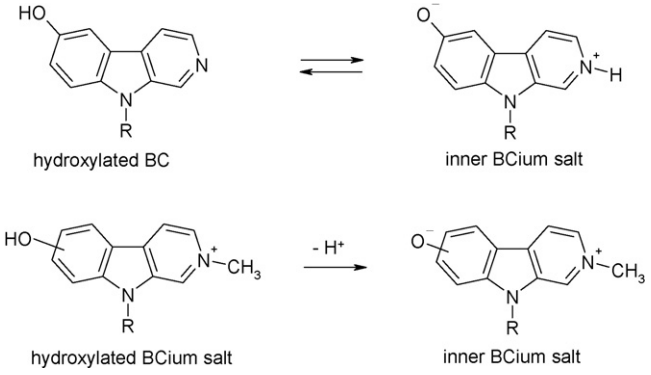


Fig. 2 – Possible formation of inner salts.

nitrogen presumably facilitates the accumulation in the matrix of mitochondria, i.e. in the proximity to the respiratory chain. The hydroxylated BC-cations were more toxic in HEK_{hDAT} than in HEK_{wt} cells. This is consistent with the notion that the property of a permanent cation of a BC represents the prerequisite for a substrate of DAT. However, the number of substituents seems to affect the transport which is concluded from the poor toxicity of compound **24**.

On the other hand, a selective toxicity was found for compound **22**, but less compared to the non-hydroxylated analogue **5**. 2-Methyl-harmolium (**26**) displayed low but selective toxicity. A complete loss of toxicity by hydroxylation was found for compound **24** (compare to **7**). The formation of polar inner salts has to be taken into consideration as outlined in Fig. 2.

3.1.4. *BCs with a methoxy group in position 6 (aromatic 6-methoxy-tryptamine derivatives)*

The experiments with the methoxy-substituted BCs demonstrated that these derivatives generally migrate into the cells and display toxicity. The cytotoxic potency of the 6-methoxylated BCs was stronger than of the respective unsubstituted BCs (compare **27–32** with **1–5** and **7**). In contrast to the BCs with a hydroxyl substituent, the methoxylated BCs are unable to form inner salts (Fig. 2), accordingly display higher lipophilicity compared to the respective serotonin derivatives and penetrate readily the plasma- and the mitochondrial membranes. This is concluded from the higher cytotoxic potency of the 2-methylated BCs (compare **22**, **24** with **31**, **32**).

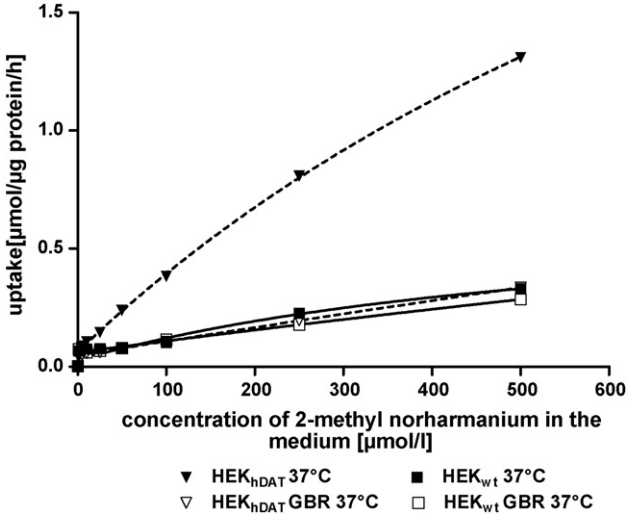


Fig. 3 – Uptake of 2-me-BC^+ in HEK_{hDAT} cells under equilibrium conditions (60 min incubation period, 37 °C, concentration range: 0–500 μM). The transport was inhibited by the selective DAT inhibitor GBR 12,909 (10 μM). No specific uptake was observed in HEK_{wt} cells.

