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SCOPAFUNGIN, AN INHIBITOR OF OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

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Abstract—Scopafungin (U-29479), an antibiotic produced by a streptomycete strain, acts as an uncoupling agent of oxidative phosphorylation in mitochondria. In addition, mitochondrial respiration is also impaired but to a lesser degree. Studies of individual reaction sequences occurring within the respiratory chain and mitochondrial difference spectra suggest that scopafungin inhibits electron flow at the flavoprotein regions associated with the oxidation of NADH and succinate.

SCOPAFUNGIN (U-29479) was isolated from the culture broth of *Streptomyces hygroscopicus var. enhygrus*. This strain produces several antibiotics commonly referred to as the endomycin complex. Crystalline scopafungin is not very soluble in water but is soluble in most organic solvents. The agent is a single chemical entity isolated from the endomycin complex of antibiotics and is non-polyenic in nature. Preparation, isolation, characterization and biological properties are described elsewhere.[1,2] Scopafungin is a very potent growth inhibitor for gram-positive bacteria, yeasts and fungi *in vitro* but has been ineffective in the systemic treatment of experimental bacterial infections in mice.

This paper describes the effect of scopafungin on oxidative phosphorylation in rat liver mitochondria and submitochondrial particles.

MATERIALS AND METHODS

Liver mitochondria were isolated from male albino rats weighing 150–250 g as described by Lardy and Wellmann.[3] Mitochondrial protein was determined with biuret reagent, with crystalline bovine albumin used as standard.[4] Mitochondrial respiration was measured manometrically.[3] Hexokinase was purchased from Cal-Biochem, Los Angeles, Calif. Inorganic phosphate was assayed by the method of Lowry and López.[5]

For the preparation of sonicated mitochondria, washed intact mitochondria suspended in 0·25 M sucrose were diluted to a concentration of 20 mg protein/ml. This suspension was subjected to sonication in a Bronwill sonicator set at 90 per cent maximum output for 90 sec. The suspension was centrifuged for 20 min at 78,000 *g*. The resulting pellet was resuspended in enough 0·25 M sucrose and homogenized in a Potter–Elvehjem homogenizer to yield a final protein concentration of 20 mg/ml.

Sub-mitochondrial fragments were prepared as described by Kielley and Bronk.[6]

Reduced nicotinamide-adenine dinucleotide (NADH)-linked ferricyanide reduction by mitochondria was followed spectrophotometrically by reading the decrease in optical density (O.D.) of the reaction mixtures at 420 nm.

Cytochrome *c* reductase and oxidase activities were assessed spectrophotometrically.[7,8] Cytochrome *c* was reduced as described by Smith[8] and had an $O.D._{550}$ to $O.D._{565}$ ratio of 5. Mitochondrial difference spectra were run at room temperature with an Unicam SP 800A double beam spectrophotometer. The exact experimental conditions are given in the Results section.

RESULTS

Mitochondrial oxidation of glutamate, succinate and β-hydroxybutyrate. The effects of scopafungin on oxidative phosphorylation associated with the oxidation of the substrates mentioned above was assessed manometrically. During the oxidation of

TABLE 1. EFFECT OF SCOPAFUNGIN ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA*

Substrate	Scopafungin (μg/ml)	Qo_2 (P)	Inhibition of respiration (%)	P : O ratio	Uncoupling (%)
Glutamate	none	41·5	0	2·6	0
	20	22·7	45·4	0	100·0
	10	39·0	6·1	1·0	61·6
	5	41·0	1·4	1·2	53·9
Succinate	none	37·6	0	2·0	0
	20	36·2	3·8	0	100·0
	10	38·0	0	0·7	66·0
	5	38·9	0	1·4	30·0
β-Hydroxybutyrate	none	23·8	0	2·0	0
	20	11·7	50·9	0	100·0
	10	20·7	13·1	1·1	45·0
	5	23·0	3·4	1·8	10·0

* Each vessel contained in a total volume of 3 ml: 6 μmoles of adenosine triphosphate; 50 μmoles of $K–PO_4$ buffer (pH 7·4); 15 μmoles of $MgSO_4$; 30 μmoles of sodium-L-glutamate; 40 μmoles of sodium β-hydroxybutyrate or 20 μmoles of sodium succinate, respectively; and 0·5 ml of mitoch-on drial suspension (20 mg of protein/ml) in 0·25 M sucrose. Hexokinase (20 μg; CalBiochem) and 50 μmoles of glucose were added from the side arms. Reactions were run for 15 min at 30°. Qo_2 (P) = microliters of O_2 uptake per milligram of mitochondrial protein per hour.

