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Assessing the molecular basis for rat-selective induction of the mitochondrial permeability transition by norbormide

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Abstract

It was recently demonstrated that the rat-selective toxicant norbormide also induces rat-selective opening of the permeability transition pore (PTP) in isolated mitochondria. Norbormide is a mixture of *endo* and *exo* stereoisomers; however, only the *endo* forms are lethal to rats. In the present study we tested both *endo* and *exo* isomers as well as neutral and cationic derivatives of norbormide to: (i) verify if the PTP-regulatory activity by norbormide is stereospecific; (ii) define the structural features of norbormide responsible for PTP-activation, (iii) elucidate the basis for the drug species-specificity. Our results show that: (i) norbormide isomers affect PTP in a rat-selective fashion; however, no relevant differences between lethal and non-lethal forms are observed suggesting that drug regulation of PTP-activity and lethality in rats are unrelated phenomena; (ii) a (phenylvinyl)pyridine moiety represents the key element conferring the PTP-activating effect; (iii) cationic derivatives of rat-active compounds accumulate in the matrix *via* the membrane potential and activate the PTP also in mouse and guinea pig mitochondria. These findings suggest that the norbormide-sensitive PTP-target is present in all species examined, and is presumably located on the matrix side. The species-selectivity may depend on the unique properties of a transport system allowing drug internalisation in rat mitochondria.

Keywords: Norbormide; Rat-toxicant; Mitochondria; Permeability transition; Species-specificity

1. Introduction

Norbormide[5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide] (NRB) is a toxic compound endowed with unique species-selective vasoconstrictor activity that is restricted to the peripheral vessels of the rat [1–6]. NRB is a mixture of eight racemic stereoisomers, which strongly differ in their vasoconstrictor activity and toxicity [7–9]. Investigations carried out *in vitro* and *in vivo* on the individual isomers of NRB demonstrated that the toxic effects of the parent compound are strongly stereospecific. Indeed, only the *endo* isomers of NRB retain the rat-selective vasoconstrictor and toxic activity elicited by the mixture [9]. The mechanisms of NRB action have yet to be clarified but available evidence suggests that the vasoconstrictor effect may be mediated by the stimulation of a number of signal transduction pathways that lead to modulation of calcium influx, presumably mediated by phospholipase C (PLC)-coupled receptors expressed in rat peripheral vascular myocytes [5].

Earlier studies demonstrated that in isolated mitochondria NRB also causes rat-selective dysfunction [10] that could be traced to the opening of the inner membrane permeability transition (PT) pore (PTP) [11], a proteinaceous channel involved in several forms of cell death [12]. It was suggested that NRB activates the PT through a transport system unique to the rat that

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allows internalisation of the drug across the mitochondrial membranes. This putative carrier was postulated to be absent in other species, or shielded from contact with the drug owing to different membrane structural arrangements [11].

The present paper was aimed at investigating whether the species-specific PTP modulation and toxicity are causally linked, and at defining the structural determinants of the effects of NRB on the PTP. To address these questions, we studied the PTP-regulatory properties of both vasoconstrictor and non-vasoconstrictor NRB isomers by following the Ca^{2+} retention capacity (a very sensitive method to detect PTP opening) in isolated rat, mouse and guinea pig liver mitochondria. In addition, a series of NRB analogues derived from the "deconstruction" of the parent molecule was evaluated in order to assess the structural features responsible for PTP activation. The mechanism of NRB action was further investigated by carrying out studies with cationic derivatives of the drug, which should accumulate in mitochondria driven by the inside-negative membrane potential.

Our results show that (i) there may be no direct link between NRB-induced mitochondrial dysfunction and lethal vasoconstriction in rats; (ii) the (phenylvinyl)pyridine subunit at C-7 is the key element conferring the PTP-activating effects to the NRB molecule; and (iii) NRB as well as its active core interacts poorly with guinea pig and mouse mitochondria unless the drug molecule is positively charged, thus allowing matrix accumulation *via* membrane potential and subsequent triggering of the PT. This latter finding indicates that the NRB-sensitive, PTP-reactive site is present in all animal species, and that the species-selectivity towards the PT depends on the unique properties of a transport system, which facilitates drug internalisation in rat mitochondria. Part of this work has been presented in Abstract Form at the 14th EBEC, Moscow, 22–27 July 2006 [13].

2. Materials and methods

Norbormide (NRB) was a generous gift of I.N.D.I.A. Industria Chimica, Padova (Italy).All isolated NRB isomers [7,8] and the following NRB analogues [14] were prepared as previously described: *endo*-7-(α-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide (DR085), 2-(1-phenylvinyl)pyridine (DR166), 2-(1-phenyl-2-ethanol)pyridine (DR282), 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-phenylbenzylidene)-5-norbornene-2,3-dicarboximide (DR488) and 5-(α-hydroxy-α-2-pyridylbenzyl)-5-norbornene-2,3-dicarboximide (DR496).

Norbornene-2,3-dicarboximide (DR067), *endo*-7-(α-2-phenylbenzylidene)-5-norbornene-2,3-dicarboximide (DR068), 2-benzylpyridine (DR303) and 2-(1-phenylethyl)pyridine (DR322) as well as the cationic derivatives: *N*-ethyl-2-(1-phenylvinyl)pyridinium trifluoromethanesulfonate (DR380),5-(α-hydroxy-α-2-pyridylbenzyl)-7-(*N*-pivaloyloxymethyl-α-2-pyridiniumbenzylidene)-5-norbornene-2,3-dicarboximide iodide (OL14), *N*-pivaloyloxymethyl-2-(1-phenylvinyl) pyridinium iodide (JS023) and *N*-pivaloyloxymethyl-2-(1-phenyl-2-ethanol)pyridinium iodide (DR656) were synthesized by Rennison, D., Laita, O., and Stäb J. (unpublished data).

