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Cyanine-related compounds: a novel class of potent inhibitors of extraneuronal noradrenaline transport*

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Summary. The neurotransmitter noradrenaline is removed from the extracellular space by neuronal and extraneuronal transport mechanisms. In the past, further functional and biochemical characterisation of the corticosterone-sensitive extraneuronal transporter was hampered by the lack of highly potent inhibitors. Here we describe a new class of selective and highly potent inhibitors of the extraneuronal noradrenaline transporter.

Clonal Caki-1 cells possess the human type of extraneuronal noradrenaline carrier. The effect of various steroids and steroid-like compounds on initial rates of specific ^{3}H-noradrenaline transport in Caki-1 cells was investigated. None of these steroids had an inhibitory potency higher than that of corticosterone which hitherto was generally accepted as the most potent inhibitor of the extraneuronal noradrenaline transport. On the other hand, a variety of quinoline and isoquinoline derivatives interacted with the extraneuronal noradrenaline transporter. Several cationic quinolines that belong to the chemical class of the cyanine dyes turned out to be very potent inhibitors of ^{3}H-noradrenaline transport in Caki-1 cells. The isocyanines, 1,1′-diisopropyl-2,4′-cyanine (disprocynium24) and 1-methyl-1′-isopropyl-2,4′-cyanine as well as the pseudoisocyanines 1,1′-diethyl-2,2′-cyanine (decynium22) and 1-isopropyl-1′-ethyl-2,2′-cyanine (iprecynium22) were most potent with IC_{50}'s of 14, 62, 16, and 18 nmol/l, respectively. The inhibitory potency on extraneuronal noradrenaline transport of 1-methyl-1′-isopropyl-2,4′-cyanine was determined also in isolated organs, namely the isolated incubated rabbit aorta and the isolated perfused rat heart. The IC_{50}'s were 740 and 100 nmol/l, respectively. By contrast, the desipramine-sensitive neuronal type of noradrenaline transporter in PC12 cells was hardly affected by the cyanine-related compounds. Decynium22 (3 μmol/l) inhibited the neuronal noradrenaline transporter of clonal PC12 cells by 14% only.

Cyanine-related compounds potently and selectively inhibit the extraneuronal transport mechanism for noradrenaline. They are expected to facilitate the functional and biochemical characterisation of the extraneuronal noradrenaline transporter.

Key words: Caki-1 cells – Decynium22 – Disprocynium24 – Iprecynium22 – Noradrenaline – Norepinephrine – Extraneuronal noradrenaline transport – Uptake$_2$

Introduction

Active transmembrane transport processes are responsible for the inactivation of various neurotransmitters such as glycine, γ-aminobutyric acid, 5-hydroxytryptamine, dopamine and noradrenaline (Uhl and Hartig 1992, Graham and Langer 1992). Noradrenaline, the neurotransmitter of the sympathetic nervous system, is removed from the synaptic cleft by transport into both the releasing neurones and adjacent non-neuronal cells. Recently, the complementary DNA that encodes the neuronal type of noradrenaline transporter has been cloned (Pacholczyk et al. 1991). The neuronal type of noradrenaline transporter is sensitive to tricyclic antidepressants (Lee et al. 1983; Schömig et al. 1988). It is generally accepted that the therapeutic effect of desipramine-like antidepressants is due to the inhibition of this transporter (Hollister 1978).

Because of the lack of potent inhibitors, much less is known about the biological role of the extraneuronal type of noradrenaline transporter which is clearly different from the neuronal type. The extraneuronal transporter has been shown to occur in various organs such as myocardium, vascular smooth muscle, salivary glands, and central nervous system (Iversen 1965; Gillespie 1976; Trendelenburg 1988; Staudt et al. 1993). Extraneuronal noradrenaline transport is not sensitive to tricyclic antidepressants but to corticosterone. Further in vivo and in

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vitro characterisation of the extraneuronal noradrenaline transporter is hampered by the lack of high-affinity ligands. Numerous functional and biochemical methods absolutely depend on the availability of highly potent inhibitors. The introduction of the human Caki-1 and FL cell lines as experimental models for extraneuronal noradrenaline transport facilitates the search for high-affinity ligands (Schömig and Schönfeld 1990; de la Lande et al. 1991). Clonal cells in culture can be grown infinitely, which makes it possible to test the inhibitory effects of numerous compounds in paired experiments. Experiments with clonal cells are not complicated by the distribution of the labelled substrate in the extracellular space, by the presence of different cell types, and by diffusion barriers. Moreover, Caki-1 and FL cells possess the human type of the extraneuronal noradrenaline transporter. Thus, we used the Caki-1 tissue culture system to search for selective and highly potent inhibitors of the extraneuronal noradrenaline transport mechanism.

