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Effect of the new matrix metalloproteinase inhibitor RO-28-2653 on mitochondrial function

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Abstract

Matrix metalloproteinases (MMPs) have recently become interesting as potential anticancer drugs. RO-28-2653 is a promising compound because of its antimetastatic and antiangiogenic activities. Due to the structural similarity of RO-28-2653 to mitochondriotoxic agents, speculation has arisen that this substance might impair mitochondrial function. We, therefore, investigated the effects of RO-28-2653 on mitochondrial enzymes and on the functional properties of isolated mitochondria and skinned muscle fibers from rat hearts. Results were compared to the action of amytal and 2,4-dinitrophenol (2,4-DNP), both of which are well documented mitochondriotoxic compounds. In contrast to 2,4-DNP, RO-28-2653 did not uncouple oxidative phosphorylation, although higher concentrations of the compound did impair mitochondrial function. Using malate/pyruvate as substrate, 50 μM of RO-28-2653 inhibited mitochondrial respiration in isolated mitochondria and skinned fibers by 23 and 11%, respectively while 2 mM of amytal elicited almost complete inhibition of the mitochondrial respiration. RO-28-2653 (50 μM) inhibited succinate-dependent respiration in both systems by 43 and 24%, respectively while 2 mM of amytal caused 41 and 23% inhibition, respectively. There was no change in the ADP/O ratios. RO-28-2653 (50 μM) did not significantly alter the activity of the respiratory chain complexes or succinate dehydrogenase, although citrate synthase (CS) was inhibited by upto 71%. This inhibition was non-competitive at a K_i of 25 ± 5 μM. Inhibitory effects in the presence of hydrophobic substances, such as BSA and Triton X-100, were significantly lower in both test systems. In conclusion, high concentrations of RO-28-2653 impair mitochondrial function, although compared to amytal and 2,4-DNP, this is rather low. The resultant impairment is less pronounced in the more complex skinned muscle fiber system, and is dependent on hydrophobic interactions.

Keywords: 2,4-Dinitrophenol; Amytal; Matrix metalloproteinases; Rat heart mitochondria; RO-28-2653

1. Introduction

MMPs have recently become important in the field of anticancer drug research. These enzymes naturally degrade extracellular proteins such as laminin, fibronectin, elastin and collagens, and play an important role in modulating the extracellular matrix [1]. Under physiological conditions, expression of these enzymes is low, except in rapidly remodeling tissues such as in menstrual endometrium, wound healing, inflammation, involuting uterus and the mammary gland [2]. The ability to degrade the extracellular matrix, including major components of basal membranes such as type IV collagen, seems to play a major role in tumor invasion and metastasis [1,3]. In particular, upregulation of MMP-2 (gelatinase A) and MMP-9 (gelatinase B), both type IV collagenases, has been found, for instance, in glioblastomas [4], gastric carcinomas [5], colorectal carcinomas [6] and breast cancer [7]. The higher the level of these enzymes, the tumor seems to be more aggressive.

Over the past few years, several inhibitors of MMPs have been developed [8] and initial clinical trials are in progress [9]. A promising compound in this field is RO-28-2653 ($M_r = 485$) (Fig. 1), which has high specificity towards MMP-2, MMP-9 and MMP-14. Good efficacy to several

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Abbreviations: 2,4-DNP, 2,4-dinitrophenol; s.w., sample weight; DMSO, dimethylsulphoxide; NCP, non-collagen protein.

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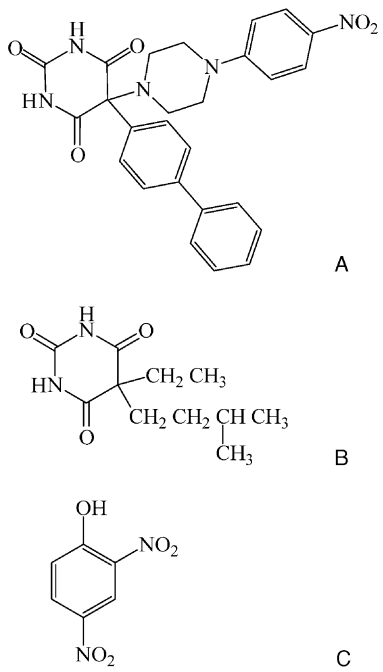

Fig. 1. Chemical structures of RO-28-2653 (A), amytal (B) and 2,4-DNP (C).

types of cancer has been reported in *in vivo* models [10], and 10 μM of RO-28-2653 completely inhibits neoangiogenesis *in vitro* [10]. RO-28-2653 has structural similarities to compounds which interfere with mitochondrial function (Fig. 1). It shares a barbiturate backbone with amytal, a known inhibitor of oxidative phosphorylation [11], and aromatic NO_2 groups with 2,4-DNP, a prototype uncoupling agent [12]. In spite of being structurally similar to 2,4-DNP, RO-28-2653 is not a weak acid and is, therefore, not be expected to be a major uncoupler.