glutamate, respiration was inhibited by 45 per cent in the presence of 20 μg of scopafungin/ml (Table 1). Lower antibiotic concentrations caused inhibitions of less than 10 per cent with this substrate. Phosphorylation was inhibited extensively. A concentration of 20 μg of scopafungin/ml caused complete uncoupling and concentrations of 10 or 5 μg/ml caused 61 and 54 per cent uncoupling respectively. Mitochondrial respiration associated with the oxidation of succinate was inhibited to an extent of less than 4 per cent in the presence of 20 μg of antibiotic/ml. Lower drug concentrations did not affect respiration. As observed during the oxidation of glutamate, phosphorylation was affected drastically. Twenty μg of antibiotic/ml caused complete uncoupling of phosphorylation. A drug concentration of 10 μg/ml caused 66 per cent uncoupling and at 5 μg/ml uncoupling amounted to 30 per cent. During the oxidation of β-hydroxybutyrate, which does not proceed via the Krebs cycle in liver mitochondria, respiration was inhibited by approximately 50 per cent in the presence of 20 μg of

scopafungin/ml; 10 μg/ml inhibited this process by 13 per cent and 5 μg/ml caused 3 per cent inhibition. Phosphorylation was substantially inhibited by scopafungin. Twenty μg of antibiotic/ml caused complete uncoupling, 10 or 5 μg/ml uncoupled to extents of 45 and 10 per cent respectively.

These results indicate that phosphorylation was generally inhibited to a larger degree by scopafungin than respiration at a given drug concentration. On the other hand, respiration was more susceptible to scopafungin inhibition during the oxidation of the NADH-linked substrates, glutamate or β-hydroxybutyrate, than during the oxidation of succinate.

In a medium deficient in inorganic phosphate, glutamate oxidation was inhibited by scopafungin to an extent of 25 per cent of the control (Table 2). In the presence of 2,4-dinitrophenol (DNP), scopafungin also proved inhibitory. The respiratory inhibition exerted by scopafungin is thus not reversed by dinitrophenol.

TABLE 2. EFFECT OF SCOPAFUNGIN ON MITOCHONDRIAL GLUTAMATE OXIDATION IN A MEDIUM DEFICIENT IN ORTHOPHOSPHATE*

Addition	Q_{O_2} (P)	% of control
Control (none)	6·3	100·0
Scopafungin (30 μg/ml)	4·7	74·6
DNP (0·15 μmole/ml)	8·9	141·2
DNP + scopafungin (30 μg/ml)	5·0	79·3
DNP + scopafungin (15 μg/ml)	5·8	92·0

* Each vessel contained in a total volume of 3 ml: 6 μmoles of Tris-HCl buffer (pH 7·4); 15 μmoles of $MgSO_4$; 30 μmoles of sodium glutamate; 0·45 μmoles of DNP when applicable; and 0·5 ml of mitochondrial suspension (20 mg protein/ml) in 0·25 M sucrose. Hexokinase (20 μg) and 50 μmoles of glucose were added from the side arms. Reactions were run for 60 min at 30°. Q_{O_2} (P) = microliters of O_2 uptake per milligram of mitochondrial protein per hour.

To elucidate further the exact point of interference of scopafungin within the respiratory chain, individual reaction sequences of the chain were studied in more detail. For these studies submitochondrial fragments were used as an enzyme source since intact mitochondria have a very low permeability for nucleotides and other compounds used in these studies.

Reduction of ferricyanide by NADH. Ferricyanide accepts electrons from the flavoprotein region during the oxidation of NADH without the participation of coenzyme Q or cytochromes. In the presence of 200 μg of scopafungin/ml, no interference with this reaction was evident (Fig. 1). This indicates that scopafungin does not interfere with NADH oxidation coupled to ferricyanide reduction.

Cytochrome c *reductase.* Cytochrome *c* reductase activity of the submitochondrial fragment fraction was assessed by measuring the increase in optical density of reduced cytochrome *c* at 550 nm.

