Inhibition of Cellular Respiration by 1,3,4-Thiadiazolo[3,2-*a*]pyrimidines[†]Masahito SUIKO, Eiji TANIGUCHI, Kazuyuki MAEKAWA*
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2-Ethylsulfonyl-7-methyl-5*H*-1, 3, 4-thiadiazolo[3, 2-*a*]pyrimidin-5-one (TPSO₂-2) showed inhibitory activity on cellular respiration of Ehrlich ascites tumor cells (E-cells) and *Escherichia coli* at 3.9×10^{-4} M. In polarographic traces of rat liver mitochondrial respiration, the agent inhibited the energy transfer of oxidative phosphorylation at 8×10^{-4} M. However, in the presence of tricine[*N*-tris(hydroxymethyl)methylglycine], the TPSO₂-2 inhibitory activity on cellular respiration disappeared. The permeability of cells was altered by TPSO₂-2 treatment, and this effect was interrupted by adding tricine. TPSO₂-2 did not inhibit the multiplication of *E. coli* in the presence of tricine but did inhibit E-cell multiplication. This means that the inhibitory activity of TPSO₂-2 on *E. coli* multiplication is attributable to respiration inhibition, whereas the cytotoxicity of TPSO₂-2 should be due to reasons other than respiration inhibition.

Some derivatives of 1,3,4-thiadiazolo[3,2-*a*]pyrimidine, a kind of purine analogs, were cytotoxic to Ehrlich ascites tumor cells (E-cells) and other experimental cells at growing phase. Strong activity was found among compounds which had an electrophilic substituent, such as alkylsulfinyl or alkylsulfonyl group at 2-position.¹⁾

2-Ethylsulfonyl-7-methyl-5*H*-1, 3, 4-thiadiazolo[3,2-*a*]pyrimidin-5-one (TPSO₂-2), the most active compound in this series, scarcely inhibited DNA polymerase, protein synthesis system and DNA-dependent RNA polymerase obtained from *Escherichia coli* K-12, but strongly inhibited DNA-dependent RNA polymerase from E-cells.²⁾ However, TPSO₂-2 suppressed the multiplication of both E-cells and *E. coli*. This suggests another mode of action of TPSO₂-2, besides RNA polymerase inhibition.

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Abbreviation: DNP, 2,4-dinitrophenol; E-cells, Ehrlich ascites tumor cells; PBS(-), phosphate buffered saline (without Ca²⁺ and Mg²⁺); TPSO₂-2, 2-ethylsulfonyl-7-methyl-5*H*-1, 3, 4-thiadiazolo[3, 2-*a*]pyrimidin-5-one; tricine, *N*-tris(hydroxymethyl)methylglycine.

In this paper the effects of TPSO₂-2 as to respiration, permeability and glycolysis are examined. It acted as an inhibitor on energy transfer for mitochondrial oxidative phosphorylation. This appears to be the mode of action of TPSO₂-2 for bactericidal activity but only a minor factor for cytotoxicity to E-cells.

EXPERIMENTAL

Materials. TPSO₂-2 was prepared as reported previously.¹⁾ ADP, ATP, phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and other reagents were purchased from the Boehringer Mannheim Yamanouchi Co. E-cells were harvested from ddN female mice inoculated 5-7 days beforehand with E-cells and washed with PBS(-). The cultured E-cells were prepared, as reported previously.¹⁾ *E. coli* K-12 was grown by shaking in liquid medium of pH 7.0 containing 0.5% glucose, 0.5% yeast extract and 2% peptone.

Cytotoxicity of TPSO₂-2 on E-cells and *E. coli*. The cytotoxicity of TPSO₂-2 on cultured E-cells was estimated by the method described previously.¹⁾ E-cells were incubated at 37°C for 3 days and counted by using the Bürkel Türk haematometer. The effect of TPSO₂-2 on growth of *E. coli* was estimated spectrophotometrically at 610 nm. Test tubes containing 6 ml of the medium described above were inoculated with 10⁸ cells of *E. coli* and cultured at 30°C for 18 hr by shaking at 115 rpm.

Cellular respiration. Respiration of E-cells and *E. coli* was determined in a Warburg manometer at 37°C. The center well of each vessel contained 0.2 ml of 20% potassium hydroxide and a piece of filter paper. Each vessel contained 2.3 ml of cell suspension and 0.5 ml of TPISO₂-2. A buffer solution was substituted for TPISO₂-2 in controls. TPISO₂-2 was added after a 20~30 min incubation and oxygen uptake was determined at 5~15 min intervals for 120 min.