Several established pharmacological substances, such as antidepressant or neuroleptic drugs [13] as well as the newly developed catechol-*O*-methyltransferase-inhibitor tolcapone [14], impair mitochondrial function. At low tissue concentrations, however, they are tolerated by the organism. The present study was designed to investigate whether or not RO-28-2653 itself impairs mitochondrial function. For this purpose, rat heart mitochondria where analyzed in terms of activity on mitochondrial enzymes and mitochondrial function. Only mild *in vitro* effects were seen at high concentrations, so that significant effects *in vivo* would appear to be unlikely.

2. Materials and methods

2.1. Animals

Investigations were performed on hearts from male Wistar rats (body weight: 200–300 g). The rats were anaesthetized with CO_2 and killed by decapitation. The beating heart was removed immediately and used for preparation of the mitochondria or skinned fibers.

2.2. Isolation of mitochondria

Rat heart mitochondria were isolated from one heart as described previously [15]. In short, the heart was roughly dissected, minced intensively with a pair of scissors, and placed into 20 mL of isolation medium A containing 10 mM of Tris, 180 mM of KCl, 10 mM of EDTA (pH 7.4) and 1 mg of trypsin. After 30 min of gentle stirring, 2 mg of trypsin inhibitor was added to block the action of trypsin. The suspension was homogenized five times in a glass–Teflon homogeniser (Type 889S, B. Braun, Melsungen, Germany) at 600 rpm. Differential centrifugation and several washing steps were applied to separate the mitochondria from the other organelles. The final mitochondrial pellet was suspended in isolation medium B containing 10 mM of Tris and 180 mM of KCl (pH 7.4), resulting in a suspension of about 50 mg of mitochondrial protein mL^{-1}. Freeze-permeabilized mitochondria were obtained by five freezing–thawing transitions between liquid nitrogen and room temperature.

2.3. Isolation and permeabilization of muscle fibers

Rat hearts were roughly dissected and washed in ice-cold 0.9% NaCl solution. For dissection using small needles, the fibers were incubated in high energy preservation solution (HEPS [16]), consisting of 10 mM of EGTA/CaEGTA buffer (free Ca^{2+} concentration: 0.1 μM), 9.5 mM of $MgCI_2$, 3 mM of KH_2PO_4, 20 mM of taurine, 5 mM of ATP, 15 mM of phosphocreatine, 49 mM of K-MES, 29 mM of imidazole–HCl and 0.5 mM of dithiothreitol (pH 7.2) on ice. Before measurement, the fibers were gently stirred for 30 min at 4° in HEPS, also containing 50 μg of saponin mL^{-1}. Prior to respirometric measurements, the fibers were washed three times in incubation medium (see in the following sections) to remove the saponin and adenine nucleotides contained in the HEPS.

2.4. Respirometric measurements

Bundles of saponin permeabilized and washed fibers were placed on filter paper to remove any adherent medium, and the sample weight (s.w.). was determined using a laboratory balance. About 1–2 mg of fibers or isolated mitochondria at a protein level of 0.1–0.3 mg mL^{-1} were used for the respirometric experiments.

Respirometry was performed at 30° in 1.5 mL of incubation medium consisting of 75 mM of mannitol, 25 mM of sucrose, 100 mM of KCl, 10 mM of KH_2PO_4, 5 mM of $MgCl_2$, 0.5 mM of Na_2EDTA and 20 mM of Tris–HCI (pH 7.4). In most experiments, 1 mg of bovine serum albumin (BSA) was added mL^{-1} of buffer. The oxygen concentration at air saturation of the medium was considered to be 233 nmol O_2 mL^{-1} at 95 kPa air pressure. An OROBOROS® oxygraph (Paar, Graz, Austria) was employed. This is a two-chambered respirometer with a peltier

