

Regular Article

Suramin Inhibits Mitochondrial ADP/ATP Carrier, Not Only from the Cytosolic Side But Also from the Matrix Side, of the Mitochondrial Inner Membrane

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Suramin was earlier reported to show inhibitory effects on the mitochondrial ADP/ATP carrier. However, two important questions, i) whether it shows a specific inhibition of the ADP/ATP carrier when applied to isolated mitochondria, and ii) whether it inhibits the mitochondrial ADP/ATP carrier only from the cytosolic side or from the matrix side, as has been observed with its canonical inhibitors of carboxyatractyloside or bongkreikic acid, remain to be answered. In the present study, we sought exact answers to these questions. As for the first question, suramin showed certain inhibitory effects on the mitochondrial respiratory chain; and at a concentration of 25 μM it showed strong inhibition of the mitochondrial ADP/ATP carrier. This property was due to its weaker inhibitory effects on the mitochondrial ADP/ATP carrier than those of carboxyatractyloside or bongkreikic acid. As for the second question, suramin inhibited the ADP/ATP carrier from both sides of the mitochondrial inner membrane. Thus, suramin was concluded to be utilizable as a new type of inhibitor for the ADP/ATP carrier; but we must pay attention to its side-effects, especially when it is applied to whole mitochondria.

Key words mitochondrial ADP/ATP carrier, inhibitor, suramin, submitochondrial particles

INTRODUCTION

Mitochondria have two membrane systems composed of the outer and the inner membranes, and these two membranes have completely distinct properties in the aspect of the permeability of small molecules. The mitochondrial outer membrane is highly permeable toward small molecules; and this property is attributable to the function of a certain protein, the so-called voltage dependent anion channel (VDAC), in the mitochondrial outer membrane (for recent reviews,¹⁻⁵). On the other hand, the mitochondrial inner membrane shows a high resistance against permeation of small molecules or ions; because an electrochemical potential of H^+ across the mitochondrial inner membrane is necessary as a driving force of ATP synthesis. However, various metabolites and ions are required to be transported across the inner membrane, because numerous metabolic reactions occur in the matrix space of mitochondria. A family of mitochondrial solute carriers, also designated as SLC25a, catalyzes the transport of molecules across the mitochondrial inner membrane (for recent reviews,⁶⁻¹⁰). The mitochondrial ADP/ATP carrier is a member of this family, and 4 isoforms have been identified in mammals. Intensive studies have been carried out on the type 1 isoform of mammals (AAC1) and the type 2 isoform of yeast (yAAC2) (for recent reviews;¹¹⁻¹³) and two specific inhibitors, carboxyatractyloside and bongkreikic acid, showing very low K_d values in the range of 10^{-8} M, were identified.¹⁴ Interestingly, these two inhibi-

tors show a selective and opposite sidedness in their actions: carboxyatractyloside inhibits the ADP/ATP carrier from the cytosolic side; and bongkreikic acid, from the matrix side. The crystal structure of the ADP/ATP carrier in complex with carboxyatractyloside was revealed in earlier studies,^{15,16} and that of it with bongkreikic acid was revealed very recently.¹⁷

Suramin, a chemical focused on in the present study, is a well known chemical used to treat the first stage of acute human sleeping sickness, caused by *Trypanosoma brucei rhodesiense*, but numerous sites of action have been reported (for recent review,¹⁸). A recent docking study using the crystal structure of the mitochondrial ADP/ATP carrier revealed that suramin shows a high affinity for the mitochondrial ADP/ATP carrier.¹⁹ Its direct inhibitory effect on the mitochondrial ADP/ATP carrier was also demonstrated by using a reconstituted mitochondrial ADP/ATP carrier. Furthermore, suramin has been referred to as a “highly selective” inhibitor of the ADP/ATP carrier based on the fact that it selectively inhibits the ADP/ATP carrier among 4 mitochondrial carriers of ADP/ATP carrier paralog 2 (AAC2), aspartate/glutamate carrier paralog 1 (AGC1), ATP-Mg/Pi carrier paralog 1 (APC1), and ornithine carrier paralog 1 (ORC1). However, regarding the actions of suramin on the mitochondrial ADP/ATP carrier, two important questions remain to be answered: i) whether it shows side effects other than the inhibitory effect on the mitochondrial ADP/ATP carrier, especially when it is applied to the isolated mitochondria; and ii) from which side of the membrane (i.e.,

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from the cytosolic side or from the matrix side), does it show its inhibitory effects on the carrier. In the present study, we sought and obtained exact answers to these questions.