1,1-Diphenylethylene (DR598) was obtained commercially (Aldrich).

Liver mitochondria from albino Wistar rats, CD1 mice and albino guinea pigs were prepared by standard differential centrifugation. The final pellet was suspended in 0.25 M sucrose to give a protein concentration of 80–100 mg/ml, as measured by the biuret method.

Mitochondrial oxygen consumption was measured in a thermostated (T=25 °C), water-jacketed vessel, using a Clark electrode connected to a recorder.

Mitochondrial PT was induced at 25 °C in a medium containing 200 mM sucrose, 10 mM Tris–Mops, 10 μM EGTA–Tris, 1 mM P_i, 0.5 μg/ml oligomycin, 5 mM succinate, 2 μM rotenone, pH 7.4 (standard medium) [15]. Ca^{2+} was used as PT inducer.

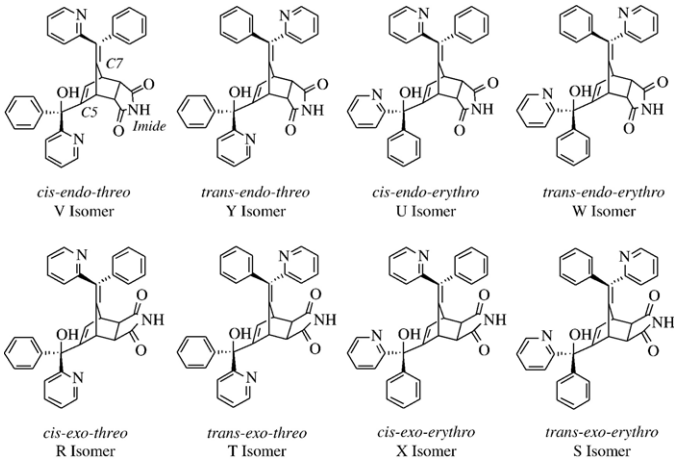
Extramitochondrial Ca^{2+} was measured fluorimetrically using Calcium Green-5 N, a membrane impermeant probe, which exhibits an increase in fluorescence emission intensity upon Ca^{2+} binding (excitation–emission λ: 480–530 nm).

Mitochondrial membrane potential ($\Delta\Psi_m$) changes were followed based on the accumulation of pyronin G (Pyr G; 3 μM) as monitored by the changes in emission fluorescence intensity at λ=580 nm (excitation λ=520 nm) [16].

Fluidity changes of mitochondrial membranes promoted by NRB and NRB analogues were evaluated by the changes in fluorescence anisotropy of 1,6-phenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluene sulfonate (TMA- DPH), which bind internal hydrophobic and polar heads/lipid backbone interfaces of the membrane lipid bilayer, respectively [17]. DPH- and TMA-DPH-labelled mitochondria were prepared by treating mitochondrial suspensions (20 mg/ml) with 300 μM probe. After 20 min (DPH) or 5 min (TMA-DPH) incubation under continuous stirring at T=25 °C, the mitochondrial suspensions were diluted to 0.2 mg/ml for anisotropy measurements. The fluorescence anisotropy (r) was collected at 340 nm (λ_{em}=460 nm) (for DPH) and 360 nm (λ_{em}=430 nm) (for TMA-DPH) by calculating the I_{VV} and I_{VH}, i.e. the fluorescence intensities polarized parallel and perpendicular to the vertical plane of polarization of the excitation beam respectively. The anisotropy (r) is defined by the equation $r=(I_{VV}-GI_{VH})/(I_{VV}+2GI_{VH})$, where $G=I_{HV}/I_{HH}$ is the correction factor for instrumental artefacts.

3. Results

NRB is a mixture of eight possible stereoisomers each of which is a racemate (see the configurations below). Since the S and U isomers are present in trace amounts, only the six most abundant isomers (V, Y, W, R, T, X) were tested for their PT-inducing activity on isolated mitochondria. Among them, only the *endo*-isomers V, Y, and W induce vasoconstriction and death in rats whereas the *exo*-forms R, T and X are ineffective [7–9].



PTP activation was tested by measuring the calcium retention capacity (CRC) of mitochondria, as detected by the fluorescence changes of the Ca^{2+} indicator, Calcium Green 5N, after 5 min incubation with the different NRB isomers (see Ref. [11]).

Fig. 1 shows the effects of 50 μM isomer V and X (as representative of the lethal and non lethal species, respectively) on the CRC of rat liver mitochondria. Untreated mitochondria