3.2. *Uptake of selected BCs into HEK_{wt} and HEK_{hDAT} cells*

The eight aromatic tryptamine derivatives among the BCs were included in this part of the study (**1–8**, Table 1). Experiments investigating the time course of the accumulation in the cell fraction revealed an equilibrium after approximately 40 min. Therefore, we chose an uptake period of 60 min because we wanted to correlate the findings of the uptake experiments with the cytotoxic potency of the BCs as determined in the cell viability assay. Under the conditions of the cell viability assay, the BCs are in an equilibrium likewise. All BCs accumulated in both cell types in a concentration-dependent manner. The uptake in HEK_{hDAT} cells was inhibited by the selective DAT inhibitor GBR 12,909 (10 μM). The transport was temperature-dependent (Fig. 3; BC^+ **5** is presented as an example).

We calculated the concentration of the BCs in the uptake experiments at 37 °C which corresponded to the EC50-value in the cytotoxicity assay for both cell lines. The calculated values are added to the respective BC and cell line as italic numbers in Table 1. It is noteworthy that the concentrations of the non-ionic BCs are in the same order of magnitude. This applies to the cationic BC as well. However, the concentration of the cationic BCs is approximately one order of magnitude lower. This means, that the cationic BCs are more toxic than the non-cationic, neutral BCs and that the difference amounts to about an order of magnitude.

On the other hand, it should be taken into account that the amount of transported BC cations differs considerably and that the easiness of the diffusion of neutral BCs differs as well. This is demonstrated in Fig. 4 which shows the correlation of the EC_{50}-values and the intracellular concentration of the BCs at a fixed medium concentration of the respective BC of 500 μM. The correlation coefficient was $r = -0.8085$ for the neutral BCs ($P = 0.015$, $r^2 = 0.654$) and $r = -0.8152$ for the cationic BCs ($P = 0.048$, $r^2 = 0.665$, compound **5** excluded). It is interesting to note, that 2-me-norharmanium (**5**) takes a position between the cationic BCs (left group in Fig. 3) and the neutral BCs (right group in Fig. 3). This is in contrast to the position of 2-me-harmanium (**6**) which is close to the other cationic BCs. The explanation could be the anhydrobase properties of **5** and **6** (Fig. 5).

4. Discussion

4.1. *BCs are cytotoxic compounds possibly contributing to the neurodegeneration in Parkinson's disease*

The *N*-methylpyridinium compound MPP^+ (**10**) causes a Parkinson-like syndrome in humans [25,26]. β-Carbolinium

Table 1 – β-Carbolines–cell viability measured by the MTS-assay, EC_{50} values

Compounds	HEK_{wt} mean [μM] ± S.D.	HEK_{hDAT} mean [μM] ± S.D.	P-values
Aromatic tryptamine derivatives and MPP^+			
9*H*-β-carboline (norharman, **1**)	717 ± 103; 2.4×10^{-6}	578 ± 171; 1.9×10^{-6}	n.s.
1-Methyl-9*H*-β-carboline (harman, **2**)	169 ± 78; 2.4×10^{-6}	215 ± 90; 1.8×10^{-6}	n.s.
9-Methyl-9*H*-β-carboline (**3**)	396 ± 8.3; 9.6×10^{-7}	764 ± 73; 1.6×10^{-6}	0.001
1,9-Dimethyl-9*H*-β-carboline (**4**)	147 ± 46; 2.7×10^{-6}	227 ± 80; 2.7×10^{-6}	n.s.
2-Methyl-9*H*-β-carboline-2-ium iodide (**5**)	710 ± 78; 4.4×10^{-7}	78 ± 26; 2.4×10^{-7}	0.002
1,2-Dimethyl-9*H*-β-carboline-2-ium iodide (**6**)	445 ± 98; 1.4×10^{-7}	85 ± 50; 8.4×10^{-8}	0.005
2,9-Dimethyl-9*H*-β-carboline-2-ium iodide (**7**)	116 ± 32; 9.0×10^{-8}	43 ± 11; 8.0×10^{-8}	0.02
1,2,9-Trimethyl-9*H*-β-carboline-2-ium iodide (**8**)	168 ± 24; 1.0×10^{-7}	48 ± 17; 7.9×10^{-8}	0.002
2-[4-(9*H*-β-carboline-2-ium-2-yl)butyl]-9*H*-β-carboline-2-ium dibromide (**9**)	1380 ± 150	15 ± 3.1	<0.0001
1-Methyl-4-phenyl-pyridinium iodide (MPP^+, **10**)	113 ± 10	0.338 ± 0.05	<0.0001
Tetrahydro-BCs-tryptamine and tryptophan derivatives			
1-Methyl-2,3,4,9-tetrahydro-1*H*-β-carboline (*rac.*) (**11**)	470 ± 113	500 ± 131	n.s.
2,2-Dimethyl-2,3,4,9-tetra-hydro-1*H*-β-carboline-2-ium iodide (**12**)	1285 ± 234	7.8 ± 5.0	0.0007
1,2,2-Trimethyl-2,3,4,9-tetra-hydro-1*H*-β-carboline-2-ium iodide (*rac.*) (**13**)	894 ± 152	118 ± 63	0.0012
R-(+)-1,2,2-Trimethyl-2,3,4,9-tetrahydro-1*H*-β-carboline-2-ium iodide (**14**)	499 ± 142	52 ± 29	<0.0001

