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# Selective dopaminergic neurotoxicity of isoquinoline derivatives related to Parkinson's disease: studies using heterologous expression systems of the dopamine transporter

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**Abstract**

Endogenous isoquinoline (IQ) derivatives structurally related to the selective dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP) and its active metabolite 1-methyl-4-phenylpyridine ($MPP^+$) may contribute to dopaminergic neurodegeneration in Parkinson's disease. We addressed the importance of the DAT molecule for selective dopaminergic toxicity by testing the differential cytotoxicity of 22 neutral and quaternary compounds from three classes of isoquinoline derivatives (3, IQs; 4, 3, 4-dihydroisoquinolines and 15, 1,2,3,4-tetrahydroisoquinolines) as well as $MPP^+$ in non-neuronal and neuronal heterologous expression systems of the DAT gene (human embryonic kidney HEK-293 and mouse neuroblastoma Neuro-2A cells, respectively). Cell death was estimated using the MTT assay and the Trypan blue exclusion method. Nine isoquinolines and $MPP^+$ showed general cytotoxicity in both parental cell lines after 72 hr with half-maximal toxic concentrations ($TC_{50}$ values) in the micromolar range. The rank order of toxic potency was: papaverine > salsolinol = tetrahydropapaveroline = 1-benzyl-TIQ = norsalsolinol > tetrahydropapaverine > 2[*N*]-methyl-salsolinol > 2[*N*]-methyl-norsalsolinol > 2[*N*]-Me-$IQ^+$ = $MPP^+$. Besides $MPP^+$, only the 2[*N*]-methylated compounds 2[*N*]-methyl-$IQ^+$, 2[*N*]-methyl-norsalsolinol and 2[*N*]-methyl-salsolinol showed enhanced cytotoxicity in both DAT expressing cell lines with 2- to 14-fold reduction of $TC_{50}$ values compared to parental cell lines. The rank order of selectivity in both cell systems was: $MPP^+ \gg$ 2[*N*]-Me-$IQ^+$ > 2[*N*]-methyl-norsalsolinol = 2[*N*]-methyl-salsolinol. Our results suggest that 2[*N*]-methylated isoquinoline derivatives structurally related to MPTP/$MPP^+$ are selectively toxic to dopaminergic cells *via* uptake by the DAT, and therefore may play a role in the pathogenesis of Parkinson's disease. 

*Keywords:* Dopamine transporter; Isoquinoline derivatives; Reticuline; 1-Methyl-4-pyridinium ($MPP^+$); Neurotoxin; Parkinson's disease

## 1. Introduction

Despite recent advances in identifying genetic causes of familial Parkinson's disease (PD) (reviewed in [7]), the etiology of the sporadic disorder remains obscure. Endogenous or exogenous toxins related to the selective dopaminergic neurotoxin MPTP and its active metabolite $MPP^+$ are potential etiologic factors.

Isoquinoline derivatives refer to IQ itself, various reduced species, like 3,4-dihydroisoquinoline and 1,2,3, 4-tetrahydroisoquinoline (TIQ), and their substituted congeners (e.g. 1-benzyl-TIQ). They are widely distributed in

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*Abbreviations:* CSF, cerebrospinal fluid; DAT, dopamine transporter; HEK-293, human embryonic kidney 293 cells; HEK-hDAT/4, dopamine transporter transfected HEK-293 cells; IQ, isoquinoline; $MPP^+$, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-2,3,6-tetrahydropyridine; Neuro-2A, mouse neuroblastoma cells; Neuro-2A-mDAT, dopamine transporter transfected Neuro-2A cells; THP, tetrahydropapaveroline; TIQ, 1,2,3,4-tetrahydroisoquinoline; wt, wild type.

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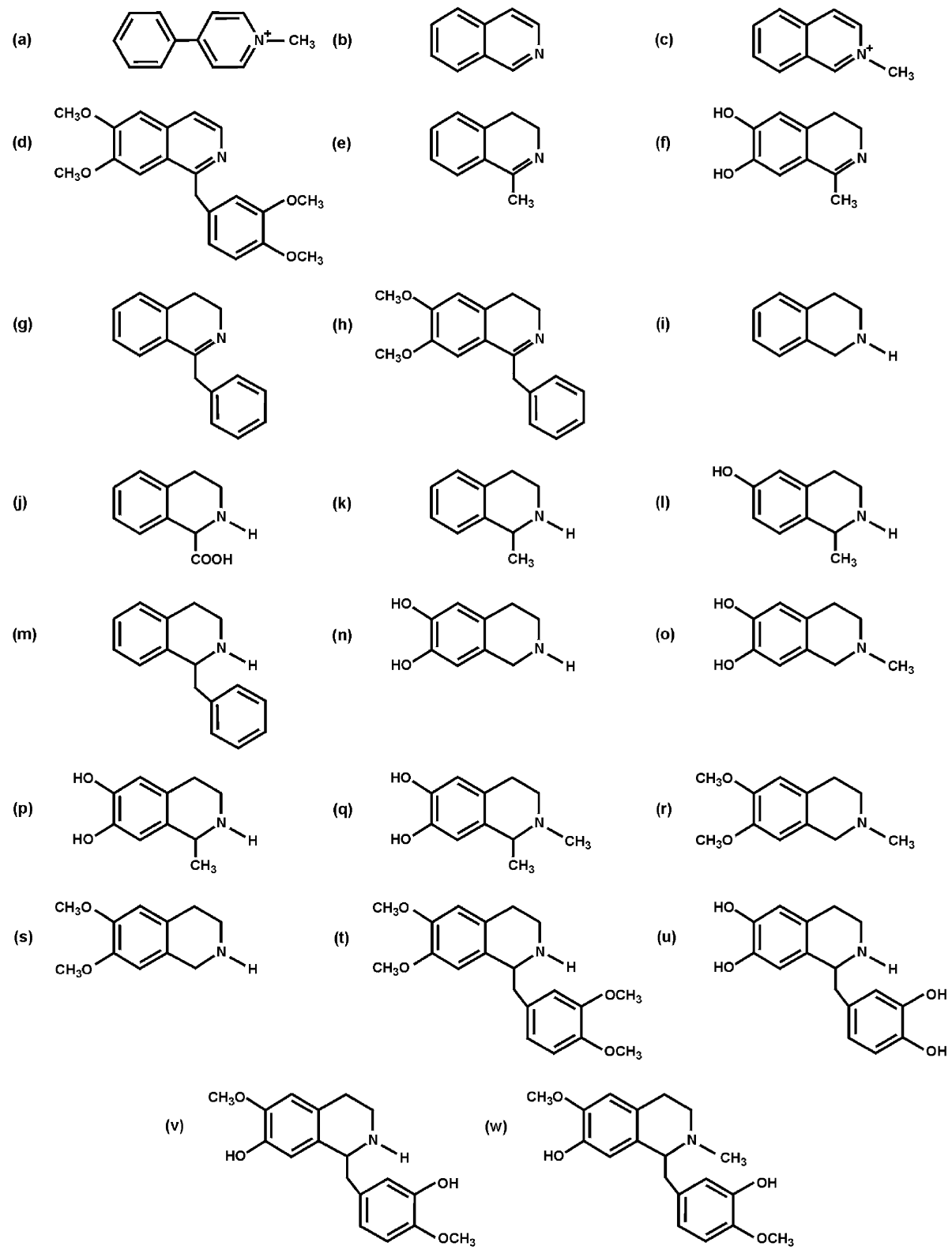