Methods

Cell culture. Caki-1 cells (ATCC HTB 46; Hay et al. 1988) were grown at 37 °C in a humidified atmosphere (5% CO_2) on plastic culture flasks (Falcon 175 cm^2, Becton Dickinson, Heidelberg, Germany) essentially as described by Schömig and Schönfeld (1990). The culture medium was McCoys medium with 16.7 mmol/l D(+)-glucose and 10% fetal calf serum (Gibco, Eggenstein, Germany). The culture medium was changed every 3 to 4 days and the culture was split every 7 days as described previously (Schömig and Schönfeld 1990). For the experiments, the Caki-1 cells were seeded on plastic culture dishes (∅ 60 mm, Nunc, Roskilde, Denmark). After 3 to 4 days, the cells formed a monolayer and each culture dish contained about 2 mg of cell protein. PC12 cells (ATCC CRL 1721; Hay et al. 1988) were grown in suspension culture as described by Harder and Bönisch (1984). The culture medium was composed of 85% RPMI 1640 medium, 10% heat-inactivated horse serum, 5% fetal calf serum (Gibco, Eggenstein, Germany), and was buffered with $NaHCO_3$ (24 mmol/l). For the experiments, the PC12 cells were seeded on plastic culture dishes (∅ 60 mm, Nunc, Roskilde, Denmark) coated with poly-L-ornithine. Reserpine (10 µmol/l) was added 24 h prior to the experiments to inhibit vesicular uptake of noradrenaline and to deplete the intracellular catecholamine stores. Each culture dish contained about 1 mg of cell protein.

Uptake of ^{3}H-noradrenaline in Caki-1 cells. Initial rates of ^{3}H-noradrenaline transport into Caki-1 cells were measured as described by Schömig and Schönfeld (1990). Caki-1 cells were preincubated at 37 °C for 20 min with buffer A (NaCl 125 mmol/l, KCl 4.8 mmol/l, $CaCl_2$ 1.2 mmol/l, KH_2PO_4 1.2 mmol/l, $MgSO_4$ 1.2 mmol/l, HEPES·NaOH 25 mmol/l (pH 7.4), D(+)-glucose 5.6 mmol/l, L(+)-ascorbic acid 1 mmol/l, pargyline 10 µmol/l, U-0521 10 µmol/l). The cells were incubated at 37 °C for 15 min with 100 nmol/l ^{3}H-noradrenaline (in 3 ml buffer A). When tested, inhibitors of ^{3}H-noradrenaline transport were present during both the preincubation and incubation period. Incubation was stopped by rinsing the cells four times with 3 ml ice-cold buffer A. The cells were solubilized by 0.1% v/v Triton X-100 (in 5 mmol/l Tris·HCl, pH 7.4). Radioactivity was determined by liquid scintillation counting. The intracellular radioactivity in absence of an inhibitor amounted to about 180 Bq per culture dish when ^{3}H-noradrenaline was used at a specific activity of $0.14 \cdot 10^{15}$ Bq/mol. Specific uptake of ^{3}H-noradrenaline was defined as that fraction of total uptake which was sensitive to 1 µmol/l decynium22. Non-specific uptake was less than 35% of total ^{3}H-noradrenaline uptake.

Uptake of ^{3}H-noradrenaline in PC12 cells. As described previously (Schömig et al. 1988), PC12 cells were preincubated at 37 °C for 20 min with buffer B (NaCl 125 mmol/l, K_2SO_4 2.4 mmol/l, KH_2PO_4 1.2 mmol/l, $MgSO_4$ 1.2 mmol/l, HEPES·NaOH 25 mmol/l (pH 7.4), D(+)-glucose 5.6 mmol/l, L(+)-ascorbic acid 1 mmol/l, pargyline 10 µmol/l, U-0521 10 µmol/l). Subsequently, the cells were incubated for 60 s with 10 nmol/l ^{3}H-noradrenaline (in 3.5 ml buffer B). When tested, decynium22 was present during both the preincubation and incubation period. Incubation was stopped by rinsing the cells four times with 3 ml ice-cold buffer B. The cells were solubilized by 0.1% v/v Triton X-100 (in 5 mmol/l Tris·HCl, pH 7.4), and radioactivity was determined by liquid scintillation counting. The intracellular radioactivity in absence of an inhibitor amounted to about 500 Bq per culture dish when ^{3}H-noradrenaline was used at a specific activity of $0.51 \cdot 10^{15}$ Bq/mol. Specific uptake of ^{3}H-noradrenaline was defined as that fraction of total uptake which was sensitive to 1 µmol/l desipramine. Non-specific uptake was less than 5% of total ^{3}H-noradrenaline uptake.

Uptake of ^{3}H-arginine in Caki-1 cells. Caki-1 cells were preincubated for 60 min at 37 °C in the absence or presence of 0.1 µmol/l decynium22 (in buffer A). Subsequently, the cells were incubated for 1 min at 37 °C in the presence of 10 µmol/l ^{3}H-arginine (in 3 ml buffer A). Incubation was stopped by rinsing the cells four times with 3 ml ice-cold buffer A. The cells were solubilized and intracellular radioactivity was determined as described above. The intracellular radioactivity amounted to about 430 Bq per culture dish when ^{3}H-arginine was used at a specific activity of $0.56 \cdot 10^{12}$ Bq/mol.