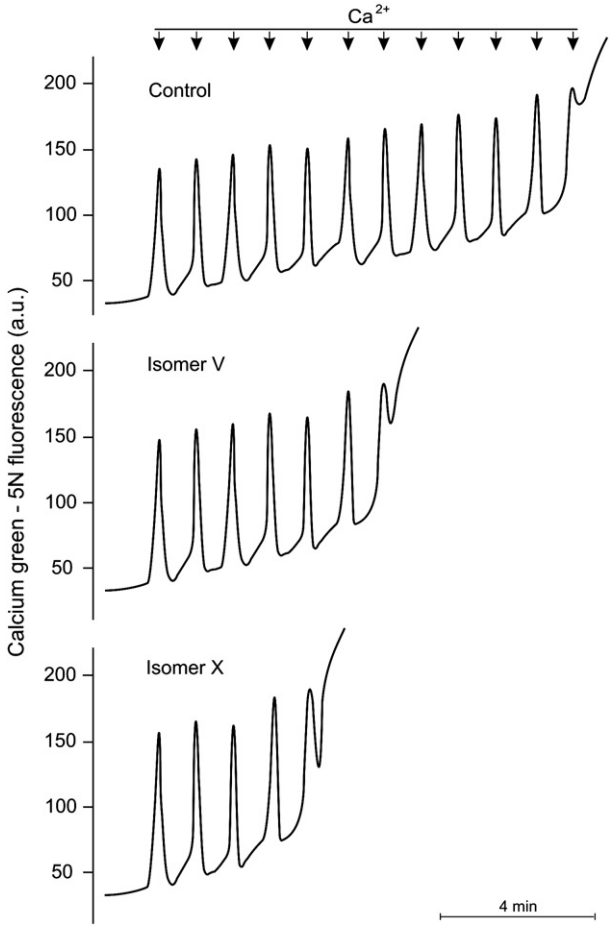


Fig. 1. Effects of the isomers X and V of NRB on the CRC of rat liver mitochondria. The experiments were started by the addition of 1 mg/ml mitochondria (not shown) to the standard incubation medium. Rat liver mitochondria were loaded with a train of 10 μM Ca^{2+} pulses at 1-min intervals. Mitochondria were pre-incubated for 5 min without (control) or with 50 μM X or V before addition of Ca^{2+}. Extramitochondrial Ca^{2+} was monitored as the fluorescence emission intensity of 0.5 μM Calcium Green-5 N ($\lambda_{excitation}$=480 nm; $\lambda_{emission}$=530 nm).

were loaded with a train of 10 μM Ca^{2+} pulses at 1-min intervals. Each Ca^{2+} addition gave rise to rapid increases of the Calcium Green 5 N fluorescence, followed by a return to the original steady-state value as Ca^{2+} was taken up by mitochondria. When the loading threshold of about 120 nmol of Ca^{2+}/mg protein was reached, a net release of Ca^{2+} into the medium was observed. Incubation with 50 μM NRB isomers V and X for 5 min decreased the Ca^{2+} load required for PTP opening to 70 and 50 nmol of Ca^{2+}/mg protein, respectively. In all cases, the addition of 1 μM cyclosporin A doubled the CRC, indicating that Ca^{2+} release was due to opening of the PTP (data not shown). Dose–response tests indicated that all NRB isomers decreased the CRC of rat mitochondria in the concentration range 5–100 μM, the maximal effect being reached at approximately 40 μM (data not shown).

Fig. 2 compares the effects of low (10 μM) and high (50 μM) concentrations of the NRB mixture and of the individual isomers on the CRC in rat, mouse and guinea pig mitochondria. Clearly, all isomers were rat-selective in inducing the mitochondrial PT, and all were effective at relatively low concentrations. Importantly, no significant difference could be detected between toxic (V, Y, W) and non toxic (R, X, T) isomers.

In order to better define the key structural features of NRB responsible for PTP activation in rat mitochondria, we evaluated several related analogues, synthesized on the basis of a selected "deconstruction" of the parent molecule (Table 1). Table 1 also shows the structure–function correlation for the PT-inducing effects on rat mitochondria as deduced from the values of CRC, expressed as percent of the control (CRC of untreated mitochondria). Substructures DR282 and DR067 exhibited marginal effects (around 20% of CRC loss), both as independent units or when combined in analogue DR496 (both in *endo* or *exo* configurations). Analogue DR085 was nearly as active as the NRB mixture while DR166 displayed the highest PT-inducing effect. All NRB substructures tested were found to have a negligible effect on mitochondria from guinea pig and mouse (Table 1).

The next set of experiments was aimed at investigating whether changes in the chemical structure of the most active compounds (NRB, DR085 and DR166) influenced their activity in rat mitochondria. To this purpose, we analyzed their analogues DR488, DR068 and DR598, in which the C-7 pyridyl ring was replaced by a phenyl ring, along with

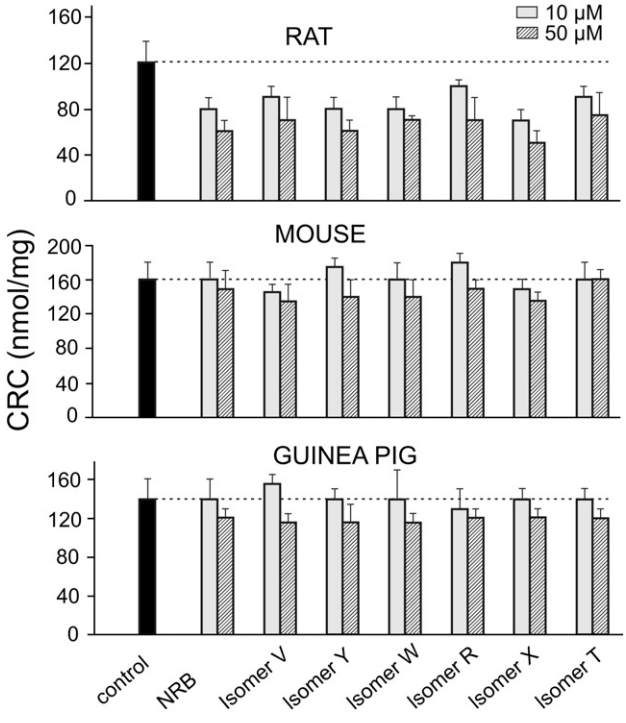
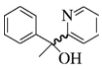
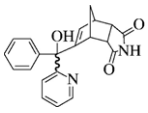
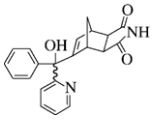
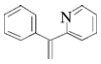
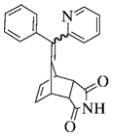
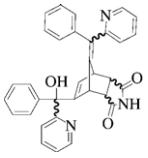