Fig. 1. Structures of isoquinoline derivatives. (**a**) 1-Methyl-4-phenylpyridinium; (**b**) isoquinoline; (**c**) 2[*N*]-methylisoquinolinium ion (2[*N*]-Me-IQ$^{+}$); (**d**) papaverine; (**e**) 1-methyl-3,4-dihydroisoquinoline; (**f**) 1-methyl-6,7-dihydroxy-3,4-dihydroisoquinoline; (**g**) 1-benzyl-3,4-dihydroisoquinoline; (**h**) 1-benzyl-6,7-dimethoxy-3,4-dihydroisoquinoline; (**i**) 1,2,3,4-tetrahydroisoquinoline; (**j**) 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid; (**k**) 1-methyl-1,2,3,4-tetrahydroisoquinoline (1-Me-TIQ); (**l**) 1-methyl-6-hydroxy-1,2,3,4-tetrahydrosioquinoline; (**m**) 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1-Bn-TIQ); (**n**) 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (norsalsolinol); (**o**) 2[*N*]-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (2[*N*]-Me-norsalsolinol); (**p**) 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol); (**q**) 1,2[*N*]-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (2[*N*]-Me-salsolinol); (**r**) 2[*N*]-methyl-6,7-dimethoxy-1,2,3,4-tetrahydrosioquinoline (2[*N*]-Me-norsalsolidine); (**s**) 6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinoline; (**t**) 1-(3′, 4′-dimethoxybenzyl)-6,7-dimethoxy-TIQ (tetrahydropapaverine); (**u**) 1-(3′,4′-dihydroxybenzyl)-6,7-dihydroxy-TIQ (THP); (**v**) 1-(3′-hydroxy-4′-methoxybenzyl)-6-methoxy-7-hydroxy-TIQ (norreticuline); (**w**) 1-(3′-hydroxy-4′-methoxybenzyl)-2[*N*]-methyl-6-methoxy-7-hydroxy-TIQ (reticuline). All compounds having chiral centers at carbon 1 were racemic except norreticuline and reticuline.

the environment, being present in many plants and foodstuffs, such as port wine, bananas, beer and milk (reviewed in [28]), and occur naturally in mammalian brain where they are synthesized by an enzymatic and non-enzymatic Pictet–Spengler condensation of biogenic amines, e.g. catecholamines, with aldehydes [34]. Isoquinolines are metabolized by cytochrome $P_{450}$, *N*-methyltransferases, as well as monoamine oxidases leading to charged quaternary forms (isoquinolinium cations) [28,42].

Since many isoquinoline derivatives are structurally similar to the Parkinson-inducing agent MPTP/$MPP^+$ (refer to Fig. 1), isoquinolines are considered as endogenous and/or exogenous dopaminergic neurotoxins playing a role in selective dopaminergic neurodegeneration in PD [28,42,47]. Interestingly, there are evidences for increased brain levels of isoquinoline derivatives, in particular dopamine-derived catecholic isoquinolines such as 1,2[*N*]-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (2[*N*]-methyl-salsolinol), and their synthesizing enzymes in drug naive *de novo* as well as levodopa-treated patients suffering from PD [18,23,38,44,52]. The most important site of action with respect to potential neurotoxicity of isoquinolines is the mitochondrial respiratory chain [28,29, 36,58]: several isoquinoline derivatives inhibit mitochondrial complex I (NADH-Q reductase) and II (succinate-Q reductase) activity as well as α-ketoglutarate dehydrogenase (α-KGDH), with several isoquinolines being more potent than $MPP^+$ [29,30,36]. Indeed, several isoquinoline derivatives show partially selective cytotoxicity in PC12 cells [31], human dopaminergic neuroblastoma SH-SY5Y cells [24–26,54,60,64], and primary dopaminergic neurons *in vitro* [9,45,46]. *In vivo* studies using experimental animals including non-human primates demonstrated that some isoquinoline derivatives are able to produce a Parkinsonian syndrome with biochemical, neurochemical and histological as well as neurobehavioral changes after chronic parental treatment (for review, see [28]).

The partial selectivity of isoquinoline derivatives towards dopaminergic cells is considered to be a consequence of cellular uptake by the dopamine transporter (DAT) as shown for $MPP^+$ [8,13,16,17,50,53]. Neutral and quaternary isoquinoline derivatives are able to partially inhibit [$^3$H]-dopamine accumulation into striatal synaptosomes from rat, but they are far less potent than $MPP^+$ [10,32,48]. The most potent blockers of [$^3$H]-dopamine uptake were derivatives of TIQ with catechol structures, such as salsolinol and its derivatives. Recently, Okada *et al.* showed that tetrahydropapaveroline (THP) and its derivatives inhibit dopamine uptake through the DAT expressed in human embryonic kidney 293 cells (HEK-293) cells [48]. Using tritiated substances, Heikkila *et al.* showed accumulation of 6,7-dihydroxy-1,2,3,4-TIQ and 1-methyl-4,6,7-trihydroxy-1,2,3,4-TIQ in rat striatal synaptosomes [10]. Takahashi *et al.* studied uptake characteristics of catechol isoquinolines in SH-SY5Y cells using a HPLC-based method [59]. They found that only (*R*)-1,2-dimethyl-6,7-dihydroxy-1,2,3,4-TIQ (2[*N*]-methyl-(*R*)-salsolinol) is transported by the DAT, while the (*S*)-isomer, 1-methyl-6,7-dihydroxy-1,2,3,4-TIQ, and 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion are not. Similarly, 2[*N*]-methylisoquinolinium ion has been shown to accumulate in rat striatal synaptosomes [12,27]. Another extensive study using rat striatal synaptosomes revealed that 2[*N*]-methylisoquinolinium, (*R*)-salsolinol and 2[*N*]-methyl-(*R*)-salsolinol are accumulated within the cytoplasm *via* the DAT [27]. Overall, the available data demonstrate that several isoquinolines, in particular those with catecholic structure, are substrates for the DAT with both moderate to low affinities at the DAT and moderate uptake velocities compared to $MPP^+$ and dopamine, respectively.

