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Tempol reduces the therapeutic effect of cyclophosphamide on an experimental tumour model

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Abstract

The nitroxyl radical 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) is reported to elicite some effects on different biological models. This paper studied the influence of Tempol on the therapeutic action of an alkylating agent cyclophosphamide (CP) in the transplantable murine lymphosarcoma LS. When administered exactly before CP, Tempol exerted no influence on efficacy of the tumour therapy but significantly reduced it under a single or multiple preliminary injections 1–3 days before CP. This regimen of Tempol administration is found to elevate the activity of aldehyde dehydrogenase in the liver, the enzyme which is known to reduce the yield of the activated metabolite(s) of CP.

Keywords: *Nitroxides, chemotherapy, cancer*

Introduction

Damage of cells by reactive oxygen species (ROS) is thought to represent one of the factors of the development and progression of malignant tumours and some neurodegenerative diseases. ROS are generated in mitochondria as a natural by-product of oxidation-reduction processes and under oxidative stress. Antioxidants reacting with ROS are assumed to be able to decrease the pathogenic effect of the latter, which was repeatedly demonstrated for well-known antioxidants such as vitamins E and C, β-carotenoids and others. Extensive literature data have accumulated to date on antioxidative properties and therapeutic effects of nitroxide radicals. For example, it was demonstrated that nitroxide radical 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (Tempol) reduces the oxidative damage of mammalian cells *in vitro* [1] and protects experimental animal's tissues (mucosa, salivary glands and hair bulbs) from radiation-induced damage *in vivo* [2]. In several experiments, the oncogenesis suppressing effect of Tempol was demonstrated as well. Thus, chronic intake of Tempol with drinking water significantly reduced the frequency of breast tumours in cancer-prone C3H mice [3] and delayed lymphoma onset in Atm-deficient mice [2,4]. Another nitroxide, 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxy, again delayed the onset of thymic lymphomas in these mice [5]. Tempo (2,2,6,6-tetramethylpiperidine-1-oxyl) in combined treatment with doxorubicin and mitoxantron enhanced apoptosis of prostate carcinoma cells and inhibited tumour growth to a greater extent than monotherapy with anti-cancer drugs [6]. A similar effect of Tempol was observed in breast cancer cells [7].

The traditional explanation is that beneficial effects of Tempol are owing to its antioxidative activity, i.e. ability to modify oxidative stress and oxidation-reduction status of the tissues. However, it is difficult to explain such a wide variety of biological effects of Tempol solely by its antioxidative action. Indeed, defending the salivary glands from radiation injury,

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Tempol produces no protective effect on radiation sensitivity of the tumour transplanted to animals [8]. If the differences in this case are not to be attributed to a different degree of oxygen retention (which mediates anti-radical activity of nitroxides [1]) in the salivary gland and tumour tissues, then in the tumour the free radical scavenging ability of Tempol has been inhibited or overwhelmed by some of its other effects. In fact, there is evidence that Tempol enhances phosphorylation and the activity of p53 protein and therefore acts as a modifier of p53-dependent genes expression [9]; it modulates the activity of the genes associated with multidrug resistance phenotype [7] and induces apoptosis in some tumour models [6]. It is quite possible that Tempol might have other, so far unknown, biological effects. The above results suggest that it deserves further study as a low-molecular compound that could find application, as such or concomitantly with anti-cancer drugs, in cancer therapy. In this work Tempol was investigated from the viewpoint of its action on the growth of transplanted murine lymphosarcoma and the effect on anti-cancer efficacy of cyclophosphamide (CP). The results indicate that Tempol attenuates the therapeutic effect of CP, presumably by enhancing its metabolic inactivation.

Materials and methods

Tempol obtained from the Experimental Chemical Facility of the Novosibirsk Institute of Organic Chemistry (Siberian Branch of the Russian Academy of Sciences) was further purified as follows: 40% Tempol solution in diethyl ether was washed in 5% potassium hydrosulphate solution, dried over $MgSO_4$, which then was filtered out. Tempol was recrystallized from diethyl ether by dropwise addition of hexane to ether solution refrigerated to 5°C at continuous stirring. The resulting sample was analysed on an Agilent 1100 HPLC system using a BioChemVack Diaspher 110-C16 column, 5 μm, 4.6×150, eluent–methanol–H_2O 20-80, elution rate 0.7 ml/min, UV detector, 242 nm. In all the samples, the base material concentration was not less than 99.4%.