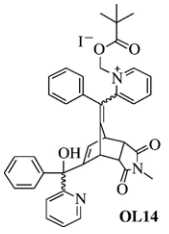
Fig. 2. CRC of rat, mouse and guinea pig liver mitochondria in the presence of the NRB isomers V, Y, W, R, X, T at 10 and 50 μM. The CRC was calculated according to the experimental procedure described in the legend to Fig. 1. Data are expressed as mean (±SD) of three independent determinations. Statistical analysis indicated that the data for rat mitochondria incubated with NRB and NRB isomers are significantly different from those of the controls (unpaired *t*-test, $P<0.01$).

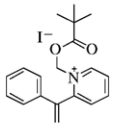
Table 1
CRC of rat, mouse and guinea pig liver mitochondria in the presence of NRB and of the substructures: DR282, DR067, DR496(endo), DR496(exo), DR085, DR166

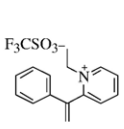
CRC (% of the control)				
Structure	Compound	Rat	Mouse	Guinea pig
	DR282	80±10	90±10	85±5
	DR067	85±10	100±5	100±5
	DR496 endo	80±20	85±10	90±10
	DR496 exo	90±10	85±20	90±10
	DR166	35±5	90±10	90±5
	DR085	55±5	90±5	90±10
	NRB	50±10	100±5	100±10

The Ca^{2+} retention capacity (expressed as % of the control) was calculated according to the experimental procedure described in the legend to Fig. 1. Drug concentration was 50 μM. Data are expressed as mean (±SD) of four independent determinations.

analogues of DR166, in which the terminal methylene group was removed (DR303) or replaced by a methyl group (DR322).


OL14


JS023


DR380

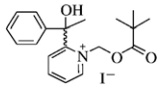
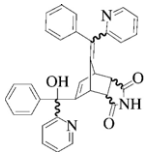
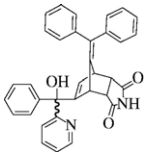
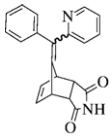
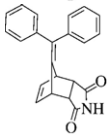
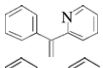
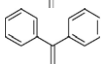
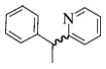
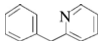

DR656

Table 2 reports the comparison between the CRC values of the modified analogues and the corresponding original molecules. Chemical modifications to the original structures of the active compounds markedly reduced their PT-inducing activity. Thus, both the C-7 pyridyl N-atom and the terminal methylene group were important in conferring high PTP activation potential to the NRB molecule. Such modified NRB analogues were also ineffective in guinea pig and mouse mitochondria (Table 2).

We next carried out a set of experiments with cationic derivatives of NRB, which should accumulate inside the mitochondria driven by the transmembrane potential (negative inside) of the inner membrane. The following cationic analogues (OL14, JS023, DR380) based on the most active compounds (NRB and DR166) towards the PT were considered together with a cationic derivative (DR656) of the inactive analogue DR282.

Fig. 3 shows the CRC of rat mitochondria obtained in the presence of the selected cationic drugs at 50 μM concentration,

Table 2
CRC of rat, mouse and guinea pig liver mitochondria in the presence of NRB and of the substructures: DR488, DR085, DR068, DR166, DR598, DR303, DR322

CRC (% of the control)				
Structure	Compound	Rat	Mouse	Guinea pig
	NRB	50±10	100±5	100±10
	DR488	80±5	90±10	90±10
	DR085	55±5	90±5	90±10
	DR068	85±10	85±10	85±5
	DR166	35±5	90±10	90±5
	DR598	85±10	90±10	95±10
	DR322	70±5	100±5	75±5
	DR303	85±10	100±5	90±10

The Ca^{2+} retention capacity (expressed as % of the control) was calculated according to the experimental procedure described in the legend to Fig. 1. Drug concentration was 50 μM. Data are expressed as mean (±SD) of four independent determinations.

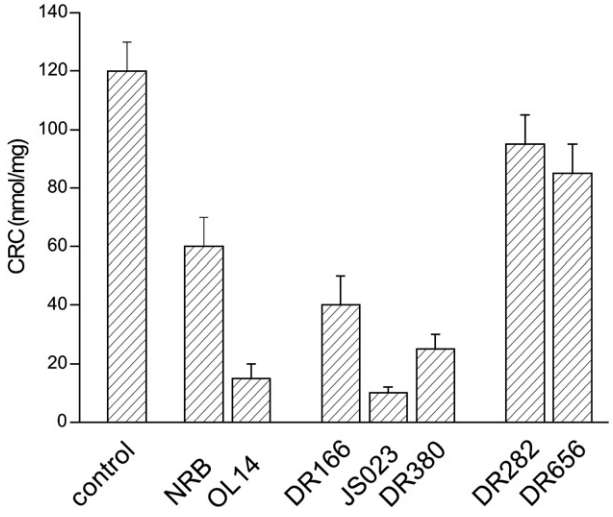


Fig. 3. Comparison between the CRC of rat liver mitochondria loaded with the cationic compounds: OL14, JS023, DR380, and DR656 and their neutral analogues: NRB, DR166, and DR282. The concentration of all NRB derivatives was 50 μM. The CRC was calculated according to the experimental procedure described in the legend to Fig. 1. Data are expressed as mean (±SD) of three independent determinations. Statistical analysis indicated that the data obtained in the presence of NRB, DR166, OL14, JS023, DR380 are significantly different from those of the control (unpaired *t*-test, $P<0.01$).