Although, uptake through the DAT has been characterized for several isoquinoline derivatives, its relevance for selective dopaminergic toxicity of these compounds remains unclear. In particular, there is no systematic study investigating whether the uptake kinetics are sufficient to increase accumulation of isoquinoline derivatives within the cytoplasm, such that concentrations needed for disruption of vital cellular structures (e.g. mitochondrial respiratory function) and subsequent cell death are reached. We now examine the potency and structural requirements of isoquinoline derivatives to exhibit DAT-dependent toxicity by studying the differential cytotoxicity of 22 neutral and quaternary isoquinoline derivatives structurally related to MPTP/$MPP^+$ from three classes of isoquinoline derivatives (3, IQs; 4,3,4-dihydroisoquinolines and 15 TIQs) listed in Fig. 1 on non-neuronal and neuronal heterologous expression systems of the DAT gene using human embryonic kidney HEK-293 and mouse neuroblastoma mouse neuroblastoma cells (Neuro-2A), respectively.

## 2. Materials and methods

### 2.1. Materials

$MPP^+$ iodide (**a**) was from Research Biochemical International (RBI); IQ (**b**), papaverine (**d**), TIQ (**i**), 1-benzyl-1,2,3,4-tetrahydroisoquinoline (1-Bn-TIQ) (**m**), 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline ((±)salsolinol) (**p**), 6,7-dimethoxy-TIQ (**s**) and tetrahydropapaverine (**t**) were obtained from Sigma. 2[*N*]-methyl-(±)salsolinol (**q**), 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (norsalsolinol) (**n**), 2[*N*]-methyl-norsalsolinol (**o**) were synthesized according to Bembenek *et al.* [2] and kindly provided by H. Thomas, Ulm, Germany, and analyzed by IR spectroscopy before use. 2[*N*]-Methylisoquinolinium ion (2[*N*]-Me-$IQ^+$) (**c**) and (±)tetrahydropapaveroline (**u**) were prepared in Matsubara's laboratory as previously reported [15,61]. 1-Methyl-3,4-dihydroisoquinoline (**e**), 1-methyl-6,7-dihydroxy-3,4-dihydroisoquinoline (**f**), 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid (**j**) and 2[*N*]-methyl-norsalsolidine (**r**) were from ACROS Organics. 1-Methyl-TIQ

(**k**) was prepared in Ohta's laboratory as previously reported [15]. 1-Methyl-6-hydroxy-TIQ (**l**) was synthesized in Wolf's laboratory from *m*-tyramine hydrochloride and acetaldehyde under physiological conditions in phosphate buffer at 50°. The reaction products were analyzed by HPLC. (*R*)- and (*S*)-enantiomers from norreticuline (**v**) and reticuline (**w**), respectively, were prepared in Michael A. Collin's laboratory. All compounds having chiral centers at carbon 1 were racemic except norreticuline and reticuline.

1-[2-[Bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]-piperazine dihydrochloride (GBR12909) were purchased from RBI. Geniticin (G418) was from Gibco. [$^3$H]-dopamine (48 Ci/mmol) and [$^3$H]MPP$^+$ (60 Ci/mmol) were purchased from Amersham International and American Radiolabeled Chemicals Inc., respectively. All other chemicals were of analytical grade and from Sigma.

### 2.2. Cell culture

Human embryonic kidney HEK-293 cells and mouse neuroblastoma Neuro-2A cells were purchased from American Tissue Type Culture Collection (reference no. CRL 1573 and CCL131, respectively; ATCC). HEK-293 cells were grown in minimum essential medium with Earl's salts and glutamine (Gibco) supplemented with 10% heat inactivated fetal bovine serum (PAA), at 37° in a humidified atmosphere of 5% $CO_2$ in air. HEK-293 cells stably transfected with the human dopamine transporter gene were prepared as previously described [53], and cultured at the conditions above except for adding 400 μg/mL geniticin (G418) to the medium. Neuro-2A cells were cultured in the medium described above supplemented with non-essential amino acids. To maintain the cells in the exponential growth phase they were harvested every 3–4 days and plated in tissue culture flasks at a density of approximately $1-2 \times 10^4$ cells/cm$^2$. One day before transfection, the cells were inoculated in 35 mm diameter petri dishes at a density of $1 \times 10^5$ cells per dish.

### 2.3. Cell line transfection

The cDNA encoding the mouse dopamine transporter (mDAT) was amplified by RT-PCR from mouse substantia nigra RNA and subcloned into the EcoRI/XhoI multiple cloning site of the eukaryotic expression vector pcDNA3 with CMV promotor (Invitrogen). Neuro-2A cells were stably transfected by calcium-phosphate DNA precipitation [6,53], and cloned by dilution in presence of geniticin G418 (1 mg/mL). The clones were then screened for [$^3$H]-dopamine and [$^3$H]MPP$^+$ uptake and the clone with highest MPP$^+$ uptake was used for further studies.

### 2.4. Uptake assays

Uptake studies with [$^3$H]-dopamine were performed as previously described [53]. In brief, the cells of interest were distributed into 24-well plates at a density of $2.5 \times 10^5$ cells per well. After 48 hr, wells were washed with 1 mL of uptake buffer (mM: 124 NaCl, 3.25 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 11 D-glucose, 10 HEPES, 0.2 mg/mL ascorbic acid and 0.2 mM pargyline to inhibit monoamine oxidase, pH 7.4 at 37°) and then incubated with 0.3 mL of buffer containing 20 nM [$^3$H]-dopamine and various concentrations of unlabeled dopamine for 10 min. Non-specific transport was determined in parallel assays conducted in the presence of 1 μM GBR12909. Uptake was stopped by adding 2 mL of ice-cold uptake buffer to each well and subsequent washing (twice) with 1.5 mL of ice-cold buffer. The incorporated radioactivity was extracted with NaOH (0.5 M) and quantified by liquid scintillation spectrometry. The 75 μL of the suspension was stored at −80° for later protein determination. For [$^3$H]MPP$^+$ uptake studies, cells were cultured in 10 cm culture dishes, washed twice with 10 mL of HBSS, and diluted to 100,000 cells/170 μL. For each test compound concentration 170 μL of cell suspension were put in an Eppendorf tube containing 20 μL test compound or HBSS (controls). After an incubation time of 15 min at room temperature 10 μL [$^3$H]MPP$^+$-solution (0.4 μCi in 10 μL HBSS; 1.1 μM) were added for another 15 min. The incubation was stopped by transferring the cell suspension into a well of a 96 well-microtiter plate containing 10 μL of 200 μM GBR12909 at 4°. The microtiter plate was centrifuged at 800 *g* in a Beckman centrifuge and washed twice with HBSS by flipping the plate to remove the supernatant. The amount of 200 μL of Microscint20 were given into each well, the plate covered with a plastic foil and shaken for 20 min. The radioactivity was counted in a Top Count Microplate Scintillation Counter (Packard). All values provided are the means ± SEM of at least three independent determination (running in duplicate).