MATERIALS AND METHODS

Materials Suramin was purchased from FUJIFILM Wako Pure Chemical Corporation (sodium salt, code 193-10611, Osaka, Japan). Adenosine 5'-diphosphate, [2,8-³H], trisodium salt (code ART-0386) was obtained from American Radiolabeled Chemicals Inc. (Saint Louis, MO, USA).

Fresh bovine hearts used for preparation of bovine heart mitochondria were a generous gift from Fujimitsu-Hasegawa Company (Fudohonmachi-3, Tokushima, Japan). Bongkreikic acid was kindly donated by Prof. Johannis A. Duine (Delft University of Technology, Delft, The Netherlands).

Preparation of Mitochondria and Submitochondrial Particles Rat liver mitochondria were isolated from the liver of adult male Wistar rats as described previously.²⁰ The animal experiments were reviewed and approved by the Instructional Animal Care and Use Committee (Approval No. T30-128). Bovine heart mitochondria were prepared according to Smith²¹ in a solution of 250 mM sucrose, 1 mM EDTA, and 2 mM Tris-HCl buffer, pH 7.4, and stored at -80°C until use. Submitochondrial particles were prepared according to Hansen and Smith²² as follows: Bovine heart mitochondria that had been stored at -80°C were thawed and suspended at 15 mg of protein/mL in medium consisting of 250 mM sucrose, 5 mM ATP, 15 mM MgCl₂ and 3 mM Tris-HCl buffer, pH 7.4. The suspension was sonicated 6 times for 30 s each time at 1-min intervals at output level 4 in a Branson Sonifier model 250D in an ice bath. Unbroken mitochondria were removed by centrifugation at 27,000 x g for 15 min at 4°C, and then submitochondrial particles were collected by re-centrifugation at 148,000 x g for 60 min at 4°C. The pellet was suspended in medium consisting of 250 mM sucrose and 3 mM Tris-HCl buffer, pH 7.4.

Measurements of Oxygen Consumption and pH Change in the Mitochondrial Suspension The overall reactions of mitochondrial oxidative phosphorylation were measured in medium consisting of 200 mM sucrose, 20 mM KCl, 3 mM MgCl₂, 10 mM succinate, 1 µg/mL rotenone and 3 mM potassium phosphate buffer, pH 7.4, in a total volume of 2.2 mL. After addition of carboxyatractyloside or suramin at the desired concentration to the medium, rat liver mitochondria were added to make a final protein concentration of 0.7 mg/mL. Oxygen consumption and pH change in the mitochondrial suspension were monitored at 25°C by using a Clark oxygen electrode (YSI Incorporated, Model YSI5331, Yellow Springs, OH, USA) and pH electrode (Toko Chemical Lab. Co., Ltd., Model PCE 105CW-SR, Tokyo, Japan), respectively. The pH changes of the incubation medium of individual experiments were calibrated by the addition of known amounts of oxalic acid, and were converted to the rates of ATP synthesis by dividing by the coefficient of 0.891.²³

Measurements of [³H]ADP Uptake by Mitochondria For measurements of [³H]ADP uptake by mitochondria, rat liver mitochondria were suspended in the above-mentioned medium (0.5 mL) to make a final protein concentration of 1.86 mg/mL. After the addition of certain chemicals (1.8 µM carboxyatractyloside, 1 µM antimycin A or suramin at the desired concentration), [³H]ADP uptake was initiated by the addition of

25 µL of 2 mM [³H]ADP (specific radioactivity of 74 kBq/µmol). After incubation for 15 s on ice, the reaction was terminated by the addition of 1.8 µM carboxyatractyloside. Then the mitochondria were pelleted by centrifugation (15,000 rpm at 4°C for 1 min in Eppendorf microfuge, model 5415R). After complete removal of the supernatant, the pellet was dissolved in 100 µL of 1% SDS solution, after which a 50-µL aliquot of it was mixed with 3 mL ACSII (GE healthcare Japan, model NACS204, Tokyo, Japan) and applied to an Aloka liquid scintillation counter, model 6100.