Table 1 (*Continued*)

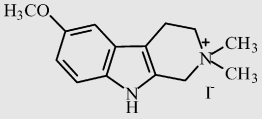
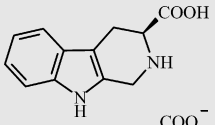
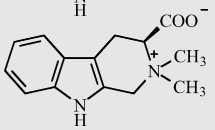
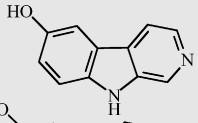
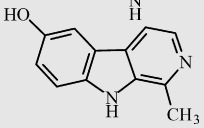
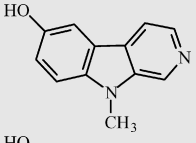
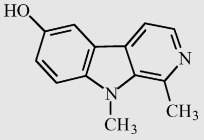
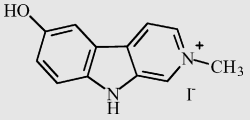
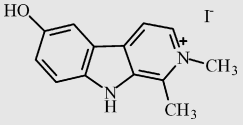
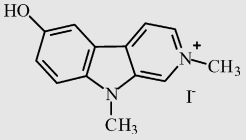
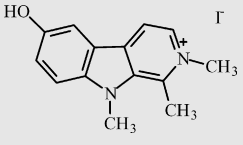
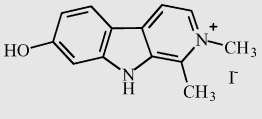
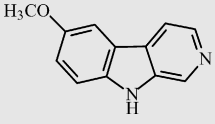
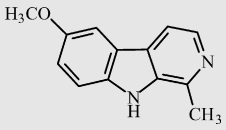
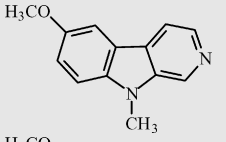
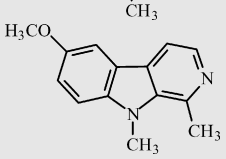
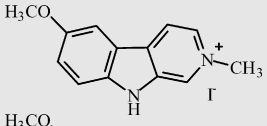
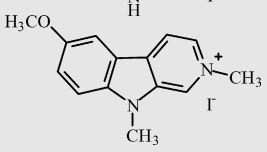
Compounds		HEK_{wt} mean [μM] ± S.D.	HEK_{hDAT} mean [μM] ± S.D.	*P*-values
6-Methoxy-2,2-dimethyl-2,3,4,9-tetrahydro-1*H*-β-carboline-2-ium iodide (**15**)		720 ± 236	44 ± 2.2	0.0077
S-2,3,4,9-Tetrahydro-1*H*-β-carboline-3-carboxylic acid (**16**)		>1000	>1000	n.d.
S-2,2-Dimethyl-2,3,4,9-tetrahydro-1*H*-β-carboline-2-ium-3-carboxylate (**17**)		>1000	>1000	n.d.
Serotonin derivatives and harmolium				
6-Hydroxy-9*H*-β-carboline (**18**)		168 ± 65	81 ± 54	n.s.
6-Hydroxy-1-methyl-9*H*-β-carboline (**19**)		53 ± 20	139 ± 45	0.039
6-Hydroxy-9-methyl-9*H*-β-carboline (**20**)		1460 ± 105	215 ± 81	<0.0001
6-Hydroxy-1,9-dimethyl-9*H*-β-carboline (**21**)		130 ± 47	97 ± 27	n.s.
6-Hydroxy-2-methyl-9*H*-β-carboline-2-ium iodide (**22**)		1315 ± 262	186 ± 90	0.002
6-Hydroxy-1,2-dimethyl-9*H*-β-carboline-2-ium iodide (**23**)		535 ± 125	73 ± 57	0.004
6-Hydroxy-2,9-dimethyl-9*H*-β-carboline-2-ium iodide (**24**)		>1000	>1000	n.d.
6-Hydroxy-1,2,9-trimethyl-9*H*-β-carboline-2-ium iodide (**25**)		1222 ± 105	150 ± 47	<0.0001
7-Hydroxy-1,2-dimethyl-9*H*-β-carboline-2-ium iodide (**26**) (2-methyl-harmolium)		450 ± 45	200 ± 29	0.0013
Methoxytryptamine derivatives				
6-Methoxy-9*H*-β-carboline (**27**)		215 ± 50	213 ± 10	n.s.