### 2.5. Toxicity assays

Cell viability was assessed by a colorimetric method, which is based on the conversion of the yellow MTT salt (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to an insoluble purple formazan dye by mitochondrial dehydrogenases of living cells, which can be measured at 570 nm spectrophotometrically [40]. Cells of interest were seeded in 96-well plates at a density of 10,000–12,500 cells per well (in 200 μL medium). The cultures were grown for 48 hr, then various concentrations of the substance of interest (with or without uptake inhibitor) in 50 μL medium were added. After incubation at 37° for 72 hr, 30 μL of MTT reagent (0.5 mg/mL MTT in PBS containing 10 mM HEPES) was added to each well and incubated in the $CO_2$ incubator for 1 hr. The medium was aspirated from each well and the culture plate was dried at 37° for 1 hr. The resulting formazan dye was extracted with acid-isopropanol (100 μL of 0.04 N HCl in isopropanol) and the absorbance was measured spectrophotometrically with a computer-operated immunoreader

(Molecular Dynamics) at a wavelength of 570 nm with reference at 630 nm. Wells without cells were used as blanks and were subtracted as background from each sample. MTT reduction was expressed as percentage of the untreated control. The data obtained with the MTT method correspond very closely to data obtained with direct cell counting using Trypan blue staining in all cell lines used (for detailed experimental procedure, see [54]; data not shown).

### 2.6. Data analysis and statistics

To evaluate toxicity, 8–10 different concentrations (0.01–1000 μM) were tested for each compound using the MTT assay. Each experiment was replicated at least three times. To compare the toxic effects of isoquinoline derivatives on different cell lines, we calculated the half-maximal toxic concentration ($TC_{50}$ value) for all compounds on wild type (wt) cell lines ($TC_{50\ wt}$) and cell lines expressing the DAT ($TC_{50DAT}$), respectively, by non-linear curve fitting (Origin, Version 5.0; MicroCal Software) according to the mathematical model $Y = A_1 + (A_2 - A_1)/(1 + 10^{(\log B - x)n_H})$, where $A_1$ is the limit when the concentration approaches 0, $A_2$ the limit when the concentration approaches the maximum, $B$ the effective concentration which leads to 50% reduction of cell viability ($TC_{50}$), and $n_H$ the Hill's slope. The ratio $TC_{50\ wt}/TC_{50DAT}$ was defined as $I_{DAT}$. The unpaired $t$-test was used to compare the $TC_{50\ wt}$ and $TC_{50DAT}$ values of a given compound.

$V_{max}$, $K_m$, and $EC_{50}$ values were also calculated using non-linear regression analysis using the iterative curve fitting program Origin (Version 5.0; MicroCal Software). Results were expressed as means ± SEM and compared using Student's unpaired or paired $t$-test.

## 3. Results

### 3.1. Characterization of dopamine transporter transfected cell lines

The uptake characteristics of the HEK-293 cell line transfected with the human DAT gene used in the present study (HEK-hDAT) are described elsewhere [53]. In brief,

Table 1
Toxicity of isoquinoline derivatives in HEK-293 (wt) and HEK-hDAT cells

| Compound tested | $TC_{50\ wt}$ (μM) | $TC_{50DAT}$ (μM) | $I_{DAT}$ |
|---|---|---|---|
| Pyridine derivatives | | | |
| $MPP^+$ | 575.0 ± 27.9 | 0.14 ± 0.02[b] | 4107 |
| Isoquinoline derivatives | | | |
| Isoquinoline | >1000 | >1000 | – |
| 2[*N*]-Me-isoquinolinium | 683.0 ± 64.9 | 49.3 ± 10.4[a] | 13.9 |
| Papaverine | 5.4 ± 2.2 | 8.2 ± 2.6 | 0.7 |
| 3,4-Dihydroxyisoquinoline derivatives | | | |
| 1-Me-3,4-dihydroisoquinoline | >1000 | >1000 | – |
| 1-Me-6,7-dihydroxy-3,4-dihydroisoquinoline | >300 | >300 | – |
| 1-Bn-3,4-dihydroisoquinoline | >300 | >300 | – |
| 1-Bn-6,7-dimethoxy-3,4-dihydroisoquinoline | >300 | >300 | – |
| 1,2,3,4-Tetrahydroisoquinoline derivatives | | | |
| 1,2,3,4-Tetrahydroisoquinoline | >1000 | >1000 | – |
| TIQ-1-carboxylic acid | >1000 | >1000 | – |
| 1-Me-TIQ | >1000 | >1000 | – |
| 1-Me-6-hydroxy-TIQ | >1000 | >1000 | – |
| 1-Bn-TIQ | 121.7 ± 4.2 | 161.2 ± 9.9 | 0.8 |
| Norsalsolinol | 154.0 ± 23.4 | 154.6 ± 27.5 | 1 |
| 2[*N*]-Me-norsalsolinol | 287.0 ± 16.3 | 64.3 ± 6.2[a] | 4.5 |
| (±)-Salsolinol | 64.8 ± 5.2 | 65.6 ± 12.8 | 1.0 |
| 2[*N*]-Me-(±)-salsolinol | 210.0 ± 42.8 | 47.4 ± 5.8[a] | 4.4 |
| 2[*N*]-Methyl-salsolidine | >1000 | >1000 | – |
| 6,7-Dimethoxy-TIQ | >1000 | >1000 | – |
| Tetrahydropapaverine | 171.3 ± 19.5 | 170.3 ± 21.1 | 1.0 |
| (±)-Tetrahydropapaveroline | 72.9 ± 10.7 | 60.3 ± 10.4 | 1.2 |
| (*R*)-Norreticuline | >300 | >300 | – |
| (*S*)-Norreticuline | >300 | >300 | – |
| (*R*)-Reticuline | >300 | >300 | – |
| (*S*)-Reticuline | >300 | >300 | – |

Each compound was tested in both HEK-293 (wt) and HEK-hDAT cell lines. The $TC_{50\ wt}$ and $TC_{50DAT}$ represent the effective concentration (μM) inducing a 50% decrease of cell viability of HEK-293 (wt) and HEK-hDAT cells, respectively, measured with the MTT assay after 72 hr of exposure. $I_{DAT}$, defined as the ratio $TC_{50\ wt}/TC_{50DAT}$, represents an index of DAT-dependent toxicity.

[a] $TC_{50DAT}$ significantly different from corresponding $TC_{50\ wt}$ at $P < 0.01$.