NADH-linked cytochrome *c* reductase activity was inhibited by approximately 60 per cent in the presence of 200 μg of scopafungin/ml (Fig. 2). Lower antibiotic

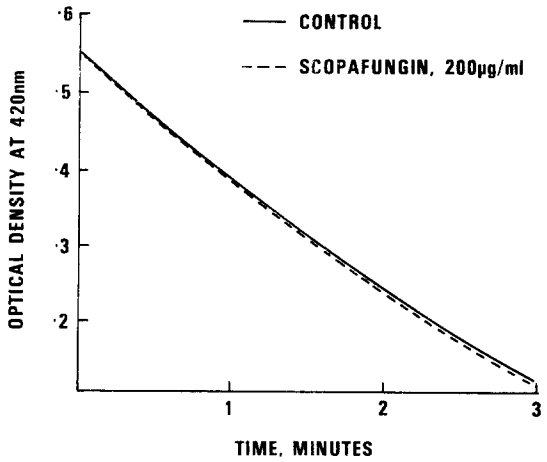

FIG. 1. NADH-linked reduction of ferricyanide by submitochondrial fragments. The reaction mixtures contained in a total volume of 3 ml: 150 μmoles of tris-(hydroxymethyl)aminomethane–hydrochloride buffer (pH 7·4); 600 μg of scopafungin; 3 μmoles of KCN; 1·5 μmoles of $KFe(CN)_6$, 5 μmoles of NADH, and 100 μg of mitochondrial protein. The reaction was started by adding NADH. The optical density was read at 420 nm against a blank cuvette containing all the reaction mixture components except NADH.

concentrations (100 or 50 μg/ml) inhibited this reaction by less than 10 per cent. The succinate-linked reduction of cytochrome *c* was completely blocked in the presence of 200 μg of scopafungin/ml (Fig. 3). In the presence of either 100 or 20 μg of antibiotic/ml, inhibition of cytochrome *c* reductase was complete during the first minute following initiation of the reaction (60 sec in the presence of 100 μg, approximately 50 sec in the presence of 20 μg of antibiotic/ml). After this initial lag period, the system recovered and cytochrome *c* reduction proceeded at near normal rates. These

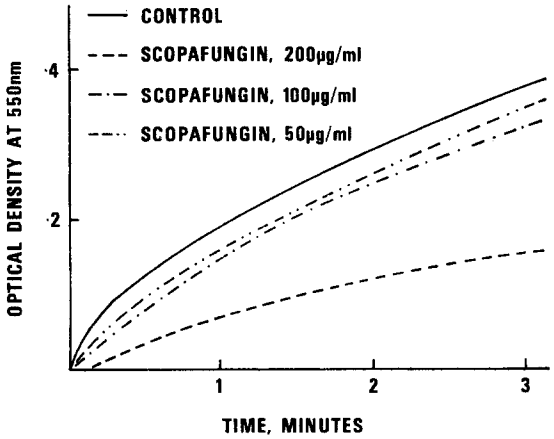

FIG. 2. NADH-linked reduction of cytochrome *c* by submitochondrial fragments. The reaction mixtures contained in a total volume of 3 ml: 150 μmoles of tris–hydrochloride (pH 7·4), 3 μmoles of KCN, 1·5 mg of cytochrome *c* (type III, Sigma), 15 μmoles of NADH, and 40 μg of mitochondrial protein. The reactions were started by the addition of NADH. The blanks contained all the reaction mixture components above except NADH.

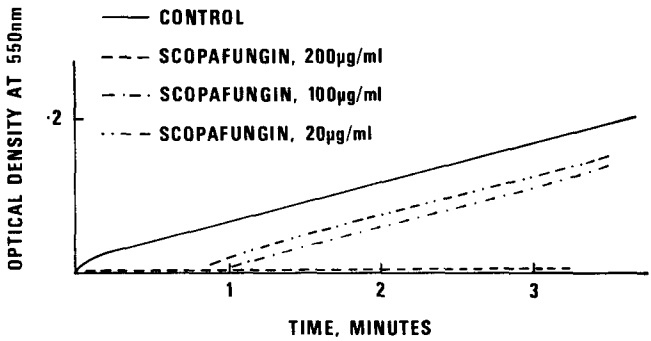

FIG. 3. Succinate-linked reduction of cytochrome *c* by submitochondrial fragments. The reaction mixtures contained in a total volume of 3 ml: 150 μmoles of tris-(hydroxymethyl)aminomethane–hydrochloride (pH 7·4), 3 μmoles of KCN, 1·5 mg of cytochrome *c*, 50 μmoles of succinate, and 200 μg of mitochondrial protein. The reaction mixtures were read against blank cuvettes containing all the reaction mixture components except substrate.

results indicate that succinate-linked cytochrome *c* reductase was somewhat more sensitive to scopafungin inhibition than NADH-associated cytochrome *c* reductase in submitochondrial fragments.