Measurement of [³H]ADP Uptake into Submitochondrial Particles For measurements of [³H]ADP uptake into submitochondrial particles, submitochondrial particles were suspended in medium containing 200 mM sucrose and 10 mM potassium phosphate buffer, pH 7.4 (0.25 mL) to make a final protein concentration of 2 mg/mL. After the addition of certain chemicals (1.6 µM carboxyatractyloside, 7.9 µM bongkreikic acid or suramin at the desired concentrations), [³H]ADP uptake was initiated by the addition of 12.5 µL of 2 mM [³H]ADP (specific radioactivity of 740 kBq/µmol). After incubation for 20 s on ice, the reaction was terminated by the addition of 7.9 µM bongkreikic acid. Then, a 100-µL aliquot of the reaction mixture was subjected to size-exclusion chromatography (Sephadex G-75 equilibrated with 135 mM glycerol in a column of 4-mm diameter and 13-cm height), after which the radioactive nucleotides taken up into the submitochondrial particles were separated from those remaining in the reaction mixture. The protein concentrations of the eluted fractions containing submitochondrial particles were measured by a BCA protocol using a Plate CHAMELEON model 425-104 (Hidex, Turku, Finland), and the radioactivities of individual fractions were measured as described above.

RESULTS

First, we tested whether suramin would show a specific inhibitory effect on the mitochondrial ADP/ATP carrier when applied to isolated mitochondria. For this test, mitochondria isolated from rat liver were suspended in medium containing succinate as a respiratory substrate and a certain concentration of a test drug such as suramin. Then, ATP synthesis was initiated by the addition of ADP to the reaction mixture. By measuring the time-dependent changes in the oxygen concentration and pH of this reaction mixture, we could evaluate the effects of the test drug on the overall reactions of oxidative phosphorylation, i.e., from the oxidation of succinate to the final phosphorylation reaction of ADP.

When the mitochondria were suspended in the medium, gradual oxygen consumption started (see **black-dotted trace** in Fig. 1A). This gradual oxygen consumption reflects the gradual oxidation of succinate to compensate the decrease in the membrane potential across the mitochondrial inner membrane by H⁺ leakage. The subsequent addition of ADP caused acceleration of the oxygen consumption and alkalization of the reaction medium (changes in the pH of the incubation medium are shown with **red-dotted trace** in Fig. 1A) accompanied with the formation of ATP (this state is referred to as "State 3", and the corresponding time period in the control experiment in Fig. 1A is shown with a rectangle). These changes spontaneously recovered to the original levels when the added ADP was completely converted into ATP, as shown with blue arrows for trace of oxygen consumption in Fig. 1A.

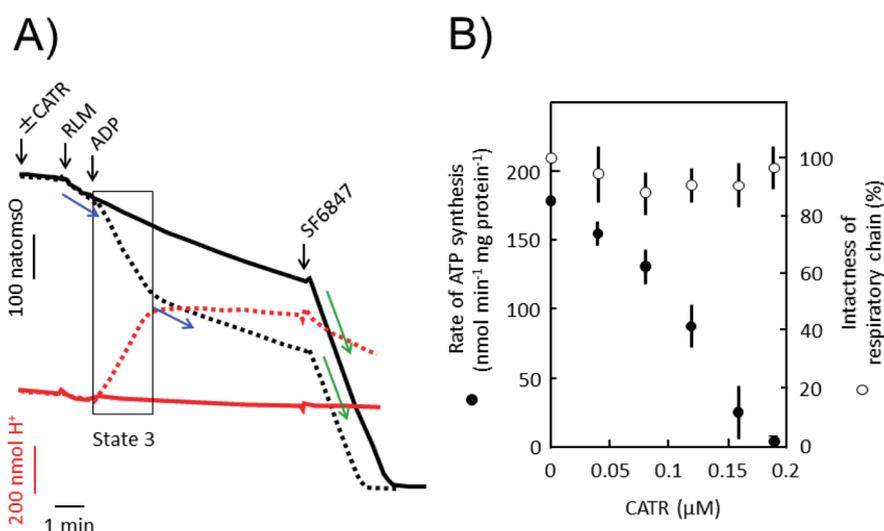


Fig. 1. Effects of Carboxyatractyloside on the pH Change and Oxygen Consumption in the Mitochondrial Suspension