Table 1 (*Continued*)

Compounds		HEK_{wt} mean [μM] ± S.D.	HEK_{hDAT} mean [μM] ± S.D.	P-values
6-Methoxy-1-methyl-9*H*-β-carboline (**28**)		93 ± 20	118 ± 56	n.s.
6-Methoxy-9-methyl-9*H*-β-carboline (**29**)		292 ± 23	247 ± 18	n.s.
6-Methoxy-1,9-dimethyl-9*H*-β-carboline (**30**)		88 ± 50	110 ± 10	n.s.
6-Methoxy-2-methyl-9*H*-β-carboline-2-ium iodide (**31**)		380 ± 25	283 ± 41	n.s.
6-Methoxy-2,9-dimethyl-9*H*-β-carboline-2-ium iodide (**32**)		53 ± 16	32 ± 2.7	n.s.

Numbers in italic of BCs **1–8** are the concentration (mol/μg protein) in the cells at the respective EC_{50}-value determined by measuring the native fluorescence. n.s.: not significant; n.d.: not determined because the value is >1000 μM.

compounds such as **5** can be considered as MPP^+ congeners which are bridged and thereby rigidized by the indole nitrogen. In this context, BCs are of further interest because contrary to MPP^+ some of them occur naturally in human brain and are considered endogenous neurotoxins (see Section 1). The concentrations of natural BCs determined in SN from brains of subjects without obvious neurodegeneration are low [6] compared to the EC_{50}-values of BCs determined in this study. Even considering that the levels of BCs in the SN of patients with PD may be higher as concluded from the findings in CSF samples of unmedicated PD patients compared to those of healthy subjects [10] and unknown effects of the postmortem period on BC-concentrations in situ, the difference remains considerable. Furthermore, the EC_{50}-value of MPP^+ and those of the most potent BCs (**12** and **9**) differ by more than an order of magnitude. However, the cells applied in this study have dopamine uptake kinetics similar to those reported for other ectopic expression systems, but differ from those of rat synaptosomes by a 5–10 times higher K_m-value [18,27]. Thus, the cell model is specific but not as sensitive as the natural DAT. The in vivo levels of the BCs are far below those required to destroy a portion of dopaminergic neurons in acute experiments (approximately 15 nmol per SN of rats; [11]). On the other hand, there is general agreement that the demise of dopaminergic neurons in PD is due to a combination of exogenous and endogenous stressors and a genetic disposition that renders cells less capable of dealing with the stress [28]. Therefore, the concentration of the endogenous stressors **5** and **7**, which have been found in human SN may very well sufficient to contribute to the degeneration of dopaminergic neurons during chronic exposure in predisposed subjects. Since neurotoxic compounds **6** and **8** occur in cerebral cortex, they may be present in SN likewise but at a concentration below the detection limit of the analytical method. Among the compounds investigated in this study, several are as potent cytotoxins as the aforementioned BCs.