Cytochrome c *oxidase.* Cytochrome *c* oxidase was stimulated approximately 2·5-fold in the presence of either 200 or 100 μg of scopafungin/ml (Fig. 4). Antibiotic concentrations of 50 or 20 μg/ml remained without effect.

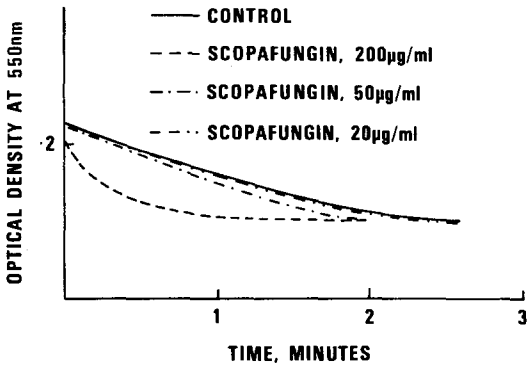

FIG. 4. Oxidation of reduced cytochrome *c* by submitochondrial fragments. The reaction mixture contained in a total volume of 3 ml: 150 μmoles of tris-(hydroxymethyl)aminomethane hydrochloride (pH 7·4), 600 μg of scopafungin, 0·5 mg of reduced cytochrome *c*, and 200 μg of mitochondrial protein. The reactions were started by the addition of mitochondria. The reaction mixtures were read against blank cuvettes containing all the reaction mixture components except mitochondria.

Mitochondrial difference spectra. For these experiments, sonicated mitochondria were used. When either NADH or succinate served as electron donors, the control spectra showed distinct absorption peaks at 605, 560 and 550 nm. These maxima correspond to the α-bands of reduced cytochrome *a*, *b* and *c* types respectively (Fig. 5, 6 top). The two peaks corresponding to the type *b* and *c* cytochromes were not resolved well from each other but the two maxima at 560 and 550 nm are discernible. The loss of absorbance located at approximately 465 nm reflects the extent of reduced flavoproteins present. This region is commonly referred to as the flavin dip. The γ-bands of

the reduced cytochromes are not visible in great detail due to the expansion of the scale in the figures.

With NADH as the reducing agent only traces of reduced flavins, cytochromes *b* and *c* and no reduced cytochromes *a* were visible in the presence of scopafungin when the sample was scanned immediately after the addition of NADH (Fig. 5). Total scanning time of the instrument was 72 sec. On rescanning the same samples 3 min after the addition of NADH, the spectrum revealed distinct peaks in the α-region of the

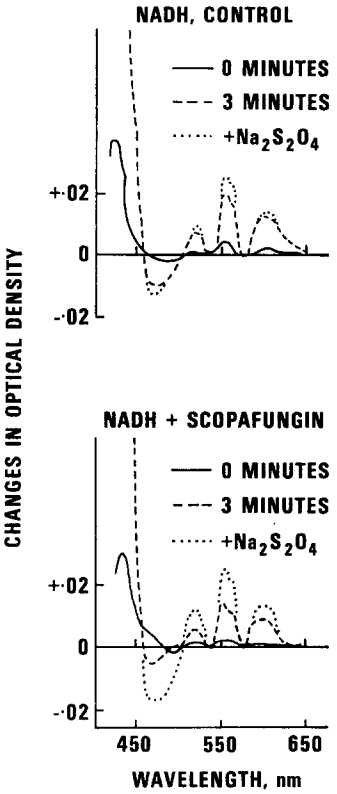

FIG. 5. Mitochondrial difference spectra with NADH. Top = control; bottom = scopafungin, 200 μg/ml. The reaction mixtures contained in a total volume of 3 ml: 200 μmoles of K–PO_4 buffer (pH 7·0); 750 μmoles of sucrose; 15 μmoles of NADH and 14 mg of mitochondrial protein. The spectra were read against a blank cuvet containing all the reaction mixture ingredients except NADH.

spectrum indicating the presence of reduced cytochromes *b*, *c*, and *a*. The flavin dip at 465 nm remained shallow. Addition of a few crystals of $Na_2S_2O_4$ to the sample cuvette resulted in a sizable enlargement of the flavin dip and some concomitant enlargement of the individual cytochrome peaks. Scopafungin at 200 μg/ml, therefore, seems to impair electron flow somewhere prior to or at the flavoprotein region associated with the oxidation of NADH.