Animals

Male and female mice of CBA strain aged 3–6 months were obtained from the vivarium of the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences. The mice were kept in plastic cages of 6–10 individuals of the same sex under natural lighting conditions and free access to water and food (granulated fodder Delta Feeds made by SPE Biopro-plus, Koltsovo, Novosibirsk region). Strain-specific transplantable Lymphosarcoma LS was used throughout the experiments. Originally the tumour was induced by nitrosomethylurea in a CBA mouse [10] and after a series of subcutaneous transplantations was transferred into ascites form and cryoconserved. The tumour is highly sensitive to induction of apoptosis by CP and undergoes complete regression after a single administration of its moderate (near 1/10 LD_{50}) doses [11]. Prior to use in the experiments, thawed tumour cells were administered to the abdominal cavity of one CBA mice. The ascites that had developed within a week after tumour inoculation were used for transplantation to experimental animals. The tumour cells (2×10^6 in 0.1 ml of saline) were inoculated in the muscles of the right thigh of mice. Eight-to-ten days after inoculation the mice were divided into experimental groups depending on tumour size. Drug batches were dissolved in distilled water (Tempol) or 0.14 M NaCl solution (CP) immediately before use and were injected intraperitoneally (i/p) or intravenously (i/v) through a lateral tail vein in amount of 1 ml of solution per 100 g of body weight. Earlier, the maximally tolerated dose of Tempol for C3H mice was found to be 275 mg/kg [12]. We used this dose in the experiments in which Tempol was administered in single injection. At multiple administration, a single dose of Tempol was 250 mg/kg, because any lethality was observed after nine daily injections of this dose of Tempol to eight mice in our previous experiment. CP (cyclophosphamide, LENSfarm, Russia) was used as an anti-cancer agent. It was administered i/v at a single dose of 20 or 30 mg/kg of animal's body weight. Tempol was administered 15 min prior to CP in some experiments and 24 hours or 72, 48 and 24 hours in others.

Tumour growth was monitored by regular volumetric measurements by caliper. The measurements were ceased when mortality of mice in the group had begun. The animals were kept under observation until their natural death, assessment was made based on life span from the moment of tumour inoculation, body and tumour weights.

Aldehyde hydrogenase activity determination

In the study of enzymes involved in CP metabolism, Tempol was administered to intact animals three times at a single dose of 250 mg/kg. A day after the last injection mice were decapitated and liver was perfused *in situ* through the portal vein and homogenized in 10 volumes of 0.15 M KCl solution. The homogenates were centrifuged after 15 min at 9×10^3 g and aldehyde hydrogenase (ADH) activity was determined in supernatants by the kinetic method [13]. The reaction mixture contained 0.1 M phosphate buffer, pH 7.4, 1 mM of pyrazole (Sigma-Aldrich), 1 mM of NAD, 5 mM of propionaldehyde and 1–2 mg of liver protein. Pyrazole was added for inhibiting possible activity of alcohol dehydrogenase

[14]. Enzyme activity was recalculated for 1 mg of protein, the concentration of which was determined by the Lowry method. The results obtained were statistically processed and the significance of the differences was evaluated in terms of Student's *t*-criterion.

Results and discussion

According to the literature, on animals *in vivo* Tempol is normally used at a dose of 275 mg/kg of body weight [8,12]. In our conditions at one single intraperitoneal injection this dose was well tolerated by all mice and did not provoke any toxic signs. At a doubled dose the injection resulted in death of 100% of the mice within 30 min after injection while a dose of 300 mg/kg turned out to be lethal for three of 22 animals (13.6%). Daily intraperitoneal administration at a dose of 250 mg/kg during 9 days was well tolerated by the mice, whose weight decreased only by 5% by the end of the experiment, indicating the absence of any pronounced cumulative toxicity of the drug.