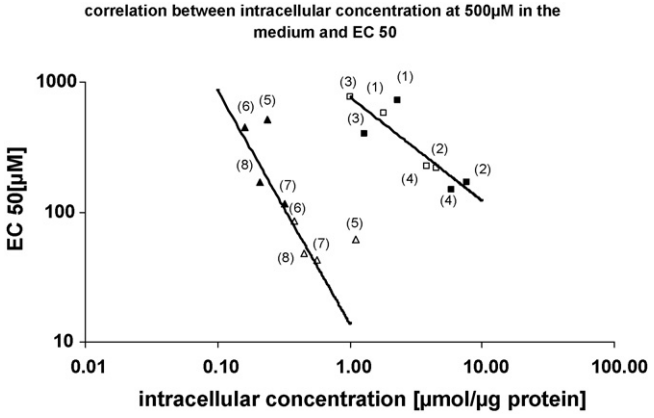


Fig. 4 – Triangles represent cationic BCs, squares neutral BCs. Filled symbols represent HEK_{hDAT} cells, open symbols HEK_{wt} cells. The numbers indicate the respective BC as listed in Table 1. Neutral BCs (1–4): Pearson's correlation coefficient $r = -0.8085$, $P = 0.015$, $R^2 = 0.654$. Cationic BCs (6–8) $r = -0.815$, $P = 0.048$, $R^2 = 0.665$. BC 5 (2-me-BC^+) is not included in the calculations because of the anhydrobase property of this compound (see discussion for details and figure). The values are the means from four to six independent experiments.

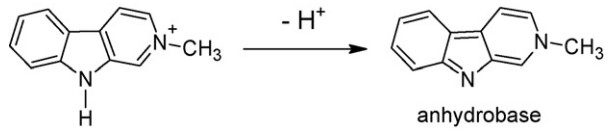


Fig. 5 – Deprotonation of cationic 2-methyl-β-carbolines to neutral anhydrobases.

Those are the tetrahydro-BCs **12**, **14**, **15**, the methoxy-BC **32**, and the bivalent norharman **9**. The first group of BCs were never investigated before despite the fact that unsubstituted tetrahydro-BCs occur in humans [1,38,39]. Thus, those compounds might occur in vivo and could contribute to the neurotoxic effects in PD.