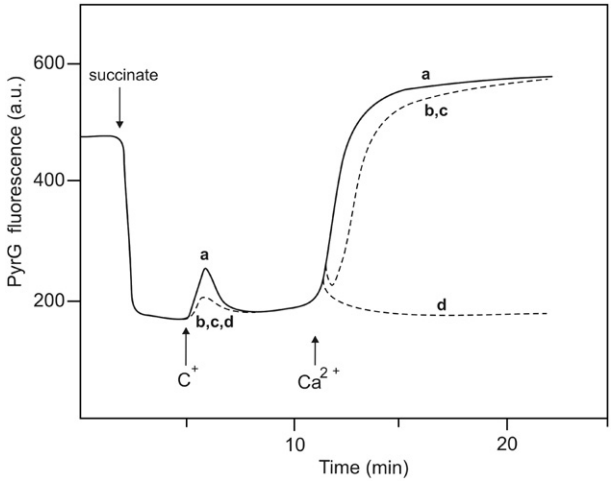


Fig. 5. Dependence of PyrG fluorescence on the mitochondrial membrane potential in the presence of cationic NRB derivatives (100 μM). Rat liver mitochondria (1 mg/ml), incubated with 3 μM Pyr G, were suspended in the standard medium in the absence of an energizing substrate. Pyr G fluorescence (in arbitrary units) was followed by exciting at 520 nm (emission=580 nm). Where indicated (arrows), succinate (5 mM), OL14 (trace a), JS023 (trace b), DR380 (trace c), DR656 (trace d) or CaCl2 (10 μM) was added.

and compares the results to those obtained with the corresponding neutral analogues. Clearly, the cationic derivatives of both the parent NRB molecule and of DR166 were much more effective in stimulating the PT. Indeed, the Ca^{2+} accumulated and retained before occurrence of the PT decreased from 40–60 nmol/mg to 10–30 nmol/mg. The cationic drugs bearing a *N*-pivaloyloxymethyl group (JS023 and OL14) displayed higher PT-inducing efficacy than displayed by the compound bearing a *N*-ethyl group (DR380), possibly as a consequence of a higher degree of lipophilicity. On the contrary, no significant changes in CRC were observed after insertion of a positive charge into DR282, thus confirming that this moiety does not affect the PT. The dramatic increase in PT-inducing potency of the most active compounds after addition of a

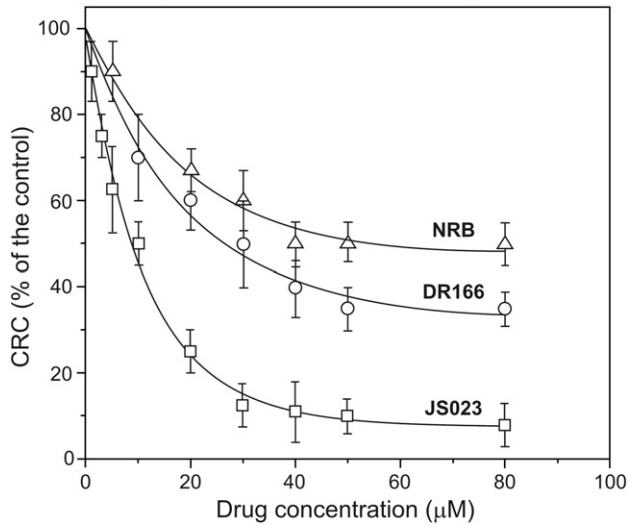


Fig. 4. CRC of rat liver mitochondria loaded with increasing concentrations of JS023, DR166 and NRB. The CRC was calculated according to the experimental procedure described in the legend to Fig. 1. Data are expressed as mean (±SD) of three independent determinations.

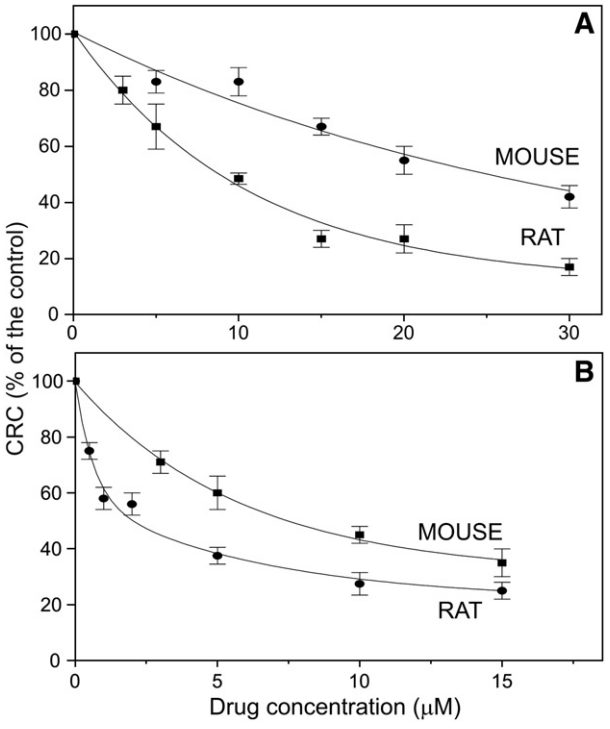


Fig. 6. CRC of rat and mouse liver mitochondria in the presence of increasing concentrations of JS023 (A) and OL14 (B). The CRC was calculated according to the experimental procedure described in the legend to Fig. 1. CRC values are expressed as percentage of the corresponding controls (untreated mitochondria). Data are means (±SD) of three independent determinations. Results similar to those of mouse were obtained for guinea pig liver mitochondria.