Preparation of mitochondria. Rat liver mitochondria was prepared according to Hogeboom's procedure³⁾ in medium containing 0.25 M sucrose and 0.1 mM EDTA and adjusted to pH 7.4 with Tris-HCl. The final mitochondrial concentrations of respiratory and ATPase measurement systems were approximately 0.8 and 0.3 mg of protein per ml, respectively.

Mitochondrial respiration. Respiration of rat liver mitochondria was followed polarographically with a Clark oxygen electrode (Yellow-Springs Instrument Co.) at 37°C (volume, 3 ml). The medium consisted of sucrose 0.25 M, KCl 0.01 M, K₂HPO₄ 0.01 M, Tris-HCl 0.01 M, MgCl₂ 2 mM and EDTA 0.2 mM and was adjusted to pH 7.4.⁴⁾

ATPase activity. The ATPase activity of liver mitochondria was assayed in the presence of an ATP-regenerating system consisting of phosphoenolpyruvate and pyruvate kinase.⁵⁾ After the reaction was stopped with trichloroacetic acid at 10% final concentration, inorganic phosphate was determined in the supernatant according to Fiske and SubbaRow.⁶⁾ Protein was determined according to Hartree.⁷⁾

Cell permeability. This was determined by staining E-cells with nigrosine^{8,9)} or erythrosine B.¹⁰⁾

Interaction of E-cells with TPISO₂-2. E-cells were boiled for 1 min and washed several times with PBS(—) or tricine buffer [tricine 50 mM, NaCl 95 mM, KCl 5 mM, MgCl₂ 2 mM, pH 7.4].¹⁰⁾ The cells and TPISO₂-2 (1.5×10^{-4} M) were incubated at 37°C for 1 or 2 hr, and centrifuged at 3,000 rpm for 10 min. Free TPISO₂-2 in the supernatant was determined spectrometrically at 300 nm.

Glycolysis. Glycolytic activity was measured in a final volume of 1 ml according to McCoy's procedure.¹⁰⁾ E-cells (4 mg protein: 8×10^6 cells) suspended in PBS(—) were incubated at 37°C for 5 min by shaking. Reactions were started by addition of 10 mM of glucose. After 30 min at 37°C, 1 ml of 10% trichloroacetic acid was added, and the mixture was centrifuged at $1,000 \times g$ for 10 min. The supernatant was subjected to lactate assay, as described by Hohorst.¹¹⁾

RESULTS

Inhibition of respiration and the effect of tricine buffer

In the manometric measurements, potassium cyanide and rotenone, well-known respiratory inhibitors, depressed the oxygen uptake of E-cells (8×10^8 cells) at 10^{-3} M and 10^{-5} M, respectively. Similarly, TPISO₂-2 in PBS(—) depressed almost completely the oxygen uptake of E-cells at a concentration of 3.9×10^{-4} M, as shown in Fig. 1.

FIG. 1. Effect of TPISO₂-2 on Respiration of E-Cells. ○—○, Control [PBS(—) or tricine buffer]; ●—●, TPISO₂-2 in tricine buffer (3.9×10^{-4} M); ▲—▲, TPISO₂-2 in PBS(—) (3.9×10^{-4} M); ↓, addition of test solution.

FIG. 2. Effect of TPISO₂-2 on Respiration of *E. coli*. ○—○, Control (tricine buffer); △—△, control (PBS(—)); ●—●, TPISO₂-2 in tricine buffer (3.9×10^{-4} M); ▲—▲, TPISO₂-2 in PBS(—) (3.9×10^{-4} M); ↓, addition of test solution.

TABLE I. EFFECT OF TRICINE BUFFER ON CELL PERMEABILITY OF TP₂SO₂-2^a

E-Cells washed with	Pretreatment	TPSO ₂ -2 (3 × 10 ⁻⁴ M) dissolved in	Rate of nigrosine stain (%)
PBS (—)	—	PBS (—)	100
PBS (—)	—	Tricine buffer	76
PBS (—)	Washed with tricine buffer	PBS (—)	90
PBS (—)	Added 2 drops of tricine buffer and incubated for 30 min	PBS (—)	30
Tricine buffer	—	Tricine buffer	0
Tricine buffer	—	PBS (—)	90
Tricine buffer	Incubated for 30 min in tricine buffer and washed with PBS (—)	PBS (—)	85
Tris-HCl buffer	—	Tris-HCl buffer	100

^a E-cells (1 × 10⁵ cells/10 μl) washed with buffer solution were added to 0.5 ml of TP₂SO₂-2 with or without pretreatment. The mixture was incubated for 2 hr at 37°C and then stained with nigrosine or erythrosine B.

Against *E. coli* (A₆₁₀=11.6), TP₂SO₂-2 in PBS(—) inhibited respiration less effectively at the same dose, but at a lower concentration respiration was not inhibited (Fig. 2).