[b] $TC_{50DAT}$ significantly different from corresponding $TC_{50\ wt}$ at $P < 0.001$.

the HEK-hDAT cells take up [$^3$H]-dopamine in a dose-dependent manner with a $K_m$ value of $23.6 \pm 3.7$ μM, a Hill coefficient of $1.05 \pm 0.13$ and $V_{max}$ value of $594 \pm 64$ pmol/min mg protein (approximately $60.0 \pm 6.5$ pmol/min $10^6$ cells). Neuro-2A cells stably transfected with the mouse DAT (dopamine transporter transfected Neuro-2A cells, Neuro-2A-mDAT) showed [$^3$H]-dopamine uptake with $K_m = 14.8 \pm 5.3$ μM, Hill coefficient close to unity and $V_{max}$ value of $9.2 \pm 2.0$ pmol/min $10^6$ cells. Uptake of $MPP^+$ by both DAT-expressing cell lines was found to be linear for 15 min at room temperature with an uptake after 15 min of $17.99 \pm 3.71$ and $5.92 \pm 0.97$% of total extracellular $MPP^+$ (30 nM) for HEK-hDAT and Neuro-2A-mDAT cells, respectively, dependent on temperature and ligand concentration. $MPP^+$ uptake in both cell types was inhibited by GBR12909 with an $IC_{50}$ value of 0.1 μM. Parental cell lines HEK-293 (wt) and Neuro-2A (wt), respectively, as well as HEK-293 cells permanently transfected with the pRc/CMV vector showed no significant GBR12909-sensitive [$^3$H]-dopamine uptake, respectively.

### 3.2. Toxic effects of isoquinoline derivatives on HEK-293 (wt) and HEK-hDAT cells

As reported earlier [53], **a** showed high potency ($TC_{50DAT} = 0.14$ μM after 72 hr) and selective toxicity ($I_{DAT} = 4107$) for HEK-293 cells expressing the human DAT gene (Table 1). DAT-dependent toxicity was completely blocked by the DAT inhibitor GBR12909 (Fig. 2A).

With concentrations up to 1000 μM, 13 isoquinoline derivatives had no cytotoxic effects on untransfected HEK-293 wild type cells, but significant dose-dependent toxicity

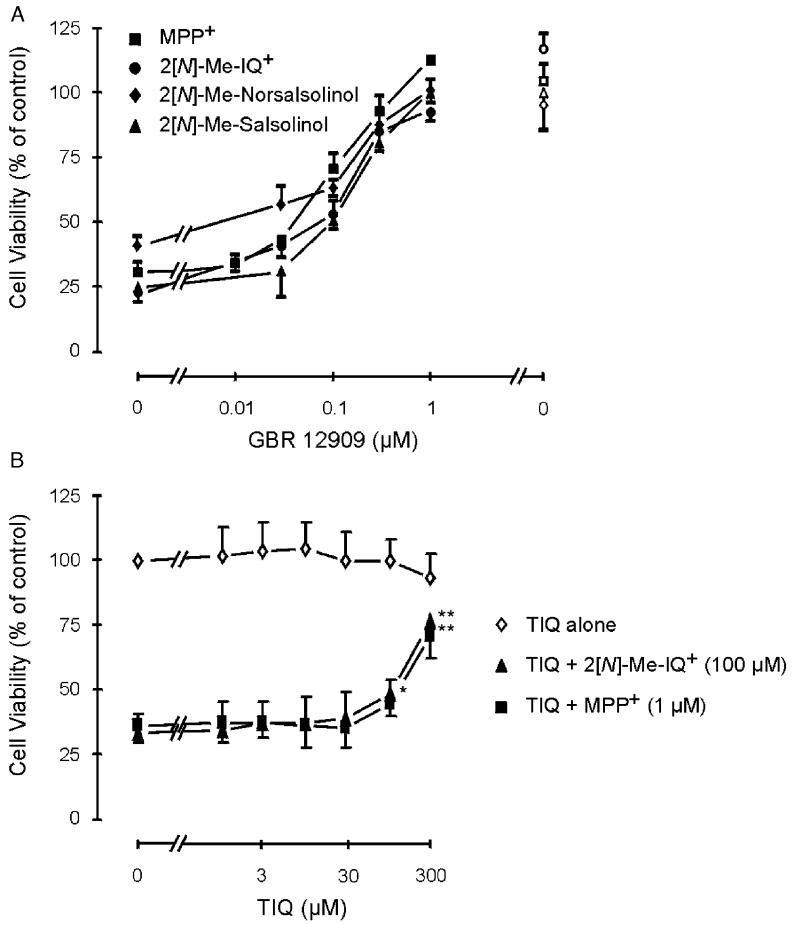


Fig. 2. (A) Effects of the dopamine transporter inhibitor GBR12909 on $MPP^+$, 2[*N*]-Me-$IQ^+$, 2[*N*]-Me-norsalsolinol and 2[*N*]-Me-salsolinol toxicity in HEK-hDAT cells (filled symbols). Cells were treated with $MPP^+$ (3 μM), 2[*N*]-Me-$IQ^+$ (100 μM), 2[*N*]-Me-norsalsolinol (100 μM) and 2[*N*]-Me-salsolinol (100 μM), respectively, in absence and presence of various concentrations of GBR12909. After a 72 hr incubation period cell viability was measured using the MTT assay. The values were normalized to the data obtained with uptake inhibitor alone (which had no effects on cell survival at concentrations up to 3 μM). For comparison, cell survival obtained with same concentrations of toxins in HEK-293 (wt) cells in absence of GBR12909 are displayed (open symbols). (B) Effects of the non-toxic TIQ on toxicity induced by $MPP^+$ (1 μM) and 2[*N*]-Me-$IQ^+$ (100 μM), respectively, in HEK-hDAT cells. Experimental conditions were essentially the same. TIQ when administered alone showed no significant toxic effects on cell survival at concentrations up to 300 μM (open symbols). Data points represent the mean ± SEM of at least three independent experiments, $*$ indicates $P < 0.05$, $**$ indicates $P < 0.01$ when compared with values obtained with respective toxin alone.

was produced by 9 isoquinolines with the following rank order of potency: **d** > **p** = **u** = **l** = **m** = **n** > **t** > **q** > **o** > **c**. The calculated $TC_{50\ wt}$ values obtained from the toxicity curves by non-linear regression analysis are summarized in Table 1. From these toxic isoquinolines, only three 2[*N*]-methylated compounds showed significantly enhanced cytotoxicity in DAT expressing HEK-293 cells (Table 1). However, potency and selectivity were less than those of **a**, with $TC_{50DAT} = 49.3$ μM and $I_{DAT} = 13.9$ for **c**, $TC_{50DAT} = 64.3$ μM and $I_{DAT} = 4.5$ for **o** and $TC_{50DAT} = 47.4$ μM and $I_{DAT} = 4.4$ for **q**. As an example for toxicity curves, Fig. 3A shows the effects of 2[*N*]-Me-$IQ^+$ on cell viability of untransfected HEK-293 and HEK-hDAT cells, respectively.