4.2. *The significance of the DAT for the cytotoxicity of BCs*

Several reports have been published dealing with the neurotoxicity of BCs with the focus on dopaminergic neurons [5,14,17,18]. The findings did not result in a consistent model which might be important for the conception that BCs are involved in the degeneration of dopaminergic neurons in PD. The reasons for obvious inconsistencies are presumably the many factors which are involved in the toxic actions of the BCs. A compound has to overcome several barriers between addition to the experimental set up and reaching the respiratory chain where it causes the primary toxic effects. Neutral and rather lipophilic BCs may pass the plasma membrane and the outer and inner mitochondrial membranes by diffusion whereas the cationic and more polar compounds are transported by the DAT and are driven by the concentration- and electrogradient to pass the outer and inner mitochondrial membranes, respectively. The extent of uptake into neuronal terminals may be the limiting factor for BC-cations by analogy to MPP^+ (**10**). In the present study, 4 of the 17 BC-cations investigated caused DAT-independent toxicity. The number of inactive cations is unexpected in view of the findings of previous reports that all BC^+cations are transported by DAT and are cytotoxic [5,17,18]. The tetrahydro-BC^+ **17**, to some extend the racemic **13**, and the fully aromatic 6-oxygenated BC^+ salts **24**, **31**, **32** did not display significant DAT-dependent toxicity. The properties of **16** and **17** indicate that amino acid derivatives, which are physicochemically very different from the other compounds, are not suited substrates for the dopamine transporter (compare **16**, **17** to **12**). Furthermore, these results and the other data from Table 1 show that methoxylation and hydroxylation in general decrease the ability of the BC^+ compounds of being transported by DAT. On the other hand, the two 6-methoxy-BC cations **31** and **32** pass the membranes easily by diffusion as both cell lines were affected about equally. The bivalent BC^+ **9**, being a much larger molecule than dopamine and the other BCs is even better transported by DAT than the monovalent BC^+ **5** and is concentrated in mitochondria which is concluded from its strong cytotoxicity in HEK_{hDAT} cells. It is noteworthy, that the toxic potency of the bivalent BC^+ is the second strongest among the 31 BCs investigated. Obviously the bis-BC^+-butan **9** turns into a conformation which generates additional binding options at the transporter protein. Similar effects have been observed for other bivalent ligands as well [29].

It is noteworthy that all 14 neutral BCs and also the 2-desaza analogue 9-me-carbazole (not shown in Table 1) caused DAT-independent cytotoxicity, though most of them at relatively high concentrations. Others have reported that the neutral BCs are much better inhibitors of the electron transport in isolated mitochondrial membranes than the corresponding cationic partners but their inhibitory activity on intact mitochondria are not enhanced because they are not actively concentrated [20].

4.2.1. *Stereoselectivity of the transport of BCs by DAT*

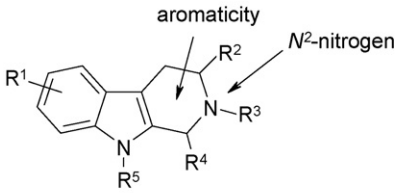
DAT-dependent toxicity of the 1,2,2-trime-$THBC^+$ dervative is much more significant for the pure *R*(+)-enantiomer **14** than for the racemate **13**. This indicates that the affinity of the transporter towards the ligands is enantioselective. In the case of **13** the *R*- is clearly favoured compared to the *S*-enantiomer. Stereoisomeric properties of the DAT have been reported previously. This function is localized in the transmembrane domain 9 through the carboxy-terminal tail [30,31].

4.3. *The role of the mitochondria for the cytotoxicity of BCs*

Once the BCs have passed the plasma membrane, the BC^+s are concentrated inside the mitochondria driven by the transmembrane electrochemical gradient (negative inside, positive outside) against their concentration gradient in analogy to MPP^+ [32]. Using several probes, up to 1000-fold accumulation of organic cations has been observed in mitochondria [20]. The accumulation within the mitochondrial matrix represents a crucial factor. 4-Phenylpyridine, the neutral unmethylated form of MPP^+, is a much more potent complex I inhibitor on the isolated respiratory chain than MPP^+, but is not neurotoxic, because it is neither a substrate of DAT nor is it concentrated inside the mitochondria [33]. The data on mitochondrial uptake suggest that the structural requirements for the accumulation of *N*-methylpyridinium cations are not very stringent [20]. This should be valid by analogy for BCs^+ as well. Indeed, we found by correlating the EC_{50}-values with the respective concentration in uptake experiments that the level of BC required to exert reduction by 50% of viable cells is approximately one order of magnitude lower for cationic BCs than for neutral BCs. The differences within the noncationic and cationic BCs, respectively, are negligible (Fig. 4).

The 2-methyl-harmolium cation inhibited NAD^+-linked respiration in rat liver mitochondria almost as potently as MPP^+ (IC_{50}: 208 μM versus 171 μM [20]). However, we did observe much weaker cytotoxic effects compared to MPP^+ suggesting that the 7-OH-substitution impairs membrane transport. On the other hand, 2-me-harminium, bearing a methoxy-substituent in 7 position is cytotoxic [18]. This is consistent with the present study which demonstrated strong cytotoxicity of methoxy-substituted BC-cations.