Trypan blue exclusion technique. Caki-1 cells were preincubated for 45 min at 37 °C in the absence or presence of 0.1 µmol/l decynium22 (in buffer A). Subsequently, the cells were incubated at 37 °C for 3 min with 2 mmol/l trypan blue in buffer E (NaCl 137 mmol/l, KCl 2.7 mmol/l, Na_2HPO_4 11.8 mmol/l, KH_2PO_4 1.5 mmol/l, pH 7.4). Incubation was stopped by rinsing the cells three times with buffer A and the cells were examined by light microscopy. Stained cells were regarded as damaged.

Uptake of ^{3}H-noradrenaline in the isolated rat heart. Male Wistar rats (180–250 g) were pretreated with heparin (5000 I.U./kg, i.p.) 20 min prior to the experiment. The isolated hearts were perfused (Langendorff technique) as described by Bönisch and Trendelenburg (1974) at 37 °C with buffer C (NaCl 137 mmol/l, KCl 2.7 mmol/l, $CaCl_2$ 1.3 mmol/l, $MgCl_2$ 1.1 mmol/l, NaH_2PO_4 0.42 mmol/l, $NaHCO_3$ 12 mmol/l, D(+)-glucose 11 mmol/l, EDTA 40 µmol/l, L(+)-ascorbic acid 280 µmol/l, pargyline 10 µmol/l, U-0521 10 µmol/l, desipramine 1 µmol/l, gassed with 95% O_2 and 5% CO_2, pH 7.4). The perfusion flow was 8 ml/(g heart weight·min). After 60 min of preperfusion, the hearts were perfused for 2 min with 100 nmol/l ^{3}H-noradrenaline and 100 µmol/l ^{14}C-sorbitol (extracellular space marker). When tested, inhibitors of ^{3}H-noradrenaline transport were present during both the preperfusion and perfusion period. After the perfusion period, the extracellular space was washed out by 2 min of perfusion with buffer C. Atria and ventricles were opened by a cut. The hearts were blotted, weighed, and transferred into 10 ml ice-cold perchloric acid (0.4 mol/l) containing EDTA (3 mmol/l), Na_2SO_3 (10 mmol/l), and L(+)-ascorbic acid (5 mmol/l). The tissues were homogenised with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). Radioactivity was measured by liquid scintillation counting. The data were corrected for extracellular space and initial rates of ^{3}H-noradrenaline uptake were calculated. Specific uptake of ^{3}H-noradrenaline in the rat heart was defined as that fraction of total uptake which was sensitive to 100 µmol/l O-methylisoprenaline.

Uptake of ^{3}H-noradrenaline in rabbit aortic strips. The initial rate of extraneuronal uptake of ^{3}H-noradrenaline in rabbit aortic strips was determined essentially as described by Henseling (1983). Rabbits (Chinchilla bastards, 2.5–3.5 kg) of either sex were pretreated with reserpine twice (1 mg/kg body weight, s.c.), 24 h and 3 h prior to the experiment. The animals were killed by cervical dislocation. The thoracic aorta was removed and cut into rings of about 20 mg weight. The tissues were equilibrated at 37 °C for 30 min in buffer D (NaCl 118 mmol/l, KCl 4.7 mmol/l, $CaCl_2$ 1.9 mmol/l, KH_2PO_4 1.2 mmol/l, $MgSO_4$ 1.2 mmol/l, $NaHCO_3$ 25 mmol/l, D(+)-glucose 5 mmol/l, L(+)ascorbic acid 5 mmol/l, EDTA 0.3 mmol/l, gassed with 95% O_2 and 5%

CO_2, pH 7.4). The aortic strips were exposed to 500 µmol/l pargyline for 30 min to inhibit monoamine oxidase. Subsequently, pargyline was washed out. The aortic strips were preincubated for another 20 min in the presence of 10 µmol/l U-0521 and 1 µmol/l desipramine to inhibit catechol-O-methyl transferase and neuronal noradrenaline uptake, respectively. The aortic strips were incubated for 10 min with 10 µmol/l ^{3}H-noradrenaline and 50 µmol/l ^{14}C-sorbitol. U-0521 (10 µmol/l), desipramine (1 µmol/l), and various concentrations of both decynium22 and compound Ic (see Table 3) were present not only during the incubation but also during the last 20 min of the preincubation. After the incubation, the aortic strips were dipped for 5 s into ice-cold buffer D and placed into perchloric acid (0.4 mol/l) to extract radioactivity. Radioactivity was measured by liquid scintillation counting. The data were corrected for extracellular space, and initial rates of ^{3}H-noradrenaline uptake were calculated. Specific uptake of ^{3}H-noradrenaline was defined as that fraction of total uptake which was sensitive to 30 µmol/l corticosterone.

Protein determination. Protein was determined as described by Lowry et al. (1951).

Calculations and statistics. For the calculation of the IC_{50}'s, the parameters of the Hill-equation for multisite inhibition (Segel 1975; eq. VIII-9) were fitted to the experimental data by a non-linear computer-assisted least square quasi-Newton method (Wilkinson 1989). The IC_{50}'s are virtually identical with the K_i-values, since non-saturating concentrations of ^{3}H-noradrenaline were used (Cheng and Prusoff 1973). The IC_{50}'s are given as geometric means with 95% confidence limits and the Hill-coefficients are given as arithmetic means with SEMs.