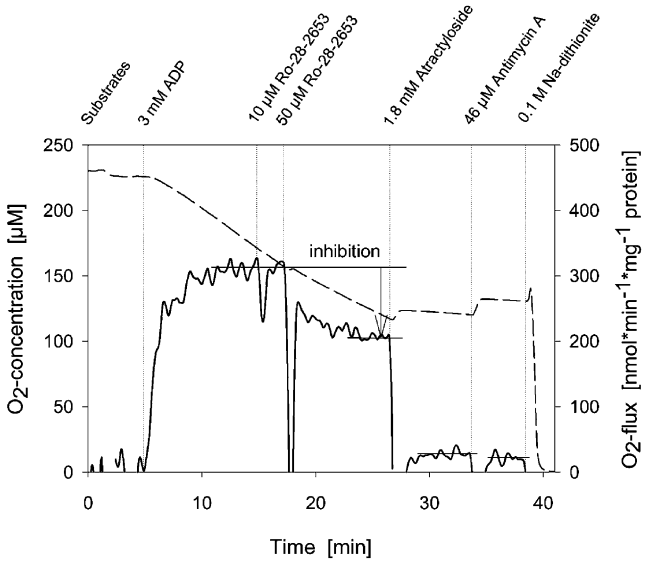

Fig. 2. Effect of RO-28-2653 on state 3 respiration of isolated rat heart mitochondria. Mitochondria were incubated in the presence of 1 mg mL^{-1} of BSA, 10 mM of pyruvate and 2 mM of malate as substrates at 30°. Active respiration was adjusted by addition of 3 mM of ADP. RO-28-2653 was added at two final concentrations. Atractyloside adjusted state 4 respiration. After inhibition of the respiratory chain with antimycine A, the remaining oxygen was exhausted by Na-dithionite for calibration purposes. Dashed line: oxygen concentration within the oxygraph. Solid line: oxygen-flux related to mg mitochondrial protein. Additions on top of the figure.

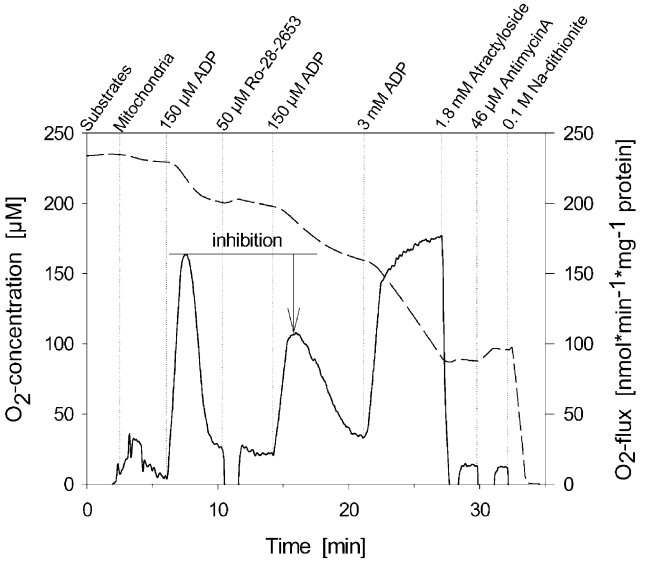

Fig. 3. Effect of RO-28-2653 on the dynamic properties of isolated rat heart mitochondria. Mitochondria were incubated in the presence of 1 mg mL^{-1} of BSA, 10 mM of pyruvate and 2 mM of malate as substrates at 30°. State 3/state 4 transitions were induced by addition of 150 µM of ADP. Then 50 µM of RO-28-2653 was added and the state 3/state 4 transition was repeated. Addition of 3 mM of ADP adjusted the state 3 respiration, which could be inhibited by atractyloside. After inhibition of the respiratory chain with antimycin A, the oxygen was exhausted by Na-dithionite for calibration purpose. Dashed line: oxygen concentration within the oxygraph. Solid line: oxygen-flux related to mg of mitochondrial protein. Additions on top of the figure.

thermostat and electromagnetic stirrers. This respirometer, also known as an ''oxygraph'', was constructed to minimize back-diffusion by using materials impermeable to oxygen [17]. Oxygen consumption rates were calculated as the time derivative of the oxygen concentration (DATLAB Analysis Software, OROBOROS®), and related to s.w. or mitochondrial protein, respectively. The action of the compounds on mitochondrial function was measured either at maximum respiration (state 3 according to Chance and Williams [18]), as shown in Fig. 2, or under dynamic conditions using state 3/state 4 transients, as described in Fig. 3. ADP/O ratios were determined from the integral of the oxygen consumption curve during dynamic state 4/state 3 transitions. To quantify uncoupling, the rate of respiration in the presence of the compounds was related to state 4 respiration in their absence.

2.5. Enzymatic measurements

Enzymatic measurements were performed in sonicated suspensions of isolated mitochondria frozen at −80° until use. Enzymatic activities were assayed spectrophotometrically at 30° using a Beckman DU 640 photometer. Assays were run in duplicate with two different quantities of sample, at least five times, to test reproducibility. Enzymatic activities were related to the mitochondrial protein concentration. NADH: ubiquinone oxidoreductase (complex I) was measured following oxidation of NADH in the presence of coenzyme Q (ubiquinone) at 340 nm using a molar extinction coefficient (ε) of 6.22 mM^{-1} cm^{-1} [19]. Succinate: cytochrome *c* oxidoreductase (complex II + III) and ubiquinone: cytochrome *c* oxidoreductase (complex III) were measured according to [20,21], respectively following reduction of ferricytochrome *c* at 550 nm using an ε of 21.1 mM^{-1} cm^{-1}. The activity of cytochrome *c* oxidase was estimated according to Wharton and Tzagaloff [22], measuring the formation of oxidized cytochrome *c* at an identical wavelength and ε. Succinate dehydrogenase was determined by reduction of the artificial electron acceptor 2,6-dichlorophenol–indophenol at 600 nm [23]. CS, as the mitochondrial marker enzyme, was determined as described in [24] with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), oxaloacetate, acetyl-CoA, but without addition of Triton X-100 at 412 nm at an ε of 13.6 mM^{-1} cm^{-1}.