To confirm the results on DAT-dependent toxicity of 2[*N*]-Me-$IQ^+$, 2[*N*]-Me-salsolinol and 2[*N*]-Me-norsalsolinol, we tested the ability of the selective DAT inhibitor GBR12909 to protect HEK-hDAT cells against their toxicity. As shown in Fig. 2A, GBR12909 is able to fully protect HEK-hDAT cells against toxicity induced by incubation for 72 hr with 100 μM **c**, 100 μM **o** and 100 μM **q**, respectively. This protection was dose-dependent with $EC_{50}$ values of $0.108 \pm 0.09$, $0.142 \pm 0.089$ and $0.179 \pm 0.015$ μM for toxicity induced by 2[*N*]-Me-$IQ^+$, 2[*N*]-Me-norsalsolinol and 2[*N*]-Me-salsolinol, respectively (no significant difference between $EC_{50}$ values). The extents of protection reach the level of cell survival seen in untransfected HEK-293 (wt) cells after treatment with the same concentration of each toxin (Fig. 2A).

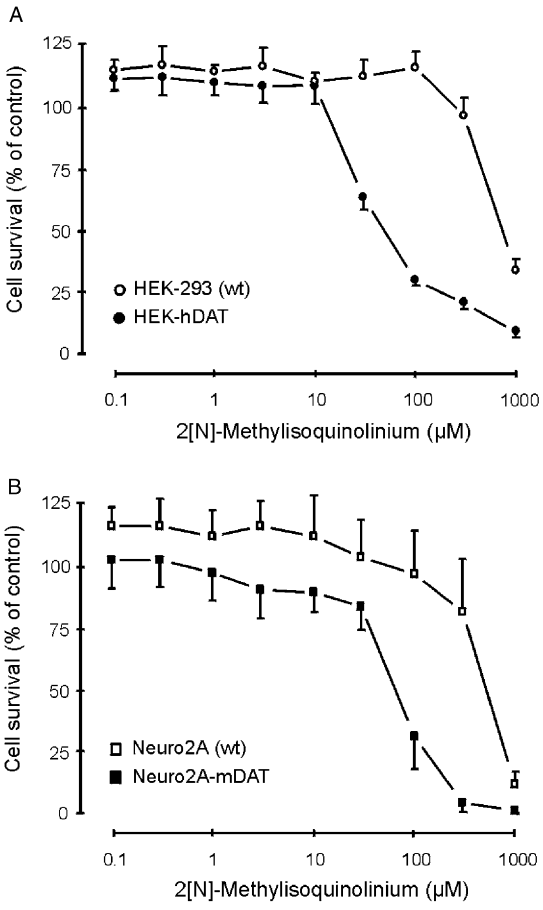


Fig. 3. Toxicity of 2[*N*]-Me-$IQ^+$ on HEK-293 (A) and Neuro-2A cells (B) permanently expressing the DAT. Wild-type cells and cells permanently transfected with the DAT gene were treated with various concentrations of 2[*N*]-Me-$IQ^+$ or vehicle for 72 hr and cell viability was assessed using the MTT assay. For half-maximal toxic concentrations ($TC_{50}$ values) see Tables 1 and 2. Data points represent the mean ± SEM of at least three independent experiments (running in triplicate).

### 3.3. Toxic effects of isoquinoline derivatives on Neuro-2A (wt) and Neuro-2A-mDAT cells

In mouse neuroblastoma, Neuro-2A cells, **a** showed high potency ($TC_{50DAT} = 1.2$ μM after 72 hr) and selective toxicity ($I_{DAT} = 73$) for Neuro-2A cells functionally expressing the mouse DAT gene (Table 2). Toxicity was completely blocked by the DAT inhibitor GBR12909 (data not shown).

We used this neuronal heterologous expression system of the DAT to investigate the DAT-dependent toxicity of isoquinoline derivatives in neuronal cells. The experimental conditions were essentially the same as already described for HEK-293 cells. Fig. 3 shows as an example the toxicity curves of 2[*N*]-Me-$IQ^+$ in untransfected and DAT-transfected Neuro-2A cells, respectively. The calculated $TC_{50}$ values obtained from the toxicity curves by non-linear regression analysis as well as the resulting $I_{DAT}$ values are summarized in Table 2. The pattern of cytotoxicity of the isoquinoline derivatives tested was similar to the pattern found in HEK-293 cells. In brief, isoquinoline derivatives with toxic potency in HEK-293 (wt) cells showed significant cytotoxicity in Neuro-2A (wt) cells with concentrations up to 1000 μM after 72 hr. From these isoquinolines, only the 2[*N*]-methylated compounds **c**, **o** and **q** showed significantly enhanced cytotoxicity in DAT-expressing Neuro-2A cells, but selectivities ($I_{DAT}$ values) were less compared to HEK-293 cells (Table 2).

### 3.4. Effects of non-toxic TIQ derivatives on toxicity of $MPP^+$ and 2[*N*]-Me-$IQ^+$

There are some isoquinoline derivatives tested in the present study showing no DAT-dependent toxicity, but inhibition of [$^3$H]-dopamine through the DAT in previous reports [32,48,59], for example **i** and **k**. We tested whether these two compounds are able to block DAT-dependent toxicity induced by 1 μM **a** and 100 μM **c**, respectively, in HEK-hDAT cells. These concentrations displayed no toxic effects on HEK-293 (wt) cells (Fig. 2A), showing that cytotoxicity at these concentrations completely depends on cellular uptake through DAT. As shown in Fig. 2B, TIQ showed dose-dependent inhibition of cytotoxicity of both $MPP^+$ and 2[*N*]-Me-$IQ^+$ with comparable potency. In contrast, 1-Me-TIQ showed no significant protection against both toxins (data not shown). Dose–response

Table 2
Toxicity of isoquinoline derivatives in Neuro-2A and Neuro-2A-mDAT cells

| Compound tested | $TC_{50\ wt}$ (μM) | $TC_{50DAT}$ (μM) | $I_{DAT}$ |
|---|---|---|---|
| Pyridine derivatives | | | |
| $MPP^+$ | 86.5 ± 8.9 | 1.2 ± 0.4[b] | 73 |
| Isoquinoline derivatives | | | |
| Isoquinoline | >1000 | >1000 | – |
| 2[*N*]-Me-isoquinolinium | 438.0 ± 62.9 | 67.3 ± 6.3[a] | 6.5 |
| Papaverine | 8.6 ± 2.6 | 10.8 ± 4.5 | 0.8 |
| 1,2,3,4-Tetrahydroisoquinoline derivatives | | | |
| 1,2,3,4-Tetrahydroisoquinoline | >1000 | >1000 | – |
| 1-Bn-TIQ | 145.5 ± 12.7 | 145.5 ± 32.3 | 0.8 |
| Norsalsolinol | 71.5 ± 10.8 | 59.0 ± 16.3 | 1.2 |
| 2[*N*]-Me-norsalsolinol | 409.7 ± 76.6 | 179.3 ± 42.1[a] | 2.3 |
| (±)-Salsolinol | 102.1 ± 21.4 | 118.2 ± 27.1 | 0.9 |
| 2[*N*]-Me-(±)-salsolinol | >1000 | 416.9 ± 42.7[a] | >2.4 |
| Tetrahydropapaverine | 178.7 ± 43.1 | 165.7 ± 38.1 | 1.1 |
| (±)-Tetrahydropapaveroline | 35.3 ± 4.7 | 36.2 ± 8.6 | 1 |