However, when tricine buffer was used instead of PBS(—), the TP₂SO₂-2 inhibitory activity on respiration of E-cells and *E. coli* disappeared (Figs. 1 and 2). A factor in tricine buffer responsible for extinguishing the TP₂SO₂-2 inhibitory activity on respiration was concluded to be the tricine molecule itself.

When E-cells were pretreated with tricine, the TP₂SO₂-2 inhibitory activity on cellular respiration disappeared. The same phenomenon was found on cell permeability of E-cells (Table I). TP₂SO₂-2 in PBS(—) altered E-cell permeability, but when TP₂SO₂-2 (3 × 10⁻⁴ M) in tricine buffer was added to E-cells which were washed with tricine buffer, the cells were not stained with nigrosine or erythrosine B.

The cytotoxic activities and the effect of TP₂SO₂-2 on respiration of E-cells and *E. coli* were tested in medium with and without tricine. The cultured E-cells were incubated for 3 days, while *E. coli* was cultured for 18 hr with shaking. The results are shown in Tables II and III. The effect of TP₂SO₂-2 on respiration of E-cells or *E. coli* was extremely distinct in the buffer used as mentioned above. The cytotoxicity of TP₂SO₂-2 in PBS(—) on E-cells appeared in 2 hr incubation, but that of TP₂SO₂-

2 in tricine buffer did not entirely. When E-cells were cultured for 3 days, however, the cytotoxic activity of TP₂SO₂-2 in tricine buffer on E-cells was the same as that in PBS(—), that is, LC₅₀ was 3 μg/ml (1 × 10⁻⁵ M) in both the buffers (Table II). On the other hand, the inhibitory activity of TP₂SO₂-2 (100 μg/ml: 3.8 × 10⁻⁴ M) on *E. coli* multiplication was not

TABLE II. CYTOTOXICITY AND EFFECT OF TP₂SO₂-2 ON RESPIRATION OF E-CELLS

TPSO ₂ -2 in	Cytotoxicity (%)		Inhibition of respiration (%) ^b (3.9 × 10 ⁻⁴ M)
	2 hr (3.9 × 10 ⁻⁴ M)	3 days (3.9 × 10 ⁻⁵ M)	
PBS(—)	100	100 ^a	100
Tricine buffer	0	100 ^a	0

^a LC₅₀ was 1 × 10⁻⁵ M.

^b Respiration activity was measured by the manometric method after 60 min.

TABLE III. EFFECT OF TP₂SO₂-2 ON GROWTH AND RESPIRATION OF *E. coli*

TPSO ₂ -2 in	Inhibition of growth (%) ^a (3.9 × 10 ⁻⁴ M)	Inhibition of respiration (%) ^b (3.9 × 10 ⁻⁴ M)
PBS(—)	100	53
Tricine buffer	0	0

^a *E. coli* (10⁸ cells) were cultured for 18 hr at 37°C with shaking. Growth inhibition was estimated from A₆₁₀.

^b Respiration activity was measured by the manometric method after 60 min.

FIG. 4. Effect of $\text{TPSO}_2\text{-2}$ on Oxygen Uptake of Rat Liver Mitochondria.
-----, Without $\text{TPSO}_2\text{-2}$; —, with $\text{TPSO}_2\text{-2}$.

present in tricine buffer but was present in PBS(—).

Interaction of E-cells with $\text{TPSO}_2\text{-2}$

The adsorption on E-cell membrane of $\text{TPSO}_2\text{-2}$ in each buffer was measured. Cells killed by boiling were used to avoid interference of $\text{TPSO}_2\text{-2}$ measurement at 300 nm with something released from living cells. As shown in Fig. 3, $\text{TPSO}_2\text{-2}$ adsorption on the surface of E-cells was inhibited by tricine molecules. The adsorbed $\text{TPSO}_2\text{-2}$ in tricine buffer

FIG. 3. Interaction of Dead E-Cells with $\text{TPSO}_2\text{-2}$ in PBS(—) or Tricine Buffer.

$\text{TPSO}_2\text{-2}$ adsorbed on E-cell (mol/E-cell) was determined from ultraviolet absorption of free $\text{TPSO}_2\text{-2}$ in supernatant at 300 nm. ●—●, in PBS(—); ○—○, in tricine buffer.

was one-third to one-fourth that in PBS(—). From this fact, tricine molecules appeared to inhibit the combining of $\text{TPSO}_2\text{-2}$ molecules to the cell membrane.