2.6. Protein determination

Protein was determined by BCA assay (Pierce, Putbus, The Netherlands) [25]. BSA was used as the standard.

2.7. Chemicals

All chemicals used were purchased from Sigma, apart from ADP and pyruvate, which were sourced from Boehringer Ingelheim. RO-28-2653 was supplied by Roche. Stock solutions of 200 mM of amytal, 6 mM of

2,4-DNP and 5 mM of RO-28-2653 were prepared in dimethylsulphoxide (DMSO).

2.8. Statistical analysis

Each parameter was proofed for normality by the Kolmogornow–Smirnow test. If this criterion was fulfilled, statistical significance was tested with the Student's *t*-test for unpaired variables. Otherwise, the Wilcoxson test (Man–Whithney *U*-test) was applied. A *P*-value of <0.05 was considered to be significant.

3. Results

3.1. Inhibition of mitochondrial respiration

While the DMSO solvent did not interfere with mitochondrial function (Table 1), amytal inhibited state 3 respiration and ADP pulse height, using malate/pyruvate as substrates, almost completely. Interestingly, succinate-dependent respiration was also affected by amytal. RO-28-2653 showed a concentration-dependent inhibition of state 3 respiration and ADP pulse height too. While pyruvate-dependent state 3 respiration was inhibited by 10 μM of this compound only at a low level, 50 μM caused significant inhibition. There was no significant time-dependency in the action of RO-28-2653. The ADP pulse height was only affected by the 50 μM concentration. Succinate-dependent respiration was also inhibited by RO-28-2653 at the same order of magnitude as that observed for amytal. Similar effects could be seen for 2,4-DNP uncoupled mitochondria. Using malate/pyruvate as substrate, 10 and 50 μM of RO-28-2653 inhibited uncoupled respiration by 8 and 27%, respectively. Using succinate as substrate, the inhibition was 7 and 40%, respectively. Only unspecific effects of RO-28-2653 were observed in freeze-permeabilized mitochondria, with respect to NADH- or succinate-stimulated respiration. In spite of inhibiting pulsed respiration after addition of ADP in excess, a sufficiently active respiration was achievable again. ADP/O ratios were not affected by RO-28-2653 with either substrate. In addition, the response of mitochondria to inhibition by atractyloside and antimycine A was normal. No effect was seen that affected the intactness of the outer or inner mitochondrial membranes (data not shown).

Using the skinned muscle fiber system, pyruvate-dependent respiration was almost completely inhibited by amytal, as observed in isolated mitochondria, while the inhibitory effect of RO-28-2653 was only about half that measured in suspensions of isolated mitochondria (Table 1). The highest rate of inhibition for 50 μM of RO-28-2653 was 24% using succinate/rotenone as substrate.

In general, the inhibitory action of 10 μM of RO-28-2653 did not significantly differ from that of DMSO solvent in either system. However, 50 μM inhibited the functional properties of the mitochondria significantly, whereby this inhibition was less pronounced in the more complex skinned muscle fiber system. In neither system, did the inhibition increase with preincubation time upto 180 min.

3.2. Uncoupling of state 4 respiration

2,4-DNP, a classic uncoupler, increased state 4 respiration to 1143% in mitochondria and 739% in skinned fibers when pyruvate/malate was used as substrate (Table 2). In the presence of succinate, the uncoupling action was lower at 323% for mitochondria and 253% for skinned fibers. This difference was mainly due to the higher absolute state 4 respiration rate when succinate was the substrate.

Uncoupling by RO-28-2653 was dependent on the concentration of the compound. In skinned fibers, it was 45% at 10 μM of RO-28-2653 and 71% at 50 μM of RO-28-2653 when pyruvate/malate was used as substrate.

Table 1
Comparison of inhibition by the test compounds on active respiration (state 3) in isolated mitochondria and skinned fibers from the rat heart[a]

Enzymes inhibitor	Substrates	Inhibition of state 3 respiration (%) in isolated mitoehondria	Inhibition of state 3 respiration (%) in skinned fibers	Inhibition of ADP pulse height (%) in isolated mitochondria
DMSO (1% (v/v))	Pyruvate/malate	3.0 ± 0.9	4.2 ± 1.5	−7.1 ± 2.4
	Succinate	0.7 ± 0.6	−2.8 ± 2.7	−1.9 ± 1.3
Amytal (2 mM)	Pyruvate/malate	99.0 ± 0.1[b]	95.8 ± 2.2[b]	92.0 ± 1.5[b]
	Succinate	41.3 ± 2.6[b]	22.8 ± 11.0[b]	36.7 ± 3.2[b]
RO-28-2653 (10 μM)	Pyruvate/malate	5.9 ± 4.1	4.4 ± 0.9	−10.7 ± 2.4
	Succinate	12.2 ± 4.0	3.1 ± 1.7	2.1 ± 2.3
RO-28-2653 (50 μM)	Pyruvate/malate	23.4 ± 2.0 [b,c]	10.7 ± 1.7 [b,c]	12.4 ± 4.4[b]
	Succinate	42.5 ± 1.0 [b,c]	23.5 ± 2.6 [b,c]	38.0 ± 2.4[b]

