Nitroxyl Antioxidant TPPA-TEMPO Increases the Efficacy of Antitumor Therapy on the Model of Transplantable Mouse Tumor

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Nitroxyl antioxidant 4-triphenylphosphonioacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl (TPPA-TEMPO) was synthesized from 4-chloroacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl chloride and triphenylphosphine. Systemic administration of TPPA-TEMPO in the subtoxic dose to mice with lymphosarcoma inhibited tumor growth, but did not prolong animal lifespan. Combined treatment with TPPA-TEMPO and cyclophosphamide increased the efficacy of antitumor therapy: it prolonged animal lifespan and increased the number of recovered mice.

Key Words: *LS lymphosarcoma; therapy; cyclophosphamide; nitroxyl radicals; TPPA-TEMPO*

Nitroxyl radicals constitute a class of stable organic radicals that easily undergo one-electron oxidation and reduction. They modulate the oxidation-reduction status of tissues by interacting with reactive oxygen radicals [8,10,12]. In the past 15 years, much attention was paid to evaluating the therapeutic and prophylactic efficacy of nitroxyl radicals in various diseases (cancer, hypertension, neurovegetative disorders, and other disturbances) whose pathogenesis results from uncontrolled free radical processes [10,11]. Mitochondria are the major source of free radicals and reactive oxygen metabolites in eukaryotic cells [1]. The methods for directed transport of various antioxidants [3,5,9,12] (*e.g.*, nitroxyl radicals [4,6,9,12,13]) into mitochondria were developed in recent years. One of these methods suggests the attachment of lipophilic cations (*e.g.*, triphenylphosphine fragment) to the antioxidant molecule. This method allows us to use the transmembrane potential of the mitochondrial and cell membrane [3,9].

Here we studied the effects of a synthetic nitroxyl radical 4-triphenylphosphonioacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl chloride (TPPA-TEMPO) on the growth of transplantable mouse lymphosarcoma (LS) and efficacy of cyclophosphamide (CP) therapy.

MATERIALS AND METHODS

TPPA-TEMPO was synthesized from 4-chloroacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl obtained as described elsewhere [2] (Fig. 1).

4-Chloroacetamido-2,2,6,6-tetramethylpiperidine-1-oxyl (25.6 mM, 6.3 g) was added to the solution of triphenylphosphine (38 mM, 10 g; Aldrich) in toluene (100 ml). This mixture was boiled in a reflux con-

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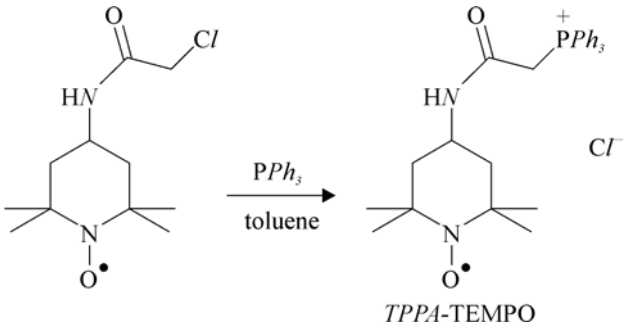

Fig. 1. Production of TPPA-TEMPO.

denser for 8 h. The reaction mixture was cooled to room temperature. The precipitate was filtered, washed with toluene, and chromatographed on a Silica gel column (chloroform as an eluate). The fractions with a triphenylphosphine derivative were combined. Chloroform was distilled at low pressure. Hot toluene (30 ml, >100°C) was added. The solution was shaken until complete dissolution and rapidly filtered. The precipitate of TPPA-TEMPO was formed after cooling to 10-15°C, filtered, and washed with toluene or benzene. The substance yield was 70%. This substance looked like rose-colored crystals with a melting point of 179-183°C. The product was dissolved on 0.14 M NaCl and injected into the peritoneal cavity of animals.

The study was performed on male CBA mice aging 4-6 months and obtained from the Laboratory of Experimental Animals (Institute of Cytology and Genetics). These animals were maintained in plastic chambers (10-11 specimens per chamber) under the natural light/dark cycle. They had free access to water and food (Chara pelleted feed, Assortiment Agro Company). Previous experiments showed that administration of TPPA-TEMPO in single doses of 90 and 70 mg/kg causes death of 100 and 83.3% mice (5 of 6 animals), respectively. The animals died 10-30 min after treatment. TPPA-TEMPO in a dose of 50 mg/kg or lower did not cause death of animals. Tenfold treatment with TPPA-TEMPO in a dose of 40 mg/kg was followed by a slight decrease in body weight (less than by 6%) and death of 1 of 8 mice. In the experiment with chronic treatment, TPPA-TEMPO was administered at a single dose of 30 mg/kg.