Materials

The synthesis of 1,1′-dimethyl-2,4′-cyanine iodide (Ia, for codes and corresponding structures see Table 3), 1-methyl-1′-isopropyl-2,4′-cyanine iodide (Ic), disprocynium24 (1,1′-diisopropyl-2,4′-cyanine perchlorate), 1,1′-dimethyl-2,2′-cyanine iodide (II), and iprecynium22 (1-isopropyl-1′-ethyl-2,2′-cyanine iodide) will be described elsewhere. Success of synthesis and purity of material were controlled by elementary-analysis (CHN) and NMR-spectroscopy. Other materials were 1,1′-diethyl-2,2′-cyanine iodide (decynium22, Atlanta, Heidelberg, Germany); 1,1′-diethyl-2,4′-cyanine iodide (Ib, Eastman Kodak, Rochester, N.Y., USA); 1,1′-diethyl-4,4′-cyanine iodide (III), 1,1′-diethyl-4,4′-carbocyanine iodide (IV), and 1,1′-diethyl-2,2′-carbocyanine iodide (V) (Aldrich, Steinheim, Germany); L-arginine hydrochloride, pargyline hydrochloride and reserpine (Sigma, Deisenhofen, Germany); U-0521 (3,4-dihydroxy-2-methylpropiophenone, Upjohn, Kalamazoo, Mich., USA); corticosterone (Fluka, Neu-Ulm, Germany); desipramine hydrochloride (Ciba Geigy, Basel, Switzerland); O-methylisoprenaline (Boehringer, Ingelheim, Germany); trypan blue (3,3′-[(3,3′-dimethyl-4,4′-biphenylene)-bisazo]-bis(5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid) tetra sodium salt, Boehringer, Mannheim, Germany); Tris (tris-[hydroxymethyl]-aminomethan) and HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (Roth, Karlsruhe, Germany). All other chemicals were either from E. Merck (Darmstadt, Germany) or from Sigma (Deisenhofen, Germmany). (−)-[7-^{3}H]-noradrenaline (NET-377, $0.51 \cdot 10^{15}$ Bq/mol) and D-[^{14}C(U)]-sorbitol (NEC-386, $12 \cdot 10^{12}$ Bq/mol) were obtained from DuPont de Nemours/NEN (Bad Homburg, Germany) and L-[2,3,4,5-^{3}H]-arginine monohydrochloride (TRK.698, $2.78 \cdot 10^{15}$ Bq/mol) was obtained from Amersham Buchler (Braunschweig, Germany).

Results

Inhibition of extraneuronal noradrenaline uptake in Caki-1 cells

Caki-1 cells were incubated for 15 min with 100 nmol/l ^{3}H-noradrenaline in order to measure initial rates of extraneuronal ^{3}H-noradrenaline uptake. Intracellular metabolism of ^{3}H-noradrenaline was avoided by the inhibition of catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO) with U-0521 (10 µmol/l) and pargyline (10 µmol/l), respectively. In agreement with earlier results (Schömig and Schönfeld 1990), the specific uptake of ^{3}H-noradrenaline was 23±2 fmol/(mg protein·min) in the absence of an inhibitor. Various steroids and steroid-like compounds were tested for inhibition of extraneuronal noradrenaline transport. All inhibited ^{3}H-noradrenaline uptake (Table 1). For dexamethasone it was not possible to determine an exact IC_{50} since the highest concentration used (10 µmol/l) caused an inhibition of less than 20%. Moreover, quinolines and isoquinolines were investigated. These compounds had IC_{50}'s below 20 µmol/l (Table 2). Interestingly enough, several cyanine-related compounds turned out to inhibit the extraneuronal noradrenaline transporter with high potency (Fig. 1). Three compounds had IC_{50}'s below 20 nmol/l. We have given these three most potent inhibitors the trivial names decynium22, iprecynium22, and disprocynium24. The other cyanine-related compounds tested had IC_{50}'s below 150 nmol/l (Table 3). The Hill-coefficients were near unity.

Table 1. Inhibition by various steroids and steroid-like compounds of the specific ^{3}H-noradrenaline uptake in Caki-1 cells. Shown are the IC_{50}'s with the corresponding 95% confidence intervals and the Hill-coefficients (n_{Hill}) with the corresponding SEM's ($n = 4$)

Substance	IC_{50} (µmol/l)	n_{Hill}
Corticosterone	0.13 (0.11, 0.17)[a]	1.22±0.08
17β-Estradiol	0.36 (0.30, 0.42)	1.11±0.07
Testosterone	1.5 (1.0, 2.2)	0.95±0.05
Progesterone	1.5 (0.8, 2.5)	1.03±0.06
Hydrocortisone	3.3 (2.8, 3.8)	1.10±0.11
Aldosterone	5.4 (3.2, 7.6)	0.96±0.08
Diethylstilbestrol	8.0 (5.7, 11.2)	1.14±0.02
Tamoxifen	26 (16, 41)	1.76±0.19
Dexamethasone	>10	