Each compound was tested in both Neuro-2A (wt) and Neuro-2A-mDAT cell lines. $TC_{50\ wt}$ and $TC_{50DAT}$ represent the effective concentration (μM) inducing a 50% decrease of cell viability of Neuro-2A (wt) and Neuro-2A-mDAT cells, respectively, measured with the MTT assay after 72 hr of exposure. $I_{DAT}$, defined as the ratio $TC_{50\ wt}/TC_{50DAT}$, represents an index of DAT-dependent toxicity. In this table only substances with $TC_{50}$ values <300 μM in HEK-293 (wt) cells (refer to Table 1) or reference compounds are displayed.

[a] $TC_{50DAT}$ significantly different from corresponding $TC_{50\ wt}$ at $P < 0.01$.

[b] $TC_{50DAT}$ significantly different from corresponding $TC_{50\ wt}$ at $P < 0.001$.

curves of $MPP^+$ and 2[*N*]-Me-$IQ^+$ toxicity obtained in absence and presence of TIQ (300 μM) showed a right-shift without changing the maximal toxic response (data not shown), suggesting a competitive inhibitory mechanism of TIQ on toxicity induced by $MPP^+$ and 2[*N*]-Me-$IQ^+$, respectively.

## 4. Discussion

The process leading to selective neurodegeneration of dopaminergic neurons within the substantia nigra pars compacta in PD remains unknown, but a growing body of evidence from morphological [5,11,49,63], molecular biological and genetic studies [21,37] indicates that the DAT protein may be responsible for the selectivity of dopaminergic cell death in this neurodegenerative disorder (for review, see [57,62]). Furthermore, impairment of mitochondrial energy production caused by reduction of mitochondrial enzyme activities is thought to be one of the most important factors leading to cell death in PD (for review, see [7,20]). Emphasizing that exogenous or endogenous toxins, which are transported into the cell by the DAT and subsequently affecting mitochondrial respiration, may play a role in the pathogenesis. Isoquinoline derivatives structurally related to MPTP/$MPP^+$ are considered as reasonable candidates with evidences for dopaminergic toxicity [9,24–26,31,45,54,60,64] and transmembrane transportation *via* the DAT [10,12,27,32,48,59].

Furthermore, increased brain levels of isoquinoline derivatives and their synthesizing enzymes were detected in drug naive *de novo* as well as levodopa-treated patients suffering from PD. The 2[*N*]-methylated catecholic isoquinoline **q**, was significantly increased in the cerebrospinal fluid (CSF) of *de novo* Parkinsonian patients compared to controls or patients with multiple system atrophy [23]. Moreover, a group of Parkinsonian patients showed an increased activity of a specific (*R*)-salsolinol *N*-methyltransferase in lymphocytes, an enzyme producing **q** [44]. These data suggest that intracerebral elevation of **q** is specific for PD. Furthermore, **m** is reported to be specifically elevated in the CSF of PD patients [18]. In PD patients under levodopa therapy several other isoquinolines are increased in the CSF [38,39] most likely reflecting increased dopamine turnover in levodopa-treated patients [39].

However, the importance of DAT-mediated cellular uptake of isoquinolines, in particular 2[*N*]-methylated derivatives, for their dopaminergic toxicity remains unclear since there has been no comprehensive study of these compounds to determine the potency, selectivity and structural requirements for DAT-dependent cytotoxicity. Therefore, we have examined the cytotoxic effects of 22 neutral and quaternary isoquinolines structurally related to MPTP/$MPP^+$ on heterologous expression systems of the DAT gene. We used non-neuronal HEK-293 and neuronal Neuro-2A cells stably transfected with the DAT gene, which show high sensitivity and selectivity for $MPP^+$-toxicity (Tables 1 and 2). The uptake kinetics are similar to those reported for other ectopic expression systems [3,17,50,51], but different to studies using synaptosomes from rat with 5–10 times higher $K_m$ values [50]. However, the susceptibility of these cells towards $MPP^+$-toxicity is essentially in the same range compared to primary dopamine neurons and other dopaminergic cell types, respectively [35,54–56]. Thus, our cell systems represent valuable tools for studying the involvement of the DAT molecule in the selectivity of dopaminergic toxicity of neurotoxins potentially related to PD.

About 9 of the 22 isoquinolines tested displayed concentration-dependent general toxicity towards both HEK-293 (wt) and Neuro-2A (wt) cells not expressing the DAT protein, with no relevant difference of toxicity ($TC_{50\ wt}$ values) between the non-neuronal HEK-293 cells and the neuroblastoma cell line Neuro-2A (Tables 1 and 2). The rank order of toxic potency ($1/TC_{50\ wt}$) towards both wild type cell lines was as follows: $\mathbf{d} > \mathbf{p} = \mathbf{u} = \mathbf{m} = \mathbf{n} > \mathbf{t} > \mathbf{q} > \mathbf{o} > \mathbf{c} = \mathbf{a}$. No clear structure–activity relationship among the IQ derivatives and their non-selective cytotoxic effects was found. Indeed, the rank order of toxic potency of isoquinolines in both parental cell lines does not correlate with their order of lipophilicity (measured by octanol/water partition coefficients [29]), nor with the potency of

inhibition of mitochondrial complexes in mitochondrial membrane fragments [29,33,58], mitochondrial respiration in intact mitochondria [33,36] or the α-KGDH complex [30]. Thus, the mechanisms of unspecific toxicity are unclear, but most likely include unspecific, DAT-independent cellular and/or mitochondrial accumulation as well as inhibition of cellular energy supply and/or production of free radicals as reported for $MPP^+$ [1,22,51,53].