[a] Experiments were performed as described in Figs. 2 and 3. Inhibition was calculated relating the rate of respiration in the presence of the effector on the rate without. Presentation of state 3 inhibition as well as inhibition of ADP-pulsed respiration in isolated mitochondria. Data as mean ± SE, $n \geq 3$.
[b] Significant difference compared to DMSO.
[c] Significant difference between fibers and mitochondria.

Table 2
Comparison of uncoupling effects of the compounds on state 4 respiration in isolated mitochondria and skinned fibers from the rat heart[a]

Enzymes inhibitor	Substrates	Uncoupling effect in isolated mitochondria		Uncoupling effect in skinned fibers	
		Absolute (% increase of state 4)	Relative to DNP (%)	Absolute (% increase of state 4)	Relative to DNP
2,4-DNP (60 μM)	Pyruvate/malate	1143.6 ± 203.7[b]	100	739.2 ± 162.4[b]	100
	Succinate	323.1 ± 50.4[b]	100	253.6 ± 86.4[b]	100
DMSO (1% (v/v))	Pyruvate/malate	18.1 ± 6.7	1.5	16.1 ± 20.5	2.2
	Succinate	0.2 ± 0.8	0.6	14.1 ± 6.2	5.6
RO-28-2653 (10 μM)	Pyruvate/malate	11.2 ± 7.1	1.0	44.8 ± 6.9	6.1
	Succinate	2.5 ± 2.6	0.8	6.9 ± 10.2	2.7
RO-28-2653 (50 μM)	Pyruvate/malate	19.3 ± 6.2	1.7	71.1 ± 17.1	9.6
	Succinate	8.4 ± 3.2	2.6	19.1 ± 7.2	7.5

[a] Uncoupling was detected in the presence of substrates without ADP (state 4) and was calculated as the percentage respiration rate after addition of the compounds and the rate before. Data as mean ± SE, $n \geq 4$.
[b] Significant difference compared to DMSO.

Interestingly, its effect in isolated mitochondria was lower than in skinned fibers at 11 and 19%, respectively. Assuming the uncoupling effect of 2,4-DNP to be 100%, the uncoupling activity of 50 μM of RO-28-2653 was only between 2.0 and 9.6%, and not significantly different from that of DMSO solvent, the uncoupling activity of which was between 0.6 and 5.4%. Therefore, the uncoupling effect of the compound should be physiologically negligible.

3.3. Effect on mitochondrial enzymes

DMSO solvent had only a slight effect on the assays (Table 3). The activity of complex I of the respiratory chain was not affected by either RO-28-2653 or 2,4-DNP, although it was almost completely inhibited by amytal (inhibition: 84%). All other respiratory chain enzymes (complexes II + III, complex III, cytochrome oxidase) were not significantly affected by RO-28-2653, amytal or 2,4-DNP. Succinate dehydrogenase, a citric cycle enzyme, was not affected either, although the activity of CS was concentration-dependently inhibited by RO-28-2653. While 10 μM of RO-28-2653 inhibited CS by 40%, 50 μM inhibited 71% of its activity. Therefore, kinetic analysis of this interaction was performed. As CS needs two substrates (acetyl-CoA and oxalacetate) to synthesize citrate, it was only possible to monitor the interaction between one substrate and RO-28-2653 at any one time. Keeping the concentration of oxalacetate at a saturated level and varying the amount of acetyl-CoA, a non-competitive inhibition of RO-28-2653 was visible (Fig. 4). A K_i of 25 ± 5 μM was estimated in the absence of hydrophobic additives. Using oxalacetate as the variable substrate a non-competitive inhibition was observed, too, and a K_i of 18 ± 6 μM could be estimated for this condition. This inhibitory effect was dependent on the mitochondrial protein concentration in the assay and the hydrophobic additives (see in the following sections). Amytal and 2,4-DNP did not affect CS.