[a] Taken from Schömig and Schönfeld (1990)

Table 2. Inhibition by various quinolines and isoquinolines of the specific ^{3}H-noradrenaline uptake in Caki-1 cells. Shown are the IC_{50}'s with the corresponding 95% confidence intervals and the Hill-coefficients (n_{Hill}) with the corresponding SEM's ($n = 4$)

Substance	IC_{50} (µmol/l)	n_{Hill}
Cyanine 863	0.16 (0.12, 0.24)[b]	1.07±0.12
Papaverine	0.23 (0.12, 0.43)[a]	1.35±0.18
Moxaverine	0.50 (0.36, 0.68)	1.32±0.14
Acridine orange	1.1 (0.6, 1.9)	0.98±0.18
Quinine	1.9 (1.4, 2.5)[b]	1.28±0.16
Chloroquine	9.0 (4.0, 20)	1.59±0.24
Emetine	10 (6.0, 16)	1.14±0.02
Quinidine	22 (15, 31)[a]	1.43±0.18

[a] Taken from Schömig and Schönfeld (1990)
[b] Taken from Russ et al. (1992)

Inhibition of extraneuronal noradrenaline uptake in the isolated incubated rabbit aorta

Rabbit aortic strips were incubated for 10 min with 10 µmol/l ^{3}H-noradrenaline. MAO, COMT, and neuro-

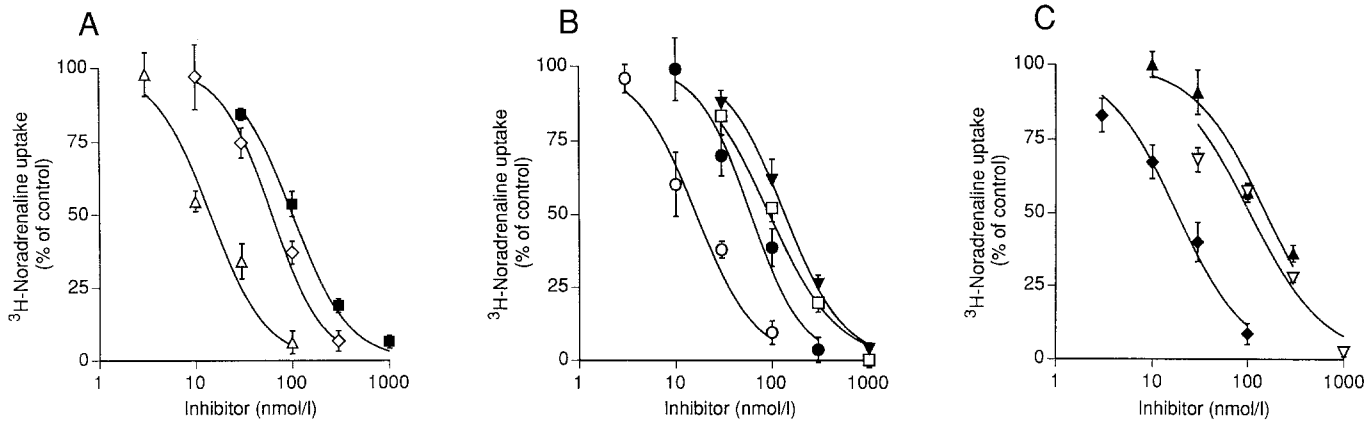
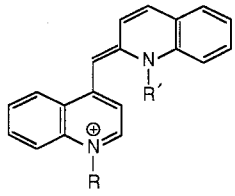
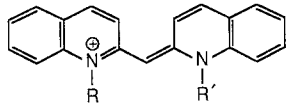
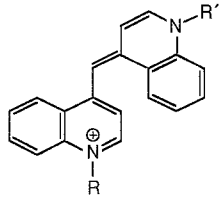
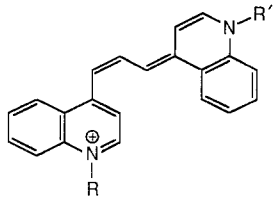
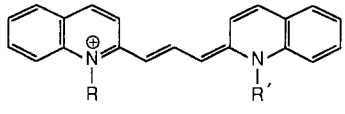


Fig. 1A–C. Inhibition of specific ^{3}H-noradrenaline uptake in Caki-1 cells. Initial rates of ^{3}H-noradrenaline (100 nmol/l) transport were determined in the presence of various cyanine-related compounds. Specific transport was defined as that fraction of total uptake which was sensitive to 1 µmol/l decynium22. Shown are means ± SEM ($n = 4-5$) of the specific uptake in the presence of the cyanine-related compounds relative to control. The codes for the cyanine-related compounds refer to the codes and their corresponding structural formulae in Table 3. **A** Disprocynium24 (△), Ic (◇), II (■). **B** Decynium22 (○), Ib (●), Ia (□), III (▼). **C** Iprecynium22 (◆), IV (▽), V (▲)