positive charge can be better evaluated by the dose-dependence data of Fig. 4, which compares, as an example, the CRC values of mitochondria supplemented with the cationic active "core" (JS023), with its neutral analogue (DR166) and with the parent molecule (NRB) (Fig. 4).

All the selected cationic NRB derivatives accumulated into the mitochondrial matrix, as tested by measuring the inner membrane potential through the fluorescence changes of Pyr G. As shown in Fig. 5, addition of succinate to rat liver mitochondria initiated a process of fluorescence quenching of Pyr G, which was due to accumulation of the probe inside energized mitochondria. Addition of the cations (C^+, 100 μM) OL14 (trace a), JS023 (trace b), DR380 (trace c) or DR656 (trace d) caused a cycle of probe release-reuptake, which indicates a transient membrane depolarization due to cation transport into the mitochondrial matrix. Addition of a small pulse of Ca^{2+} caused collapse of the membrane potential in mitochondria treated with OL14, JS023 and DR380. The collapse of membrane potential was not due to the addition of Ca^{2+} *per se* (e.g., trace d) but rather to PTP opening, as confirmed by the effect of CsA, which prevented the Ca^{2+}-dependent fluorescence increase (not shown). In contrast, only a transient depolarization due to Ca^{2+} transport in the matrix was observed with DR656. Similar results were obtained for mouse and guinea pig mitochondria (results not shown).

The availability of active cationic derivatives which accumulate in the matrix provides a unique opportunity to test whether the lack of effects of NRB in mouse and guinea pig mitochondria depends on species-specific differences in PTP structure or rather in drug transport. We therefore compared the effects of JS023 (Fig. 6A) and OL14 (Fig. 6B) on the PT of rat and mouse mitochondria. Strikingly, both cationic drugs were able to induce opening of the PTP also in mouse mitochondria, although higher concentrations were necessary to obtain effects similar to those observed in rat mitochondria. Similar results were observed with guinea pig mitochondria (not shown).