Effect of $\text{TPSO}_2\text{-2}$ on mitochondrial respiration

The inhibitory action mode of $\text{TPSO}_2\text{-2}$ on rat liver mitochondrial respiration was investigated. The results are illustrated with polarographic traces, shown in Fig. 4. When $\text{TPSO}_2\text{-2}$ (8×10^{-4} M) was added to the incubation mixture in state 4 oxidation of succinate, the addition of ADP (2×10^{-4} M) did not increase the oxygen uptake rate. However, the addition of 2,4-dinitrophenol (DNP) (5×10^{-5} M), an uncoupling agent,¹²⁾ apparently increased the rate of oxidation. Next, $\text{TPSO}_2\text{-2}$ (8×10^{-4} M) added in state 3 oxidation, gradually depressed the rate of oxidation. Neither ADP nor DNP increased it, but 2-phenylpropylguanidino-4,6-dimethylpyrimidine (PPG-2) (2.4×10^{-4} M), an uncoupling agent reported by Shuto *et al.*,¹³⁾ increased the oxidation.

Effect of $\text{TPSO}_2\text{-2}$ on ATPase activity

The effect of $\text{TPSO}_2\text{-2}$ on ATPase of rat liver mitochondria is shown in Table IV. $\text{TPSO}_2\text{-2}$ had no effect on ATPase activity itself, but inhibited DNP action on ATPase activity. This finding suggests that $\text{TPSO}_2\text{-2}$

TABLE IV. EFFECT OF $\text{TPSO}_2\text{-2}$ ON RAT LIVER MITOCHONDRIAL ATPASE^a

Additions	$\mu\text{mol Pi/mg protein/min}$
None	0.34
+ 3.8×10^{-4} M $\text{TPSO}_2\text{-2}$	0.35
+ 1.2×10^{-4} M $\text{TPSO}_2\text{-2}$	0.33
+ 3.8×10^{-5} M $\text{TPSO}_2\text{-2}$	0.35
+ 5.0×10^{-5} M DNP	0.49
+ 5.0×10^{-5} M DNP + 3.8×10^{-4} M $\text{TPSO}_2\text{-2}$	0.39

^a ATPase activity of rat liver mitochondria was determined at 37°C for 15 min using the Kagawa method.⁵⁾

TABLE V. EFFECT OF $\text{TPSO}_2\text{-2}$ ON GLYCOLYSIS OF E-CELLS

Addition	Lactate formation ^a ($\mu\text{mol}/30 \text{ min/mg protein}$)	Inhibition (-) or promotion (+) (%)
None	0.30	
+ Dextran sulfate (2.7×10^{-5} M)	0.20	-33.4
+ DNP (5.4×10^{-4} M)	0.36	+20.0
+ $\text{TPSO}_2\text{-2}$ (7.7×10^{-4} M)	0.30	0

^a E-cells (8×10^6 cells) were incubated with the indicated concentration of each compound for 30 min at 30°C.

acts in a manner similar to that of oligomycin, the inhibitor of energy transfer of oxidative phosphorylation.^{14,15)}

Effect of $\text{TPSO}_2\text{-2}$ on glycolysis

The result shown in Table V indicates that glycolysis of E-cells is not inhibited even at such a high concentration of $\text{TPSO}_2\text{-2}$ as 7.7×10^{-4} M.

DISCUSSION

$\text{TPSO}_2\text{-2}$ showed inhibitory activities on cellular and mitochondrial respiration at 3.9×10^{-4} M and 8×10^{-4} M, respectively. Its mode of action was found to be inhibition of energy transfer of oxidative phosphorylation. However, in the presence of tricine the $\text{TPSO}_2\text{-2}$ inhibitory activity on cellular respiration dis-

appeared, and the alteration of cell permeability also disappeared. $\text{TPSO}_2\text{-2}$ adsorption on the E-cell membrane decreased in the presence of tricine molecules. However, the effect of $\text{TPSO}_2\text{-2}$ on rat liver mitochondrial respiration did not disappear by addition of tricine. Thus, it can be presumed that certain binding sites for $\text{TPSO}_2\text{-2}$ may be present on the cell surface and that binding with $\text{TPSO}_2\text{-2}$ may be inhibited by the tricine molecules.

$\text{TPSO}_2\text{-2}$ did not inhibit the multiplication of *E. coli* even in the presence of tricine molecules, but did inhibit E-cells and had no effect on glycolysis. Therefore, the inhibitory activity of $\text{TPSO}_2\text{-2}$ on *E. coli* multiplication is attributable to respiration inhibition. On the other hand, for the inhibition of E-cell multiplication, another mechanism of action than respiratory inhibition could be responsible. As reported previously,²⁾ the inhibition of RNA synthesis can be thought as the most reasonable mode of action for cytotoxicity; $\text{TPSO}_2\text{-2}$ inhibits RNA polymerase at a much lower concentration (5×10^{-5} M) than respirations of cells (4×10^{-4} M) or mitochondria (8×10^{-4} M).

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