Table 3. Inhibition by various cyanine-related compounds of the specific ^{3}H-noradrenaline uptake in Caki-1 cells. Shown are the structural formulae, the codes, the IC_{50}'s with the corresponding 95% confidence intervals, and the Hill coefficients (n_{Hill}) with the corresponding SEM's ($n = 4-5$)

Chemical class	Structural formula	R	R′	Code	IC_{50} (nmol/l)	n_{Hill}
Isocyanines		-methyl	-methyl	Ia	100 (70, 150)	1.46 ± 0.17
		-ethyl	-ethyl	Ib	60 (35, 110)	1.63 ± 0.32
		-isopropyl	-methyl	Ic	62 (39, 98)	1.68 ± 0.40
		-isopropyl	-isopropyl	disprocynium24	14 (8, 25)	1.51 ± 0.28
Pseudoisocyanines		-methyl	-methyl	II	94 (56, 160)	1.25 ± 0.09
		-ethyl	-ethyl	decynium22	16 (9, 30)[a]	1.42 ± 0.11
		-isopropyl	-ethyl	iprecynium22	18 (10, 32)	1.20 ± 0.21
Cyanines		-ethyl	-ethyl	III	140 (100, 190)	1.44 ± 0.20
Carbocyanines		-ethyl	-ethyl	IV	110 (70, 150)	1.08 ± 0.09
		-ethyl	-ethyl	V	160 (100, 240)	1.17 ± 0.16

[a] Taken from Russ et al. (1992)

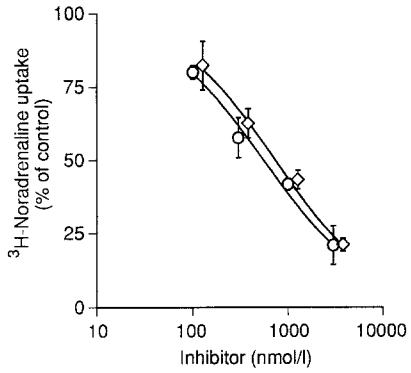


Fig. 2. Inhibition of specific ^{3}H-noradrenaline uptake in the incubated rabbit aorta. Strips prepared from the rabbit thoracic aorta were incubated for 10 min with 10 µmol/l ^{3}H-noradrenaline in the absence and presence of various concentrations of decynium22 (○) and compound Ic (◇). Specific uptake was defined as that fraction of total ^{3}H-noradrenaline uptake which was inhibited by 30 µmol/l corticosterone. Shown are means ± SEM ($n = 4$) of the specific uptake in the presence of the inhibitor relative to control

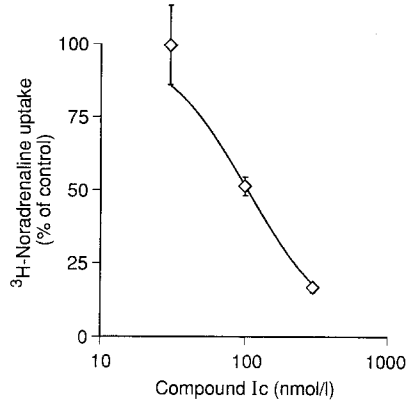


Fig. 3. Inhibition of specific ^{3}H-noradrenaline uptake in the isolated perfused rat heart. Isolated rat hearts were perfused with 100 nmol/l ^{3}H-noradrenaline for 2 min in the absence and presence of various concentrations of compound Ic. Specific uptake was defined as that fraction of total uptake which was sensitive to 100 µmol/l O-methylisoprenaline. Shown are means ± SEM ($n = 4$) of the specific uptake in the presence of the inhibitor relative to control

nal noradrenaline transport were inhibited by pargyline (10 µmol/l), U-0521 (10 µmol/l), and desipramine (1 µmol/l), respectively. In the absence of an inhibitor, the initial rate of specific ^{3}H-noradrenaline transport was 420 ± 48 fmol/(mg tissue·min). The effect of two cyanine-related compounds on ^{3}H-noradrenaline transport was tested. Decynium22 and compound Ic potently inhibited extraneuronal noradrenaline transport. The IC_{50}'s were 560 (240, 960) nmol/l and 740 (310, 1280) nmol/l, respectively ($n = 4$). The corresponding Hill-coefficients were 0.79 ± 0.05 for decynium22 and 0.82 ± 0.12 for compound Ic (Fig. 2).

Inhibition of extraneuronal noradrenaline uptake in the isolated perfused rat heart

In order to measure initial rates of uptake, isolated rat hearts were perfused for 2 min with 100 nmol/l ^{3}H-noradrenaline. MAO, COMT, and neuronal noradrenaline uptake were inhibited by pargyline (10 µmol/l), U-0521 (10 µmol/l), and desipramine (1 µmol/l), respectively. Specific uptake was defined as that fraction of total uptake which was sensitive to 100 µmol/l O-methylisoprenaline. Specific uptake amounted to 2.83 ± 0.08 pmol/(mg tissue·min) ($n = 4$). The IC_{50} of compound Ic for the inhibition of extraneuronal noradrenaline transport in the perfused rat heart was 100 (70, 140) nmol/l ($n = 4$). The Hill-coefficient was 1.45 ± 0.20 ($n = 4$) (Fig. 3).