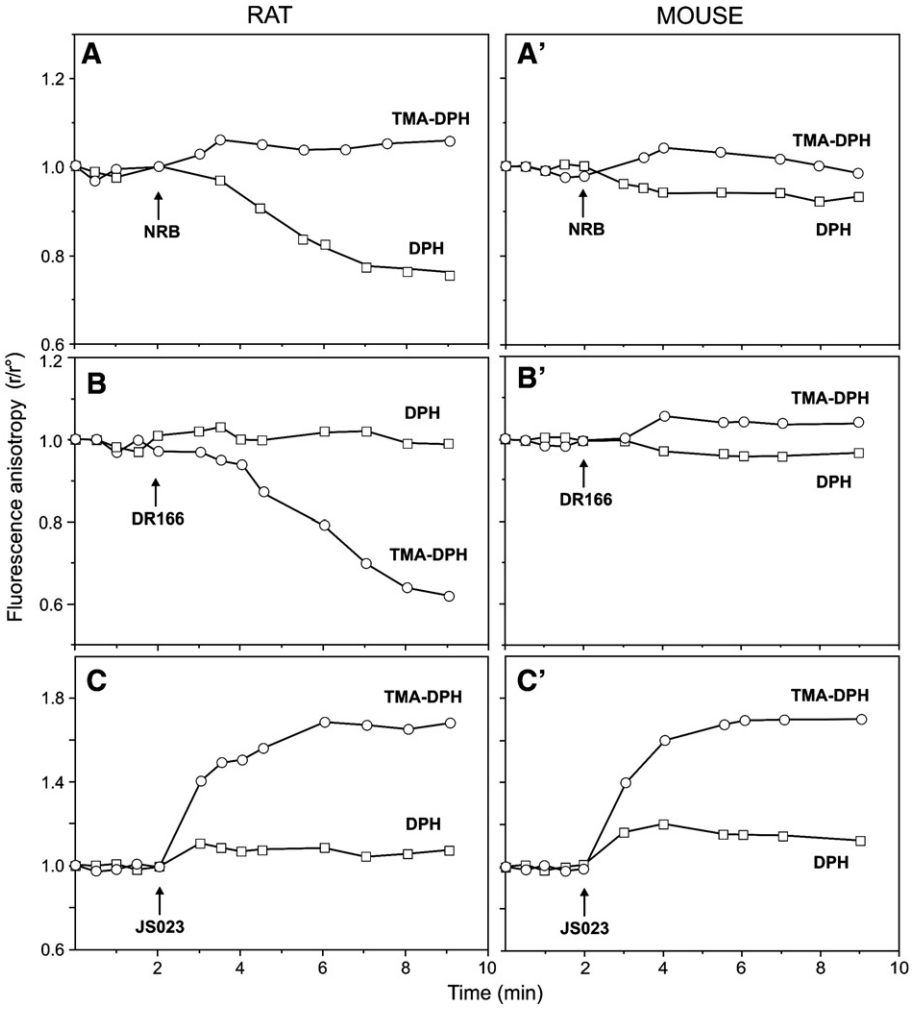


Fig. 7. Effects of NRB, DR166 and JS023 on the fluorescence anisotropy of DPH-, and TMA-DPH-labelled rat and mouse mitochondria. DPH- and TMA-DPH-labelled mitochondria were prepared by treating mitochondrial suspensions (20 mg/ml) with 300 μM probe. After 20 min (DPH) or 5 min (TMA-DPH) incubation under continuous stirring at T=25 °C, the mitochondrial suspensions were diluted to 0.2 mg/ml for anisotropy measurements. The fluorescence anisotropies, r, were collected at 340 nm (λ_{em}=460 nm) (for DPH) and 360 nm (λ_{em}=430 nm) (for TMA-DPH). All anisotropy intensities were normalized to those ($r°$) observed before addition of 50 μM NRB (A, A'), DR166 (B, B'), JS023 (C, C') (for rat mitochondria, $r°$s of DPH and TMA-DPH were, respectively, 0.14±0.01 and 0.09±0.02; for mouse mitochondria the corresponding $r°$s were: 0.16±0.02 and 0.1±0.02).

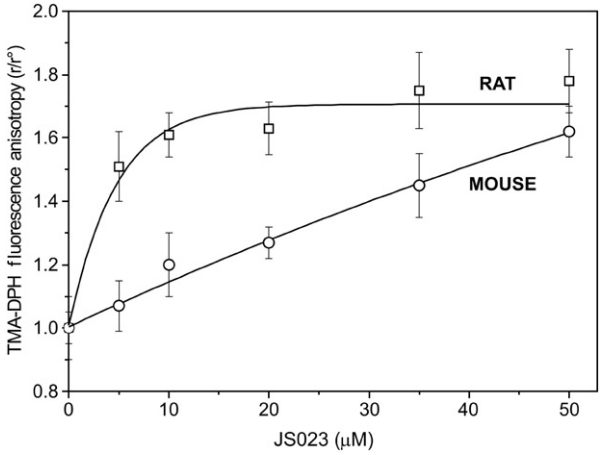


Fig. 8. Effects of increasing concentrations of JS023 on the fluorescence anisotropy of TMA-DPH-labelled rat and mouse mitochondria. The experimental procedure was the same as described in the legend to Fig. 6. All anisotropy intensities were normalized to those (r^o) observed before addition of JS023 (see legend to Fig. 6). Data are means (±SD) of three independent determinations.

In order to identify which membrane domains were perturbed by NRB and NRB analogues, we measured the fluorescence anisotropy changes of DPH- and TMA-DPH-labelled mitochondria (in the presence of CsA to prevent opening of the PTP). In particular, we investigated whether the increase of PT-inducing efficacy correlated with a change in the distribution pattern of the drug in the membrane. We compared the effects of the whole molecule with those of its active subunit, DR166, along with the cationic derivative JS023, at 50 μM concentrations (Fig. 7). In rat mitochondria (panel A), NRB mostly affected the hydrophobic core of the lipid bilayer, as detected by the changes in DPH anisotropy. The decline of anisotropy intensity is indicative of an increase in lipid fluidity [11,17]. No relevant structural modifications of the polar heads/lipid backbone interface regions, as monitored by TMA-DPH anisotropy [17], were apparent. These data suggest a preferential accommodation of NRB in the interior of the lipid bilayer. In contrast, DR166 (panel B) mostly perturbed the lipid domains sensed by TMA-DPH, which suggests a preferential accumulation of this fragment in the lipid tail/polar heads border areas. Such lipid domains should mainly reside in the matrix-exposed leaflet of the inner membrane bilayer since we found that TMA-DPH is transported in the inner membrane interior by charge effects (data not shown). Accumulation of the cationic drug JS023 in the matrix-exposed regions caused a relevant increase of TMA-DPH anisotropy (panel C), which reflects a structural immobilization of these membrane domains, probably due to ionic interactions between JS023 and the negatively charged phospholipid heads. Similar effects were observed with other cationic NRB analogues, such as OL14 and DR380 (data not shown).

In mouse mitochondria (Fig. 7, panels A′, B′), DPH and TMA-DPH reported only minor changes in the membrane dynamic properties upon interaction with the neutral compounds, NRB and DR166; on the other hand, the cationic analogue JS023 (panel C′) exhibited effects similar to those observed in rat mitochondria. It is noteworthy that rat and mouse mitochondria displayed a marked difference in TMA-DPH anisotropy changes at increasing concentrations of JS023 below 50 μM (Fig. 8). With mouse mitochondria, higher concentrations of cationic drug were necessary to induce increases of the probe anisotropy comparable to those obtained in rat mitochondria. Strikingly, the dose-dependent anisotropy trend closely matched that obtained for PT induction (Fig. 8, compare with Fig. 6A).

4. Discussion

The mechanisms underlying the rat-selective vasoconstriction effect and toxicity of NRB are still poorly understood. Detailed studies of the individual stereoisomers of NRB demonstrated that both drug-induced contractile activity and lethality in rats are strongly modulated by the molecular isomerism, only the *endo* configurations retaining the pathological effects elicited by the mixture [7]. These results indicate that binding of NRB *in vivo* is stereospecific, thereby suggesting the existence of a drug receptor uniquely expressed in the myocytes of the rat peripheral vessels. Moreover, recent experiments on the rat caudal artery with selected fragments of the parent molecule demonstrated that groups at all three sites (C-7, C-5 and imide) must be retained for NRB-type vasoconstriction, the "*endo*-type" isomerism not being sufficient to cause toxic effects *per se* [14].