3.4. Role of hydrophobic interactions

Respirometric investigations are usually performed in the presence of low concentrations of BSA. In its absence, the inhibitory action of RO-28-2653 on mitochondrial function was significantly higher than in its presence (Fig. 5). This difference was obvious, especially when the concentration of RO-28-2653 was 10 μM. Another fact is the lower inhibition of RO-28-2653 with respect to

Table 3
Comparison of inhibitory action of the test compounds on mitochondrial enzymes[a,b]

Enzymes inhibitor	Complex I	Complex II + III	Complex III	COX	SDH	CS
Control	127 ± 8 (100%)	779 ± 85 (100%)	1234 ± 205 (100%)	210 ± 20 (100%)	1425 ± 131 (100%)	1303 ± 30 (100%)
DMSO (1% (v/v))	137 ± 16 (108%)	828 ± 3 (108%)	985 ± 72 (79%)	218 ± 27 (104%)	1109 ± 61 (78%)[c]	1285 ± 24 (88%)
Amytal (2 μM)	21 ± 7 (16%)[c]	794 ± 36 (103%)	1020 ± 221 (82%)	185 ± 5 (88%)	1225 ± 43 (86%)	1490 ± 5 (114%)[c,d]
2,4-DNP (60 μM)	125 ± 6 (98%)	800 ± 24 (104%)	946 ± 225 (77%)	208 ± 10 (99%)	1226 ± 48 (86%)	1399 ± 94 (107%)
RO-28-2653 (10 μM)	122 ± 5 (96%)	657 ± 156 (85%)	9801 ± 16 (79%)	183 ± 15 (87%)	1335 ± 66 (94%)	786 ± 81 (60%)[c,d]
RO-28-2653 (50 μM)	127 ± 6 (102%)	685 ± 98 (89%)	932 ± 114 (76%)	194 ± 14 (92%)	1382 ± 89 (96%)	380 ± 67 (29%)[c,d]

[a] Activities were measured in disintegrated rat heart mitochondria without BSA. Enzyme activities are given in U g^{-1} of mitochondrial protein as mean ± SE, $n \geq 5$. Relative activities vs. controls are listed in the parenthesis.
[b] NADH: ubiquinone oxidoreductase (complex I); succinate: cytochrome *c* oxidoreductase (complex II + III); ubichinol: cytochrom-*c* oxidoreductase (complex III); cytochrome *c* oxidase (COX); succinate dehydrogenae (SDH); citrate synthase (CS).
[c] Significant difference compared to controls.
[d] Significant difference compared to DMSO.

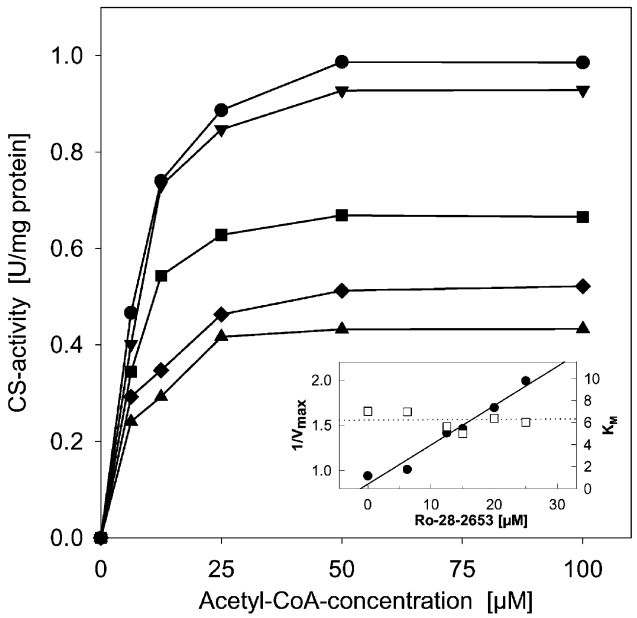

Fig. 4. Dependency of CS activity on the concentration of its substrate acetyl-CoA at different concentrations of RO-28-2653 and excess of oxalacetate (0.5 mM). Measured photometrically at 30° and pH 7.4. (●) control without inhibitor; (▼) 6.25 µM; (■) 12.5 µM; (◆) 20 µM; (▲) 25 µM of RO-28-2653. Insert: dependency of the reciprocal maximum CS activity (●) and K_M-values in µM (□) on the concentration of RO-28-2653, as derived from Lineweaver–Burk plots.

mitochondrial respiration in skinned fibers vs. isolated mitochondria. In addition to mitochondrial proteins, skinned fibers contain many more extramitochondrial proteins and lipids which can act as hydrophobic binding sites for RO-28-2653, making this comparable to adding BSA to the medium.

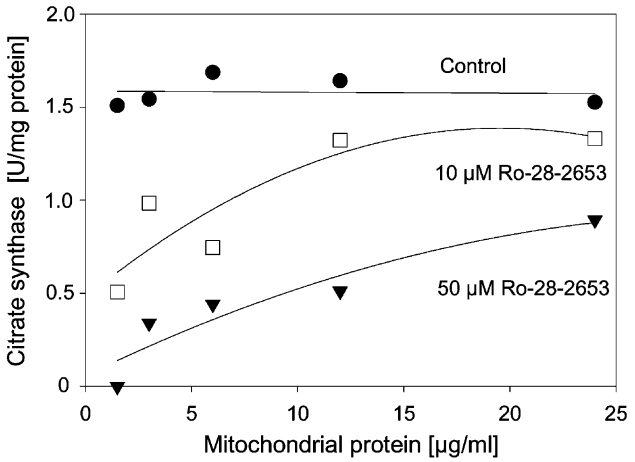

Fig. 6. Activity of CS relative to the concentration of RO-28-2653 and protein content. The activity of CS was measured in ultrasonificated suspensions of isolated rat heart mitochondria, at increasing concentrations of mitochondrial protein. The assay mixture did not contain any other proteins.