The experiments measuring pH change and oxygen consumption were highly reproducible (see error bars in panel B). Typical traces of time-dependent oxygen consumption (black traces) and pH change (red traces) of multiple runs are shown in **panel A**. “RLM” represents the addition of rat liver mitochondria. Dotted lines and solid lines represent the results of control experiments without the addition of carboxyatractyloside and those observed with samples containing 0.18 μM carboxyatractyloside (shown as CATR), respectively. The rates of ATP synthesis and intactness of respiratory chain were evaluated from the rate of pH change observed after addition of ADP and the rate of oxygen consumption observed after addition of 100 nM SF6847, respectively; and their dependences on the concentration of carboxyatractyloside are shown in **panel B**. The results obtained at individual concentration were shown as mean value ± SD of 3 independent runs.

Further addition of an uncoupler of oxidative phosphorylation, SF6847, led to the maximum rate of mitochondrial oxygen consumption; as this uncoupler renders the mitochondrial inner membrane permeable to H⁺.

As carboxyatractyloside has been well established as one of the “specific” inhibitors of the mitochondrial ADP/ATP carrier,¹⁴ prior to evaluation of the effects of suramin, we first examined the actions of carboxyatractyloside on the overall reaction of oxidative phosphorylation. When 0.18 μM carboxyatractyloside was added to the mitochondrial suspension, the induction of State 3 caused by the addition of ADP, i.e., the acceleration of the oxygen consumption and the alkalinization of the reaction medium, was completely suppressed (see **solid traces** in Fig. 1A). However, subsequent addition of SF6847 (final concentration of 100 nM) caused a remarkable acceleration of the oxygen consumption; and this rate of the oxygen consumption was almost the same as that observed in the absence of carboxyatractyloside (as shown with green arrows in Fig. 1A), indicating that carboxyatractyloside at this concentration did not show side effects on the electron transport system.

We carried out these experiments at various concentrations of carboxyatractyloside; and the dose/response curves of carboxyatractyloside for i) the rate of ATP synthesis (determined by the rate of the pH change of the incubation medium) and ii) the intactness of the electron-transport system (determined by the rate of the maximum oxygen consumption) are shown by closed and open circles, respectively, in Fig. 1B. It was evident that ATP synthesis could be completely suppressed with 0.18 μM carboxyatractyloside. ATP synthesis can be easily suppressed not only by inhibition of the ADP/ATP carrier, but also by inhibition of ATP synthase or the electron-transfer system. However, we could exclude the side effects of carboxyatractyloside on the mitochondrial electron-transfer system, because carboxyatractyloside at this concentration had almost no effect on the maximum rate of the oxygen consumption

(Fig. 1B, open circles).

We next examined the effects of 11 μM suramin on these reactions and obtained the results shown in Fig. 2A (likewise as in Fig. 1A, control traces of the oxygen consumption and the pH change in the absence of suramin are shown by **black and red dotted traces**, respectively; and those obtained in the presence of suramin are shown by **solid traces**). In the presence of 11 μM suramin, ATP synthesis was largely (65%) inhibited, but there was also a moderate inhibitory effect (26%) on the electron-transport system. These inhibitory effects of suramin on both ATP synthesis and the electron-transport system occurred in a dose-dependent manner, as shown in Fig. 2B. From these results, we could not clearly conclude that suramin inhibited the mitochondrial ADP/ATP carrier, because ATP synthesis could be inhibited not only by an inhibition of the mitochondrial ADP/ATP carrier, but also by inhibition of the electron-transport system.

Thus, we next conducted an experiment to determine whether suramin directly inhibited the mitochondrial ADP/ATP carrier. For this test, we performed an ADP uptake experiment using [³H]ADP as a tracer. As shown in Fig. 3A, 90% of the [³H]ADP uptake was suppressed by 1.8 μM carboxyatractyloside, but not influenced by antimycin A, an inhibitor of electron transport system. Therefore, even though suramin showed an inhibitory effect on the electron-transport system, it did not influence the ADP uptake; and, hence, we could directly evaluate the effect of suramin on the mitochondrial ADP/ATP carrier by using this experimental system. As shown in Fig. 3B, suramin showed a direct inhibitory effect on the mitochondrial ADP/ATP carrier; and its IC₅₀ was determined to be about 5 μM. This result clearly indicated that suramin inhibited the mitochondrial ADP/ATP carrier from the cytosolic side.