Intriguingly, NRB was recently shown to cause rat-selective opening of the PTP in isolated mitochondria [11], suggesting that *in vivo* mitochondrial dysfunction could be a potential physiological pathway leading to death in rats. To test this hypothesis, we evaluated the PT-inducing effects of rat-toxic (*endo*-isomers V, Y, W) and non toxic (*exo*-isomers R, X, T) compounds on isolated liver mitochondria. The results obtained from our experiments show that both lethal and non lethal NRB isomers display comparable stimulatory effects on the PT. Yet, all PTP-active isomers maintained a strict species-selectivity for the rat.

Although a direct correlation between NRB-induced mitochondrial dysfunction and lethal vasoconstriction is not obvious from these data, the possibility of PTP being a cause of toxicity in the rat cannot be totally dismissed. As a matter of fact, rat-selective PTP opening in isolated mitochondria suggests that NRB may specifically affect rat mitochondria *in vivo* as well. Indeed, studies on mitochondria isolated from liver of rats previously subjected to NRB stress showed that mitochondria were damaged to some extent and that energy-dependent reactions were impaired (10). Very importantly, all compounds lethal to rats are also PTP-active in isolated mitochondria. Although we observed induction of the PT in isolated mitochondria also by non-lethal NRB isoforms, our findings indicate that transport may be a key factor determining bioavailability to mitochondria *in situ*. Thus, the possibility exists that the exo-isomers do not reach mitochondria in the tissues that are critical for toxicity. An alternative hypothesis is that mitochondrial damage could play a role in NRB toxicity as a secondary, potentiating event.

In order to test whether the whole NRB molecule is necessary (as found for the vasoconstriction effect) or whether a specific active core is sufficient to account for the NRB effects

on mitochondrial function, we assayed the PT-inducing efficacy of various NRB analogues, probing the parent molecule's three key structural features (substituents at C-5, C-7 and the imide unit). Our results indicate that neither the group at C-5 nor the imide group play a critical role in PT induction, whereas the (phenylvinyl)pyridine subunit at C-7 remains active, being sufficient for PTP activation. The possibility that this subunit is the key element conferring toxicity to NRB molecule also *in vivo* cannot be ruled out: actually, molecular integrity and *endo*-type stereoisomerism could be necessary for properly modulating the binding of NRB to its cell receptors, the whole molecular scaffold then functioning as a vector for the active feature responsible for the rat-unique effects. Clearly, further studies are necessary in order to better understand the mode of action and selectivity of NRB and the possible correlations between the different pathological effects.

If the hypothesis of a direct link between NRB-induced mitochondrial dysfunction and vasoconstriction is correct, a plausible mechanistic explanation should take into account the unique behaviour of the rat mitochondrial system towards the drug. The selectivity of PTP opening in isolated mitochondria, in fact, would suggest that the interaction mode of NRB in rat mitochondria is different from that taking place in mitochondria from other animal species *in vivo* as well. This difference could be due to a different pore structure/target or to a different modality of drug internalisation. To better clarify this point, we used cationic derivatives of the drug which were shown to enter mitochondria driven by the inside-negative potential of the inner membrane. CRC experiments demonstrated that cationic NRB analogues can activate the PTP, provided that they bear the active core of the molecule, i.e. the (phenylvinyl)pyridine subunit. Under these conditions, mouse and guinea pig mitochondria are also affected by the drug. These findings clearly indicate that: i) NRB as well as NRB substructures are endowed with an intrinsic ability to cross mitochondrial membranes in all animal species. This is not unexpected as the lipid composition of the mitochondrial membranes is similar between the different species, ii) NRB target on the PTP is the same in all animal species since it specifically recognizes the (phenylvinyl)pyridine moiety. The need of higher drug concentrations for PT induction in mouse and guinea pig mitochondria, as compared to rat mitochondria, can be ascribed to a different distribution pattern of the drug in rat and non-rat species, as it can be deduced from the results of fluorescence anisotropy experiments.

The fluorescence anisotropy experiments, carried out with selected reporters of different membrane domains, indicate that the target of NRB action is localized on the matrix side of the mitochondria, which is in agreement with a preferential stimulation by NRB of the PTP-activating capacity of the internal Ca^{2+} site, as previously suggested [11]. Actually, the order of drug efficacy, NRB < DR166 < cationic NRB, correlates very well with a gradual shift of drug distribution from the core of the lipid bilayer to matrix-exposed mitochondrial domains (Fig. 7). Furthermore, there is a strict correspondence between the extent of drug-induced perturbation of matrix lipid domains and the efficiency of PTP-activation (compare Figs. 8 and 6). Similar correlations between membrane-perturbing effects and PTP triggering by cationic NRB derivatives can be observed in mouse and guinea pig mitochondria, which again suggests that the selectivity of NRB action on mitochondria does not concern the target of the drug on the pore. Rather, the higher doses of cationic drug necessary to induce perturbation of the matrix domains and PT activation, together with the inertness of neutral compounds, clearly indicate that NRB does not easily gain access to the mitochondrial matrix in any species other than the rat.

Taken together, these findings suggest that the amount of NRB taken up by mitochondria *via* passive diffusion may not be sufficient to perturb mitochondria and stimulate the PT. Thus, as previously suggested [11], the occurrence of PTP activation specifically in rats probably involves a transport system allowing a deeper penetration of the drug in the inner membrane.

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