To test whether or not the inhibitory action of RO-28-2653 on CS also depends on hydrophobic interactions, the protein concentration in the cuvette was varied by a factor of 10 while keeping the concentration of RO-28-2653 constant. As can be seen from Fig. 6, enzymatic activity of the controls remained unchanged, which indicates good reproducibility and proportionality of the measurement. Enzyme activity increased in the presence of RO-28-2653, as the protein concentration increased, which clearly indicates a dependency between the inhibition seen and the protein concentration. Similar results can be achieved by the introduction of artificial hydrophobic binding sites. In

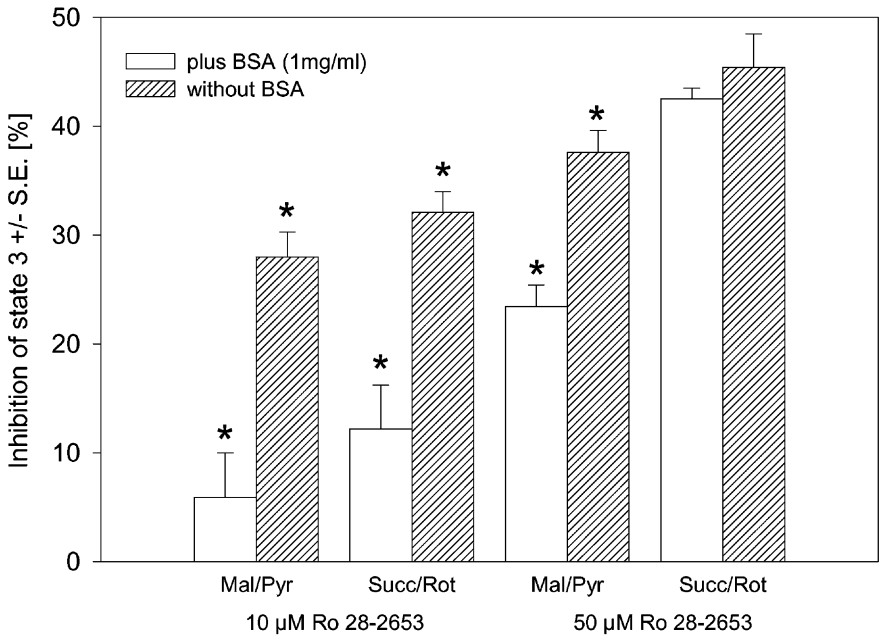

Fig. 5. Effect of BSA on the inhibition of state 3 respiration by RO-28-2653 in isolated rat heart mitochondria. Experimental procedures are listed in the legend to Fig. 2; Mal/Pyr: pyruvate/malate; Succ/Rot: succinate/rotenone. Single asterisk indicates statistically significant differences ($P < 0.05$) between the presence and absence of BSA.

this way, 0.5% of Triton X-100 (v/v) decreased the inhibition of 10 and 50 μM of RO-28-2653 on CS from 40 and 71%, respectively to 4 and 14%, respectively; this was not significantly different in the controls.

4. Discussion

The use of isolated mitochondria is a standard procedure to test the effects of newly developed compounds on mitochondrial function. Using this system, 50 μM of RO-28-2653 elicited upto 43% inhibition of mitochondrial respiration, while the effect of 10 μM of RO-28-2653 was not significantly different to that observed with DMSO solvent.

The system of isolated mitochondria has several disadvantages, such as low mitochondrial yield and a consistent, broken mitochondrial subpopulation due to the preparation process used. To overcome these limitations, the skinned fiber technique was introduced [16,26,27]. Here, the mitochondria remain in their physiological environment within the cell. In this case, 50 μM of RO-28-2653 inhibited state 3 respiration by not more than 24%. This is only about half the rate of inhibition observed in isolated mitochondria, and is probably mainly due to unspecific binding of RO-28-2653 with non-mitochondrial proteins, thus, decreasing the activity of the inhibitor since it is a hydrophobic compound. It was possible to simulate this effect by varying the BSA concentration in the medium for respirometric measurements using isolated mitochondria (Fig. 5).

In contrast to the inhibition of state 3 respiration, the uncoupling ability of RO-28-2653 is of minor importance since its uncoupling effect is less than 10% of that of the 2,4-DNP reference, and is not significantly different to that of DMSO solvent. This very low uncoupling activity is in line with the chemical structure of RO-28-2653 (Fig. 1). An uncoupler has to cross from the intermembrane space, through the inner membrane, into the matrix space as an acid, and has to return as an anion [28]. For that purpose, the uncoupler must equilibrate with the aqueous phases on both sides of the inner membrane. Therefore, all known uncouplers are weak acids. The only potential acidic hydrogen group of RO-28-2653 is part of the keto–imino group of barbituric acid. However, the acidity of this hydrogen group is almost zero due to the attached aromatic cycles. The high hydrophobicity of RO-28-2653 further decreases its potential ability for uncoupling the electrochemical proton gradient across the membrane. Accordingly, the real uncoupling effect of RO-28-2653 in vivo should be physiologically negligible.