Regarding the inhibition of the mitochondrial ADP/ATP carrier by suramin, we had one more intriguing question to answer: whether suramin inhibits the mitochondrial ADP/ATP carrier only from the cytosolic side or from the matrix side,

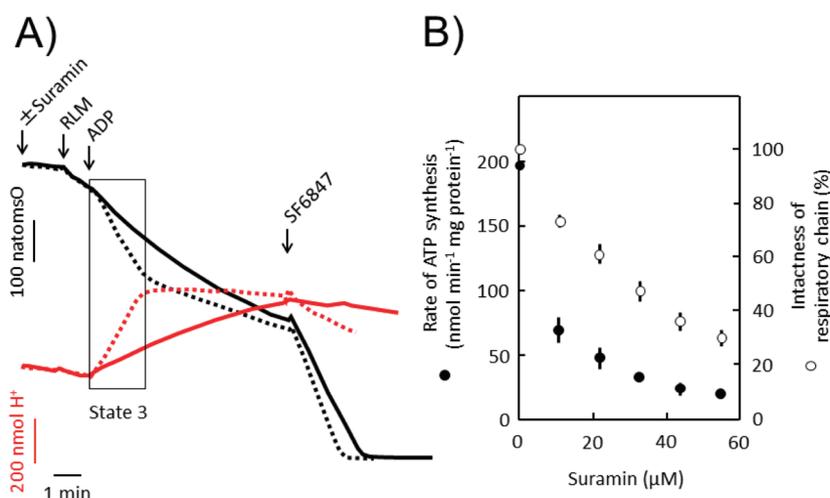


Fig. 2. Effects of Suramin on the pH Change and Oxygen Consumption in the Mitochondrial Suspension

Typical traces of time-dependent oxygen consumption (black traces) and pH change (red traces) of multiple runs are shown in **panel A**. "RLM" represents the addition of rat liver mitochondria. The rates of ATP synthesis and intactness of the respiratory chain were evaluated from the rate of pH change observed after the addition of ADP and the rate of oxygen consumption observed after that of 100 nM SF6847, respectively; and their dependences on the concentration of suramin are shown in **panel B**. The results obtained at individual concentration were shown as mean value \pm SD of 3 independent runs.

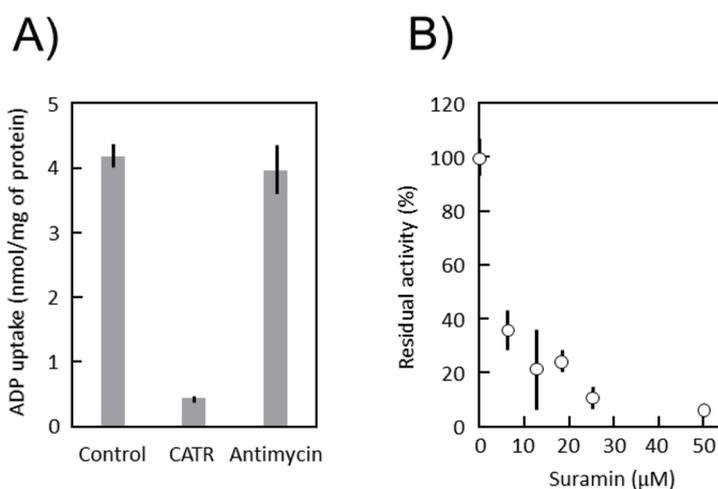


Fig. 3. Effects of Suramin on the Mitochondrial ADP Uptake

To characterize the uptake of ADP via the ADP/ATP carrier, we measured mitochondrial ADP uptake in the absence or presence of 1.8 μ M carboxyatractyloside (CATR) or 1 μ M antimycin A, as shown in **Panel A**. **Panel B** shows the dose-dependent inhibition of ADP uptake by suramin. Note that the amount of ADP uptake observed in the presence of carboxyatractyloside (0.4 nmol/mg mitochondrial protein) was subtracted from the counts of individual samples in Panel B as backgrounds. Data represent mean value \pm SD of three independent runs.