We used a transplantable strain of LS lymphosarcoma (Institute of Cytology and Genetics). This strain is highly sensitive to alkylating compounds [7]. The tumor was transplanted to 76 animals (2×10^6 tumor cells intramuscularly into the right thigh). After this procedure, the mice were randomized into 7 groups (Table 1). Group 1 animals served as the control. The mice of groups 2, 5, and 7 received daily intraperitoneal injections of TPPA-TEMPO. CP in a dose of 30 mg/kg was injected intraperitoneally to animals of groups 4, 5, 6, and 7 on day 10 after tumor transplantation. TPPA-TEMPO was injected daily to mice of

TABLE 1. Groups of Experimental Animals

Group	Treatment before therapy	Therapy	Treatment after therapy
1	Control	–	–
2	TPPA-TEMPO intraperitoneally, 30 mg/kg daily, days 1-10 after tumor transplantation	–	–
3	–	–	TPPA-TEMPO intraperitoneally, 30 mg/kg daily, days 11-17 after tumor transplantation (days 1-7 after administration of CP)
4	–	CP intraperitoneally, single dose 30 mg/kg, day 10 after tumor transplantation	–
5	TPPA-TEMPO intraperitoneally, 30 mg/kg daily, days 1-10 after tumor transplantation	CP intraperitoneally, single dose 30 mg/kg, day 10 after tumor transplantation	–
6	–	CP intraperitoneally, single dose 30 mg/kg, day 10 after tumor transplantation	TPPA-TEMPO intraperitoneally, 30 mg/kg daily, days 11-17 after tumor transplantation (days 1-7 after administration of CP)
7	TPPA-TEMPO intraperitoneally, 30 mg/kg daily, days 1-10 after tumor transplantation	CP intraperitoneally, single dose 30 mg/kg, day 10 after tumor transplantation	TPPA-TEMPO intraperitoneally, 30 mg/kg daily, days 11-17 after tumor transplantation (days 1-7 after administration of CP)

groups 6 and 7 over 8 days after administration of CP. The scheme of TPPA-TEMPO treatment under these conditions was similar to that before administration of CP. Group 3 mice received TPPA-TEMPO in the same periods as animals of groups 6 and 7. However, group 3 mice were not pretreated with CP. The size of tumor nodes was estimated with a caliper. These measurements were performed from the start of therapy until death of mice. The average volume of tumors was calculated in each group of animals. The animals were examined until death. The lifespan was recorded after tumor transplantation. Body weight and tumor weight were measured. The mean values of these parameters were calculated. The significance of differences was evaluated by Student's *t* test.

RESULTS

After administration of CP, the average volume of tumor transplants in TPPA-TEMPO-receiving mice of groups 2, 5, and 7 was much lower than in animals of groups 1, 3, 4 and 6 not treated with the chemical agent (1.30±0.08 and 1.80±0.09 cm^3, respectively; $p<0.001$). Body weight (including the weight of tumor) in untreated mice and TPPA-TEMPO-receiving animals was shown to increase by 10 and 4%, respectively. Body weight gain in these mice was 4 and 0%, respectively. Our results show that chronic administration (10 days) of TPPA-TEMPO in a dose not causing the decrease in body weight gain by more than 4% was followed by a 30% inhibition of tumor growth. These data illustrate a certain specificity of drug action on the tumor. However, the effect of TPPA-TEMPO on tumor growth (Fig. 2) and lifespan of animals was nearly undetected after drug withdrawal. Group 2 mice receiving TPPA-TEMPO over the first 10 days died from tumor progression in the follow-up period (similarly to control animals of group 1). TPPA-TEMPO increased the lifespan of animals only by 14% (Table 2).

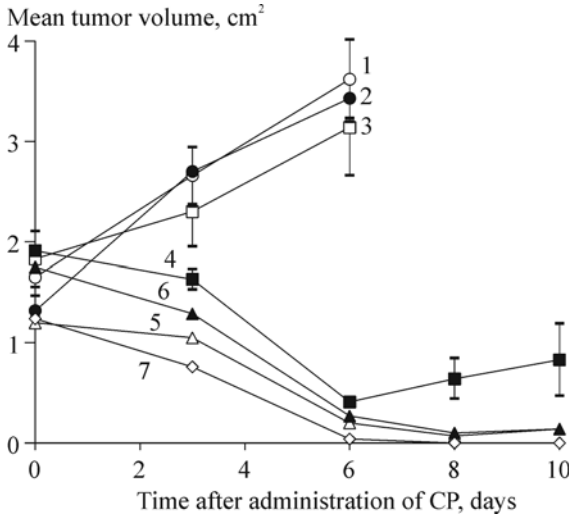

Fig. 2. Effect of TPPA-TEMPO on the growth of LS lymphosarcoma transplants and therapeutic effect of CP on this tumor; various schemes of treatment with TPPA-TEMPO. Control group (*1*); administration of TPPA-TEMPO over the first 10 days after tumor transplantation (*2*); administration of TPPA-TEMPO on days 11-17 after tumor transplantation (*3*); single administration of CP on day 10 after tumor transplantation (*4*); administration of TPPA-TEMPO over the first 10 days after tumor transplantation followed by single treatment with CP (*5*); single administration of CP on day 10 after tumor transplantation followed by 8-fold treatment with TPPA-TEMPO (*6*); administration of TPPA-TEMPO over the first 10 days after tumor transplantation, single treatment with CP, and 8-fold administration of TPPA-TEMPO (*7*).