With succinate as a reducing substrate, only traces of reduced flavins and cytochromes were apparent in the presence of scopafungin when the samples were scanned immediately after the addition of the substrate or 3 min later (Fig. 6 bottom). Full

reduction, effected by the addition of $Na_2S_2O_4$, produced a sizable increase of the flavin dip and distinct peaks indicating the presence of reduced cytochromes *b*, *c*, and *a*. These results show that scopafungin also induces substantial inhibition of flavoprotein and cytochrome reduction during the oxidation of succinate. Thus, the mitochondrial difference spectra prepared with either NADH or succinate as substrates show that scopafungin at 200 μg/ml interferes with electron transfer at the flavoprotein regions in both cases. However, the inhibitory effect induced by the antibiotic was more substantial when succinate rather than NADH served as substrate.

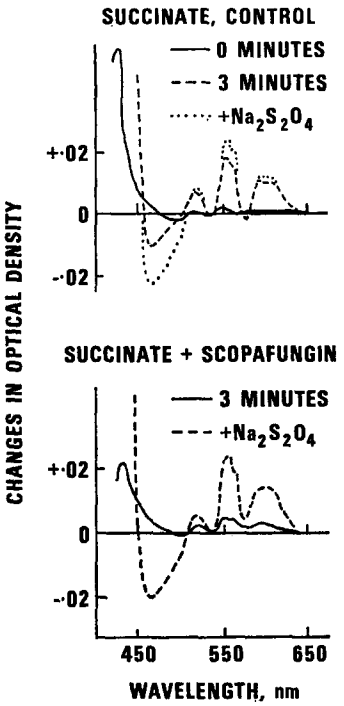

FIG. 6. Mitochondrial difference spectra with succinate. Top = control; bottom = scopafungin, 200 μg/ml. The reaction mixtures contained in a total volume of 3 ml: 200 μmoles of K–PO_4 buffer (pH 7·0); 750 μmoles of sucrose; 50 μmoles of Na-succinate and 14 mg of mitochondrial protein.

DISCUSSION

Scopafungin acts as an uncoupler of respiration-associated phosphorylation in mitochondria. Complete (100 per cent) uncoupling occurred during the oxidation of glutamate, succinate and β-hydroxybutyrate in the presence of 20 μg of scopafungin/ml. At this antibiotic concentration, NAD-linked respiration was only partially inhibited (approximately 50 per cent). Phosphorylation was thus impaired to a much larger extent than respiration at a given antibiotic concentration. Scopafungin, therefore, acts primarily as an uncoupler of oxidative phosphorylation.

Studies of individual reaction sequences occurring within the respiratory chain showed that NADH-ferricyanide reductase activity was not inhibited by scopafungin.

Cytochrome *c* reductase was inhibited substantially by high concentrations of scopafungin. The reaction was more sensitive to scopafungin inhibition if succinate

rather than NADH served as the reducing agent. It is of interest that scopafungin, present in concentrations which induce only partial inhibition of succinate–cytochrome *c* reductase, blocked this reaction completely for a short period of up to 1 min immediately after addition of substrate. After this initial lag period, the system recovered and cytochrome *c* reduction proceeded at rates comparable to the control.

Cytochrome *c* oxidase was stimulated in the presence of high concentrations of scopafungin (100–200 μg/ml). Lower concentrations had no effect. The stimulatory effect of scopafungin in this reaction is difficult to interpret. However, within the fully integrated electron transport chain, this stimulatory effect would be obscured since the cytochrome *c* reductase function, strongly inhibited by the antibiotic, immediately precedes the cytochrome *c* oxidase function.

Difference spectra showed that scopafungin reduces the extent of mitochondrial NADH and succinate-linked reduction of flavins and cytochromes. The flavoprotein-reducing regions are located prior to the cytochrome reduction sites within the respiratory chain. This suggests that scopafungin in higher concentrations, interferes with electron transport of the respiratory chain at the flavoprotein regions associated with NADH and succinate oxidation. In these studies with high concentrations of scopafungin and submitochondria particles, the flavoprotein region associated with the oxidation of succinate appears more sensitive to scopafungin inhibition than the corresponding region linked to NADH oxidation. This is contrary to the sensitivity of respiration in intact mitochondria.

NADH-ferricyanide reductase was not inhibited by scopafungin as discussed above although it is assumed that ferricyanide diverts electrons from the flavoprotein region during the oxidation of NADH. This indicates that the inhibition site of scopafungin within the flavoprotein region is located after the ferricyanide reduction site.

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