as has been observed with its canonical inhibitors of carboxyatractyloside or bongkreikic acid. To answer this question, we next assessed the ADP uptake into submitochondrial particles, in which ADP/ATP carrier exists in an "inside out" direction in comparison with those in the mitochondrial membrane. When [³H]ADP was added to the submitochondrial particles from bovine heart, the uptake of a certain amount of [³H]ADP was observed (Fig. 4A). We confirmed that this uptake was not influenced by the addition of carboxyatractyloside, indicating that any contamination by right-side-out particles was negligible. On the contrary, this uptake was largely suppressed (85%) by the addition of 7.9 μ M bongkreikic acid, even at neutral pH, indicating that the preparation of submitochondrial particles was successful. Suramin was also effective for suppressing the [³H]ADP uptake by the submitochondrial particles, and its IC₅₀ value was determined to be about 10 μ M (Fig. 4B).

DISCUSSION

There are two important preceding papers describing the actions of suramin on the functions of isolated mitochondria.^{19,24} In the earlier paper, Calcaterra *et al.* showed that suramin inhibits the ADP- or the uncoupler-stimulated oxidation of malate/glutamate by rat liver mitochondria but that the former is more sensitive than the latter. These results are consistent with ours (see Fig. 2B), but the concentrations of suramin causing 50% inhibition of the ADP-stimulated respiration and the uncoupled electron transport (about 100 μ M and higher than 400 μ M, respectively) were more than 10 times higher than those observed in the present study (7.5 μ M and 30 μ M, respectively). The reason causing such differences is uncertain. More importantly, based on the results of kinetic studies, they described that suramin behaved as a mutually

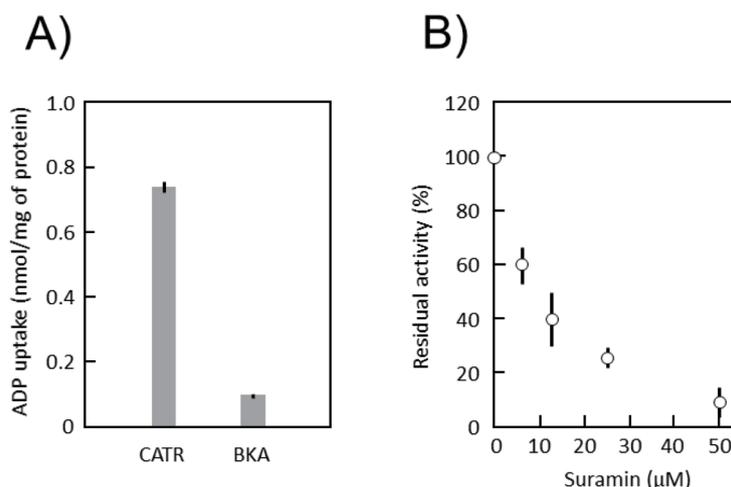


Fig. 4. Effects of Suramin on ADP Uptake into Submitochondrial Particles

To characterize the uptake of ADP via the ADP/ATP carrier, we measured ADP uptake into submitochondrial particles in the presence of 1.6 μM carboxyatractyloside (CATR) or 7.9 μM bongkreic acid (BKA); and the results are shown in **Panel A**. **Panel B** shows the dose-dependent inhibition of ADP uptake by suramin (in the presence of 1.6 μM carboxyatractyloside). Note that the count observed in the presence of bongkreic acid (0.1 nmol/mg submitochondrial particles) was subtracted from the counts of individual samples in Panel B as backgrounds. Data represent mean value \pm SD of three independent runs.

exclusive inhibitor with respect to atractyloside and suggested that a primary site of action of suramin is the ADP/ATP carrier,²⁴⁾ but this possibility has not yet examined.