The $THBC^+$ did not differ much from the fully aromatic analogues regarding cytotoxicity. Injection of the *N*-monomethylated THBC into mice and monkeys was toxic without a decrease in dopamine levels in the striatum. This suggested to the authors that this MPTP analogue is unlikly to cause



R^1: *H < OH < OCH_3;* R^2: *H << COO^-;* R^3: *should produce a cationic N^2, in quaternary THBCs the R-enantiomer is favoured;* R^4: *no significance in aromatic compounds;* R^5: *CH_3 ~ H;* N^2: *essential (compare to 9-me-carbazole)*; aromaticity: *no obligatory feature.*

Fig. 6 – Synopsis of the structure–activity relationships with regard to the transportation of BCs by the high affinity dopamine uptake.

idiopathic PD [35,36]. These findings are important because they suggest that the *N*-methylated THBC is neither further *N*-methylated nor oxidized in vivo. Both reactions would yield a BC-cation. The latter point is explained by the report that N^2-me-THBC is no substrate of monoamine oxidase [37]. Furthermore, the THBCs are specifically cytotoxic only as cations and are taken up by the DAT as demonstrated in this study.

4.3.1. *Formation of neutral anhydrobases*

An additional issue in the structure–activity relationship (SAR) discussion of BCs^+ is the option of 2-me-BC^+ form neutral anhydrobases after dissociation of the proton in 9-position as outlined in Fig. 5 for compound **5**. As a consequence of the equilibrium of the charged carbolinium and its neutral anhydrobase, the BC is concentrated to a lesser extent in mitochondria as expected. Differences in acidity of **5** and **6** may be responsible for the differences with regard to cytotoxicity (see Fig. 4). Norharman in the harman derivative **6** is sterically shielded by the methyl in position 1. Calculations of pK_a values (Advanced Chemistry Development Inc., Version 5.12) yielded a higher acidity 12.87 ± 0.4 for **5** compared to 13.30 ± 0.4 for **6**. Accordingly, the norharman derivative **5** looses its cationic properties more easily than **6** and therefore resembles the non-cationic BCs **1–4** much more than **6**. This can be consistently found, e.g. compare toxicity of 2-me-BC^+ (**5**) with 2,9-dime-BC^+ (**7**) or 1,2-dime-BC^+ (**6**) and 1,2,9-trime-BC^+ (**8**). In all examples, the 2-methylated BCs are less cytotoxic than the 2,9-dimethylated cations which bear no hydrogen in 9-position, though the difference does not reach significance for the HEK_{hDAT} cells. Spectroscopic studies with 2-me-norharmanium (**5**) and 2-me-harmanium (**6**) indicated some formation of the respective anhydrobase at neutral pH [34]. The deprotonated forms would rapidly partition across the mitochondrial membrane. Reprotonation would then occur within the mitochondrial matrix.

4.4. *Conclusion*

Comparing the cytotoxicity of the groups of BCs, it is striking, that the neutral serotonin derivatives are generaly slightly more toxic than the respective tryptamine derivatives. Whether this depends on the easiness of diffusion or on the potency of inhibition of complex I remains to be elucidated. In general it seems that every part of the molecule that disturbs the planarity of the indole part of the BC structure, reduces the ability of being transported by the DAT. For the fully aromatic compounds with a methoxy group, **31** and **32**, which are unexpectedly DAT inactive, the methyl group attached to the oxygen is able to rotate free out of the planarity. With respect to the 2,2-dimethylated THBC derivatives **12** and **15**, the compound with a methoxy group (**15**) is taken up 10 times less readily than the unsubstituted counterpart **12**. The substituents attached to the BC molecule and the stereoisomerism affect the transport by the DAT (Fig. 6). The size of the molecule is of minor importance. With respect to the inhibition of the respiratory chain, the primary mechanism of toxicity of the BCs, the cationic property of the molecule is more important than structural features.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.06.046.

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