Effect of decynium22 on cell viability

The effect of decynium22 on the viability of Caki-1 cells was investigated to exclude the possibility of a non-specific cytotoxic effect. Caki-1 cells were incubated for 45 min with 100 nmol/l decynium22 (6-fold IC_{50} for the inhibition of extraneuronal noradrenaline transport). Decynium22 did not reduce the incorporation of the cationic amino acid L-arginine (10 µmol/l) in Caki-1 cells. The rate of incorporation was 350 ± 30 pmol/(mg protein·min) in the absence and 340 ± 30 pmol/(mg protein·min) in the presence of decynium22 ($n = 3$). In addition, the viability of the cells measured by the trypan blue exclusion method was not affected by decynium22. Less than 3% of cells were stained.

Effect of decynium22 on the neuronal noradrenaline uptake in PC12 cells

In order to answer the question whether the cyanine-related compounds selectively block the extraneuronal noradrenaline transporter, the effect of decynium22 on the neuronal noradrenaline transporter was determined. PC12 cells were incubated for 1 min with 10 nmol/l ^{3}H-noradrenaline in order to measure initial rates of uptake. MAO and COMT were inhibited by pargyline (10 µmol/l) and U-0521 (10 µmol/l), respectively. Vesicular storage of catecholamines was avoided by preincubation of the cells with reserpine (10 µmol/l). Specific uptake was defined as that fraction of total ^{3}H-noradrenaline uptake which was sensitive to 1 µmol/l desipramine. It was 0.60 ± 0.01 pmol/(mg protein·min) ($n = 6$). The highest concentration of decynium22 tested (3 µmol/l – about 200-fold IC_{50} for the inhibition of the extraneuronal noradrenaline uptake) inhibited ^{3}H-noradrenaline uptake in PC12 cells by 15 ± 3%, only ($n = 6$).

Discussion

High-affinity ligands with dissociation constants below 100 nmol/l are valuable tools for both functional and biochemical characterisation of membrane-bound proteins. On the one hand, research on numerous membrane-bound receptors and ion channels strongly depends on the availability of selective and potent inhibitors. Various of these high-affinity ligands eventually turned out to be therapeutically useful compounds. On the other hand, only few high-affinity ligands exist for transmembrane transport systems, such as the tricyclic antidepressants which inhibit the neuronal transporters for biogenic

amines. The steroid corticosterone and the isoquinoline alkaloid papaverine were generally accepted as the most potent inhibitors of extraneuronal noradrenaline transport (Iversen and Salt 1970; Bönisch et al. 1982). In Caki-1 cells, the inhibition constants (K_i's) are 130 nmol/l and 230 nmol/l, respectively (Schömig and Schönfeld 1990). However, these affinities are insufficient for a variety of functional and biochemical methods. Thus, we looked for selective and more potent inhibitors of extraneuronal noradrenaline transport. Corticosterone and papaverine served as the starting points for this search.

In Caki-1 cells, we tested the effect of various steroids and steroid-like compounds on extraneuronal noradrenaline transport. With the exception of dexamethasone, all tested steroids as well as the steroid-like compounds, diethylstilbestrol and tamoxifene, inhibited extraneuronal noradrenaline transport (Table 1). The rank order of the inhibitory potencies was virtually identical with the rank order reported by Iversen and Salt (1970) who examined the inhibition by steroids of extraneuronal noradrenaline transport in the perfused rat heart. There was no relation between the potencies of the tested steroids to inhibit noradrenaline transport in Caki-1 cells and their antiinflammatory or sodium-retaining effects. The potent antiinflammatory steroid dexamethasone and the sodium-retaining steroid aldosterone were characterised by K_i's above 3 μmol/l. None of the tested steroids inhibited the extraneuronal noradrenaline transport more potently than corticosterone.

Interestingly enough, all quinoline and isoquinoline derivatives tested in this study interacted with the extraneuronal noradrenaline transporter. The K_i's ranged from 160 nmol/l for cyanine863 to 22 μmol/l for quinidine (Table 2). The remarkable diversity in the chemical structures of these compounds suggests that the presence of a quinoline or isoquinoline moiety per se is sufficient for a significant interaction with the extraneuronal noradrenaline transporter. Another common characteristic of the quinolines and isoquinolines listed in Table 2 is the positive charge at physiological pH. From various receptor ligand binding systems, it is known that most of the high-affinity ligands are quite lipophilic. Thus, for the search of high-affinity ligands, we focused on compounds which fulfil three criteria: the compound should (1) possess a quinoline or isoquinoline moiety, (2) bear a positive charge at physiological pH and (3) be characterised by some degree of lipophilicity.

The cyanine-related compounds prototypically met these criteria. The main characteristic of these compounds are two quinoline ring systems. In the case of the cyanines, the isocyanines, and the pseudoisocyanines, the bicyclic systems are connected by a methine bridge. In the case of the carbocyanines the bridge is lengthened by one vinylene unit (see Table 3). Cyanine-related compounds are permanently positively charged. Moreover, they are quite lipophilic, since the positive charge delocalizes over both bicyclic systems and the connecting bridge.