In a recent study, Todisco *et al.* carried out a docking-based virtual screening by using the crystal structure of the mitochondrial ADP/ATP carrier and a chemical library selected from the KEGG ligand database (criteria used for selection of ligands is “greater than 10 atoms and lighter than 1200 g/mol”).¹⁹⁾ With this approach, they successfully identified 100 chemicals expected to show a certain affinity for the mitochondrial ADP/ATP carrier. So, by using a bacterially expressed and reconstituted type 2 human mitochondrial ADP/ATP carrier, they selected 13 commercially available chemicals and examined whether these chemicals would show inhibitory effects on the mitochondrial ADP/ATP carrier. As a result, of these 13 chemicals tested, suramin and chebulinic acid were found to show inhibitory effects on the mitochondrial ADP/ATP carrier, and their IC_{50} values were determined to be approximately 0.8 μM and 4 μM , respectively. Their IC_{50} value of suramin (0.8 μM) was about 6 times lower than the one we observed in the present study (5 μM); but this difference could have been due to the difference in the experimental systems used between the two studies: they used a purified and reconstituted ADP/ATP carrier, whereas we used mitochondria isolated from rat liver. The exact interpretation of the inhibitory action of suramin in their study, especially the question as to from which side of the carrier does suramin inhibit would be very difficult; because they used the reconstituted ADP/ATP carrier. However, from the facts that membrane-impermeable carboxyatractyloside inhibited approximately 90% of the transport activity of the ADP/ATP carrier (Fig. 5a in ref.¹⁹⁾) and that bongkreic acid showed pH-dependent inhibitory effects (fourth paragraph in section 3.4 in ref.¹⁹⁾), the reconstituted ADP/ATP carrier seemed to show the orientation of “right side out.” Based on this interpretation, the inhibitory effect of suramin on the reconstituted ADP/ATP carrier would seem to reflect the inhibition of the ADP/ATP carrier from the cytosolic side. Therefore, the intriguing question regarding from which side of the membrane suramin inhibits the

mitochondrial ADP/ATP carrier was not yet answered by them.

In the present study, we successfully demonstrated that suramin inhibited the mitochondrial ADP/ATP carrier from both sides of the mitochondrial inner membrane. Our achievements do not simply contribute for the therapeutic usage of suramin, but may contribute for a better understanding of the features of mitochondrial ADP/ATP carrier. We observed slightly different IC_{50} values of suramin for ADP uptake by mitochondria and submitochondrial particles (5 and 10 μM , respectively). This difference may indicate different affinities of suramin for the cytosolic and matrix sides of the ADP/ATP carrier, but to validate this possibility, further careful experiments using mitochondria and submitochondrial particles prepared from identical animal species and identical tissues would seem to be necessary.

As numerous papers described that suramin shows inhibitory actions on ATP-related enzymes or proteins such as Na^+ , K^+ -ATPase,²⁵⁾ protein kinase C,²⁶⁾ prinoceptors,²⁷⁾ adenylyl cyclase²⁸⁾ or pyruvate kinase²⁹⁾ (see also ref. 18 for a recent review article on the action of suramin), suramin may be expected to be recognized by the ADP/ATP carrier as an analogue of adenine nucleotides. The ligand-accessible ADP/ATP carrier, i.e., both c-state and m-state carriers, were reported to show positively charged cavities toward intermembrane and matrix spaces, respectively.¹⁷⁾ Suramin may bind to these positive cavities of ADP/ATP carrier as a nucleotide analogue. However, it cannot be transported by the carrier, due to its structural differences from the transportable genuine substrates, ADP or ATP, and therefore inhibits the carrier. Moreover, different from the canonical and specific inhibitors of the ADP/ATP carrier, i.e., carboxyatractyloside or bongkreic acid, both of which can interact with the multiple amino acid residues in the ADP/ATP carrier,^{15,17)} suramin does not have such structural properties. This difference would be the reason why suramin does not show high affinity for the ADP/ATP carrier.

In conclusion, we concluded suramin to be utilizable as a new type inhibitor of the mitochondrial ADP/ATP carrier, effective from both sides of the inner mitochondrial membrane; but we must pay attention to possible side-effects,

especially when it is applied to whole mitochondria. When we were finalizing the preparation of our manuscript, a new paper describing the inhibitory action of suramin on the ADP/ATP carrier was published.³⁰⁾ The authors carefully evaluated the effects of candidate inhibitors on the ADP/ATP carrier by employing multiple assay systems: i) cellular metabolic profiling, ii) thermostability shift assay, and iii) reconstituted ADP/ATP carrier. Their IC₅₀ value of suramin obtained with the reconstitution assay (2.4 μM) was not markedly different from ours. More importantly, they also emphasized the importance of employing a direct measurement system to avoid false-positive effects.

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Conflict of interest The authors declare no conflict of interest.

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