Among the group of isoquinoline derivatives tested in this study, only the three 2[*N*]-methylated compounds **c**, **o** and **q** displayed significantly enhanced toxic effects against both HEK-293 and Neuro2A cells permanently expressing the DAT compared to the parental cell lines, although they were less potent and less selective than **a**. The selective toxicity of these compounds was completely blocked by the DAT inhibitor GBR12909. These results demonstrate DAT-mediated toxicity of 2[*N*]-methylated isoquinolines in non-neuronal and neuronal cells. In general, HEK-hDAT cells showed higher selectivity for all three substances as well as $MPP^+$ when compared to the Neuro-2A-mDAT cell line. These differences are most likely due to much higher expression of the DAT protein in HEK-hDAT cells when compared to Neuro-2A-mDAT cells as shown with uptake studies [3,17,50,51]. However, differences in ATP synthesis, and in mitochondrial and hexose kinase pathways may also account for the differences in the susceptibility to the toxins between HEK-293 and Neuro2A cells, respectively. The rank order of selectivity ($I_{DAT}$ values) in both cell types was as follows: $\mathbf{a} \gg \mathbf{c} > \mathbf{o} = \mathbf{q}$. This order of selectivity is similar to that reported for the affinities ($1/K_m$) of **a** and several isoquinolines, namely **c** and **q**, for the DAT-mediated uptake into rat striatal synaptosomes [27]. Taking into account all available data on DAT-mediated uptake of the isoquinolines tested in this study, only the isoquinoline derivatives showing DAT-mediated toxicity also display cellular uptake *via* the DAT and vice versa [12,27,59]. Only the studies on DAT-mediated uptake of **p** revealed conflicting results: Matsubara *et al.* demonstrated DAT-mediated influx of (*R*)-salsolinol into rat striatal synaptosomes [27], while Takahashi *et al.* did not find any accumulation into human SH-SY5Y cells by the DAT [59]. The reason for this discrepancy is unclear, but may arise from different methods and/or cell systems used in this uptake studies.

Many more isoquinolines inhibit DAT-mediated [$^3$H]-dopamine uptake, for example **i**, **k**, and **u** without being transported into the cytoplasm through the DAT [27,32,48,59]. Thus, they act like most DAT inhibitors. Conclusively, there is no correlation between the inhibitory potency of DAT function and DAT-dependent toxicity. The compound **i**, which did not show significant DAT-mediated toxicity and uptake, respectively, was able to block DAT-mediated toxicity induced by **a** or **c**. In contrast, **k** did not show any protective effects. The reason for this discrepancy is unclear, but is most likely due to different binding affinities and/or bindings sites at the DAT molecule.

Using primary dopaminergic cells from rat, **c** exerted dose-dependent toxicity towards dopaminergic, but not GABAergic neurons, which was partially blocked by DAT inhibitors [45]. Another 2[*N*]-methylated isoquinoline, which showed DAT-mediated toxicity in the present study, namely **q**, shows partial dopaminergic toxicity *in vitro* and is able to produce an animal model of PD in rats [24,25,43]. Furthermore, McNaught *et al.* showed differential toxicity of slightly different isoquinolines towards PC12 cells [31], which was directly correlated with their potency to inhibit high affinity [$^3$H]-dopamine uptake [32], but not with their potency to inhibit mitochondrial complex I activity or mitochondrial respiration [29,30,33]. In contrast to our results, McNaught *et al.* (1996) did not find significant toxicity of 2[*N*]-Me-$IQ^+$ towards PC12 cells [31]. Other studies using primary mesencephalic cultures from rat showed partially selective dopaminergic toxicity of **i**, **d** and **t** [9,46], which did not show any significant DAT-mediated toxicity in the present study. From the other compounds showing no or only general toxicity towards both parental and DAT-expressing cells, only **i** and **m** are reported to display partial dopaminergic toxicity *in vivo* [18,19,41]. The reasons for these discrepancies are unclear, but may be due to different uptake kinetics of the DAT in different cell types and distinct properties of different cell types leading to different susceptibilities independent from DAT expression. This view is supported by the observation that the order of dopaminergic toxicity of 6,7-dimethoxy isoquinolines (**d** and **t**) in primary mesencephalic cultures correlates with their inhibitory effects on mitochondrial complex I activity and respiration [9]. Interestingly, both stereo-isomers of reticuline (**w** and **w′**), which are proposed to play a role in atypical Parkinsonism among residents of Guadeloupe in French West Indies [4], did not show general or DAT-mediated cytotoxicity.

The group of IQ derivatives used in the present study for analyzing DAT-mediated toxicity does not allow to measure quantitative structure–activity (selectivity) relationships, but some qualitative trends are apparent. The DAT-mediated toxicity was restricted to 2[*N*]-methylated compounds, since structurally identical compounds without 2[*N*]-methylation lacking selective toxicity towards DAT-expressing cell lines (see Fig. 1). Thus, **c**, which has the closest structure-based relationship to $MPP^+$ within the compounds tested in this study (no side chains; equivalent charge of the N-atom of the pyridine ring), showed the highest selectivity towards DAT-expressing cell lines. However, all isoquinoline derivatives were less potent and selective compared to $MPP^+$, most likely due to topographical reasons: The intramolecular distance between the N-atom and the centroid of the benzene ring is shorter in isoquinolines compared to $MPP^+$, in which this distance is close to that in the extended form of dopamine [27,32]. All modifications of the original structure of **c** resulted in partial or complete loss of selective toxicity towards DAT-expressing cells. Among the *N*-methylated IQ derivatives

used in this study, catecholic fully oxidized isoquinolines (TIQ derivatives) showed less selectivity for DAT-expressing cells compared to **c**. In agreement with Kawai *et al.* [14], methoxylation of carbonyl residues 6 and 7 of the benzene ring, as in **r**, completely abolished DAT-mediated toxicity. In addition, large side chains at position 1 of the pyridine ring as that in reticuline (**w** and **w′**) seems to inhibit DAT-dependent toxicity, but methylation at position 1 did not alter selectivity for DAT-expressing cells [compare **o** and **q**].

We suggest that the DAT molecule is responsible for the partial selectivity of cytotoxicity induced by *N*-methylated isoquinolines towards dopaminergic neurons, at least *in vitro*. The low affinity and uptake velocity of isoquinolines at the DAT may be a rate-limiting factor for selective dopaminergic toxicity. Conclusively, high concentrations of and/or prolonged exposure to isoquinolines may be necessary to produce significant dopaminergic toxicity. This does not rule out the possible involvement of isoquinoline derivatives in the pathophysiology of PD since the slow progression of this disease suggest a mild and prolonged degenerative process. However, the present results together with morphological and genetic evidences for the involvement of the DAT in the etiopathogenesis of PD (for review, see [57]) further support the hypothesis that selective toxic insults towards dopaminergic neurons *via* DAT-mediated cellular uptake of exogenous and/or endogenous 2[*N*]-methylated isoquinolines, in particular 2[*N*]-Me-salsolinol, play a pivotal role in dopaminergic neurodegeneration in PD [28,42,47]. However, studies have shown that the selective dopaminergic toxicity of $MPP^+$ is due, at least in part, to its relatively high affinity for the DAT and poor affinity/uptake velocity at other transporters, such as the norepinephrine or serotonin transporter [3,51]. Therefore, future studies are warranted to determine the affinity and uptake velocity of isoquinolines at other neurotransmitter transporters, as wells as the respective transporter-mediated toxicity.

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