The cyanine-related compounds turned out to be very potent inhibitors of the extraneuronal noradrenaline transporter. Disprocynium24, iprecynium22, and decynium22 inhibited initial rates of specific noradrenaline transport into Caki-1 cells with K_i's below 20 nmol/l (Table 3). In other words, disprocynium24 – the most potent inhibitor – inhibited the extraneuronal noradrenaline transport one order of magnitude more potently than corticosterone or papaverine. In most ligand binding systems, the rate of association (k_{on}) is diffusion-limited, being about $5 \cdot 10^7$ l/(mol·min) for ligands with relative molecular weights of about 300 (Benveniste and Mayer 1991; Kishino et al. 1991). By assuming this rate constant for association, the rate constant for dissociation (k_{off}) of disprocynium24 at the extraneuronal noradrenaline transporter can be estimated, being 0.7 min^{-1} (Hulme and Birdsall 1992). Hence, the half-life of the disprocynium24-carrier complex is about 1 min. A half-life of the ligand-binding site complex of over 30 s is an important requirement for binding studies. It allows the separation of bound and free ligand by standard separation techniques (Bennett and Yamamura 1985). In other words, ligand-binding experiments at the extraneuronal noradrenaline transporter should now be possible with the highly potent cyanine-related compounds.

It was also of interest to investigate the effect of some of the cyanine-related compounds in more conventional models for the investigation of extraneuronal noradrenaline transport, namely the isolated incubated rabbit aorta and in the isolated perfused rat heart (Bönisch and Trendelenburg 1974; Henseling 1983). Based on the availability of the compounds at that time, we chose decynium22 and compound Ic for the experiments with the isolated incubated rabbit aorta and compound Ic for the experiments with the isolated perfused rat heart. In both organs, the cyanine-related compounds inhibited noradrenaline transport with potencies markedly higher than those of the most potent inhibitors known so far (Iversen 1965; Salt 1972; Bönisch et al. 1982). In the incubated rabbit aorta, however, the K_i's of compound Ic and decynium22 were higher than in Caki-1 cells by a factor of about 12 and 35, respectively. This discrepancy is most likely due to limited diffusion in the incubated organ. In an incubated organ, active transmembrane transport systems and metabolising enzymes produce steady state concentration gradients for substrates within the extracellular space (Green 1976; Schömig et al. 1992). Beneath the surface of an incubated organ, the substrate concentration equals the concentration in the incubation medium. Towards the centre of the tissue, the extracellular substrate concentration declines. Under these circumstances, the use of a transport inhibitor has several consequences. On the one hand, uptake decreases due to direct inhibition of the transporters. On the other hand, the blockade of transporters tends to flatten the concentration gradient with the effect that transporters near to the centre of the tissue are exposed to higher substrate concentrations than in the absence of an inhibitor. The latter phenomenon counteracts the effect of an inhibitor and causes an underestimation of inhibitory potencies. Schömig and Schönfeld (1990) found that the inhibitory potencies of various compounds for the inhibition of extraneuronal noradrenaline transport were consistently about 10 times lower in the isolated incubated rabbit aorta than in the

Caki-1 tissue culture system. In Caki-1 cells and in the perfused organ, on the other hand, diffusion barriers are negligible. The K_i of compound Ic for the inhibition of extraneuronal noradrenaline transport in the perfused rat heart corresponded with the K_i in Caki-1 cells.

The finding that decynium22 affected neither the viability of Caki-1 cells, measured by the trypan blue exclusion method, nor the incorporation of the cationic amino acid L-arginine excluded the possibility of a non-specific cytotoxic action. To test the selectivity of the cyanine-related inhibitors, the effect of a representative compound, decynium22, on the desipramine-sensitive neuronal type of noradrenaline transporter was investigated in clonal rat phaeochromocytoma cells (PC12). PC12 cells express many properties characteristic of noradrenergic neurones including the neuronal uptake mechanism for noradrenaline (Greene and Tischler 1982; Harder and Bönisch 1984). Up to the highest concentration tested (3 μmol/l), decynium22 affected the neuronal type of noradrenaline transporter by a very small margin only. Recently, surprising similarities between extraneuronal transport of noradrenaline and renal transport of organic cations were reported (Schömig and Schönfeld 1990). The fact that decynium22 also inhibits the renal transporter with high potency further supports the view of a close relationship (Schömig et al. 1993).

Cyanine-related compounds are a new class of selective and highly potent inhibitors of the extraneuronal noradrenaline transporter. Even the weakest members of this new class of inhibitors were as potent as corticosterone which hitherto was generally accepted as the most potent inhibitor of extraneuronal noradrenaline transport. We expect the highly potent cyanine-related inhibitors to be helpful for both functional and biochemical characterisation of the extraneuronal noradrenaline transporter. Regarding the high affinities of some of these compounds, ligand binding studies should be possible in future. The use of cyanine-related inhibitors in in vivo experiments may help to define the physiological role of extraneuronal noradrenaline transport more precisely.

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