TABLE 2. Lifespan and Tumor Weight in Mice with LS Lymphosarcoma after CP Therapy in Combination with Preliminary and/or Further Treatment with TPPA/TEMPO (*M*±*m*)

Group	Number of mice	Number of died animals with tumor	ALS of died animals with tumor, days	Average weight of tumor, g		Lifespan of animals without tumor, days
				per tumor-bearing mouse	per mouse (mean value)	
1	11	11	17.0±1.2	3.90±0.58	3.90±0.58	–
2	10	10	19.4±1.8	4.30±0.58	4.30±0.58	–
3	11	11	16.4±2.0	3.10±0.26	3.10±0.26	–
4	11	11	27.9±3.5**	3.90±0.45	3.90±0.45	–
5	11	6	47.1±4.9**++	4.00±0.76	2.20±0.74	45; 60; >100; >100; >100
6	11	9	34.8±5.5**	2.6±0.5	2.1±0.52*+	58; >100
7	11	4	41.0±4.6**++	3.60±0.75	1.3±0.59**++	16; 36; 37; 42; 48; 50; 66

Note. ALS, average lifespan. *$p<0.05$ and **$p<0.01$ compared to group 1 (control); +$p<0.05$ and ++$p<0.01$ compared to group 4 (CP monotherapy).

A slight decrease in the volume of tumor transplants was observed after administration of TPPA-TEMPO at the late stage of tumor growth (Fig. 2). Therefore, monotherapy with TPPA-TEMPO is much less effective. TPPA-TEMPO should be used only in combination with other antitumor drugs for therapy of tumor patients.

Single treatment with CP was followed by tumor regression in mice of all groups (Fig. 2, Table 2). Preliminary and further treatment with TPPA-TEMPO (after administration of CP) was followed by a more significant and long-term decrease in tumor volume. Tumor growth was repeatedly observed 8 days after single administration of CP. After combined treatment with CP and TPPA-TEMPO, the volume of tumors was shown to decrease progressively. In experiments with CP monotherapy (group 4), the mice died from tumor growth on day 27.9±3.5 after tumor cell transplantation. More than 50% animals of group 5 (6 of 11 specimens) receiving CP after treatment with TPPA-TEMPO died from tumor growth in the later period. Complete recovery was achieved in 3 mice. These animals survived for more than 100 days with no tumor recurrence. Group 6 mice received TPPA-TEMPO after treatment with CP. Nine of eleven animals with tumors died under these conditions. One of two mice survived for more than 100 days with no tumor recurrence. Group 7 mice received TPPA-TEMPO before and after treatment with CP. Only 4 animals with tumors died. The remaining mice without tumors died in the same period. Body weight of these animals was 80% of the basal level. Paresis of the hindlimbs was revealed in 3 mice of group 7.

Our results show that TPPA-TEMPO causes a moderate inhibition of tumor growth. After administration of TPPA-TEMPO in combination with CP, this agent increases significantly the efficacy of antitumor therapy. It is manifested in a significant increase in the lifespan of animals and number of recovered specimens. These effects of TPPA-TEMPO depend differently on the duration of treatment. Tenfold treatment with TPPA-TEMPO before single administration of CP was followed by recovery of 45.5% animals. However, the average lifespan of mice was shown to increase by 2.5 times (more than 63 days). At the same time, 18-fold treatment with TPPA-TEMPO was followed by recovery of 63.6% animals. The average lifespan of these mice (41.0±4.6 days) was 1.5 times longer than that of CP-receiving animals. Therefore, the advantage of long-term treatment with TPPA-TEMPO is a greater rate of recovery from tumor growth. By contrast, an increase in the lifespan of animals is less significant under these conditions.

These features are probably related to the side effects of TPPA-TEMPO on animals. Our experiments were performed with the same scheme of treatment with TPPA-TEMPO in one dose. Another scheme of treatment with TPPA-TEMPO will be probably accompanied by a greater antitumor action and smaller adverse effect in the body. It should be emphasized that mitochondrion-addressed antioxidants attract much attention. These data indicate that the antitumor effect and other biological properties of TPPA-TEMPO (as one of these compounds) should be studied in details.

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