At the enzyme level, amytal, as expected, inhibited the complex I of the respiratory chain almost completely, but did not affect the other enzymes. DMSO solvent and 2,4-DNP were inert in respective to all the mitochondrial enzymes determined. Surprisingly, RO-28-2653 did not affect complex I of the respiratory chain, although it exhibited a strong inhibitory action on CS. RO-28-2653 acts as non-competitive inhibitor to CS (Fig. 4).

Assuming the inhibition of CS is responsible for the inhibition of pyruvate-dependent respiration, the question arises as to what could be the reasons for the different sensitivity of inhibition at a functional and enzymatic level. One explanation is the metabolic control theory, which explains threshold effects on the functional level caused by enzyme inhibition [29]. Since the maximum activity of single enzymes normally exceeds the activity of the maximum metabolic flux, remarkable amounts of most enzymes can be inhibited without any effect on flux. The second explanation is based on the different concentrations of mitochondria and test compound in the respirometric and enzymatic incubations. Due to the higher sensitivity of spectrophotometric measurements, incubation mixtures contain about 30 times less protein than mixtures for oxygraphic measurement. Therefore, the relative excess of test compound to mitochondrial protein is much greater in enzymatic measurement. This relative excess of RO-28-2653 could explain the higher degree of inhibition in enzymatic measurement. Inhibition of CS by RO-28-2653 was shown to be dependent on the ratio of protein to the concentration of the test compound (Fig. 6), and to hydrophobic additives such as Triton X-100. It should be mentioned that enzymatic assays were performed without BSA; therefore, the activity of the test compounds should be higher in incubations for enzymatic assays. However, it cannot be ruled out that the enzyme itself is not affected to a greater extent by the drug in the assay of the single enzyme than in the more physiological environment within the intact mitochondria of skinned muscle fibers. Finally, interactions between RO-28-2653 and other substrates or proteins might also contribute to the effects observed at a functional level.

All these reasons can cause differences in the inhibition of pyruvate-dependent respiration and the inhibition of CS. This cannot, however, explain the high degree of inhibition of succinate-dependent respiration. Since uncoupled succinate respiration was also inhibited by RO-28-2653, but not oxidation of succinate in freeze-permeabilized mitochondria, it can be assumed that the dicarboxylate carrier may be the target for inhibition. This could also be the explanation for the inhibition of succinate-dependent respiration by amytal, which was of the same order of magnitude as that observed for RO-28-2653. The fact that high concentrations of amytal inhibited not only pyruvate-dependent respiration, but also succinate-dependent respiration is surprising, since amytal is known to be a selective inhibitor of complex I of respiratory chain, and succinate is metabolized only via complex II–IV, with complex I not being involved. In contrast to amytal, rotenone, the most selective inhibitor of complex I, specifically inhibited complex I, but did not affect complex II at μM concentrations. Apart from the fact that no reliable

assay exists to test the dicarboxylate carrier, this is of minor importance since succinate *in vivo* is not the preferred substrate for oxidative phosphorylation. In addition, it was possible to rule out F_0F_1-ATPase and the AdN translocator being affected by RO-28-2653, since uncoupled respiration could be inhibited under conditions where neither enzyme contributed to the respiration flux.

While *in vitro* studies clearly show the biological activity of RO-28-2653, e.g. the complete inhibition of neoangiogenesis at 10 μM [10], inhibitory effects on mitochondria at this concentration are of minor importance. Even at a maximum concentration of 50 μM in aqueous media, only small alterations in mitochondrial function were observed in our most complex system of skinned muscle fiber. Compared to the action of amytal and 2,4-DNP, the effect of RO-28-2653 on mitochondrial function is much lower. Therefore, it can be speculated that the action of RO-28-2653 *in vivo* is physiologically negligible. In addition, slight interactions could be compensated by mitochondrial adaptation processes, in the case of physiological relevance concomitant to chronic treatment with RO-28-2653.

This is in close agreement with the fact that RO-28-2653 is well tolerated *in vivo*: in several mouse tumor models, RO-28-2653 has displayed excellent antitumor and antimetastatic efficacy at doses ranging from 12.5 to 90 mg kg^{-1} per day. Toxicological studies in mice, rats and monkeys have not shown any adverse effects upto 4 weeks at RO-28-2653 doses of upto 360 mg kg^{-1} per day.[1]

The skinned fiber technique has proven to be a useful tool for simulating the *in vivo* effects of drugs on mitochondrial function *in vitro*.

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