In the study of possible anti-cancer effects of Tempol, the latter was administered once at a dose of 275 mg/kg or four times every other day at a single dose of 250 mg/kg to mice with intramuscular transplants of LS. At one single administration Tempol produced no effect on tumour growth: life span of the mice with the tumour was 19.1 ± 1.74 days in control group ($n = 9$) and 18.0 ± 1.04 days in the group of Tempol-treated animals ($n = 10$); the tumour weight in both groups was virtually the same (5.6 g and 5.7 g, respectively). A therapeutic effect was observed at a four times administration of Tempol—in one experiment tumour growth attenuation was 20%, in another 40% (tumour weight at the end of the experiments was 3.5 ± 0.22 g vs 4.4 ± 0.23 g in control in the first case and 2.7 ± 0.28 g vs 4.5 ± 0.66 g in control in the other case). Nonetheless, the life span of the mice treated with Tempol was not longer but even shorter (in the latter experiment by 25%) than in the control group of animals with tumour (10 mice in each group).

Therefore, the results obtained are evidence in support of low activity of Tempol as an anti-cancer agent at least in our model. However, prior to switching to other models it had to be found whether or not the tumour treatment would be more efficacious when the anti-cancer drugs were used in combination with Tempol. For this reason in the next series of experiments on the same model (lymphosarcoma LS) we investigated the effect of Tempol on therapeutic efficacy of CP, to which tumour used is highly sensitive [11].

Since radioprotective and other biologically significant effects of Tempol were demonstrated at its injection 10–15 min before radiation [8] or immediately after chemical [15] impact in the study of the effect of Tempol on tumour chemotherapy efficacy we began the experiments with a single administration of Tempol at a dose of 275 mg/kg 15 min before CP injection.

In one of the experiments, CP at a dose of 30 mg/kg was administered to tumour bearing mice of group 1 (control) and to mice of group 2, but 15 min after the injection of Tempol. The mice were kept under observation for more than 2 months, until all animals with tumour perished. In both groups the same number of animals perished (six of 13 and 12 animals, respectively). However, in group 1 the life span was 47 ± 5.2 days and in group 2 only 32 ± 1.4 days (statistically significant differences, $p < 0.05$). Dynamics of tumour regression and recurrence in a similar experiment is presented in Figure 1, indicating that at virtually the same rate and degree of regression, recurrence of the tumour in a group of mice treated with CP in combination with Tempol began significantly (2 weeks) earlier than in mice treated with CP only. Therefore, combined use with CP Tempol did not increase but slightly decreased the efficacy of anti-cancer therapy.

In respect to the tumour used in the experiments the efficacy of CP was not enhanced by Tempol treatment 24 h before CP (20 mg/kg) administration. As can be seen in Figure 2, while in CP-treated mice the tumour regression still continued, in the mice under combined treatment with CP and Tempol, after a short regression the tumour progression resumed. On day 7 after treatment, average tumour size in CP and CP + Tempol groups was 1.5 ± 0.4 cm^3 and 4.4 ± 0.8 cm^3, respectively (statistically significant differences, $p < 0.05$). Later tumour growth resumed in CP-treated mice (on day 10 the

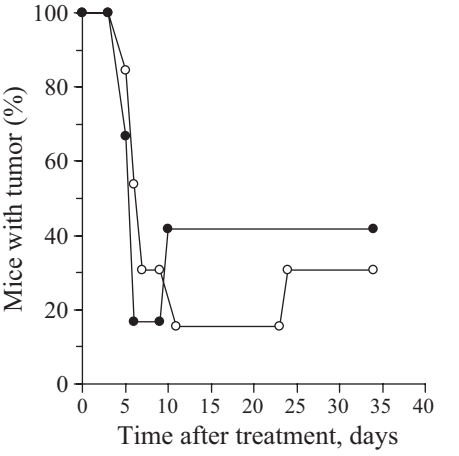

Figure 1. Regression and recurrence of solid transplants of LS in mice after administration of CP (○) or CP with Tempol (●). CP at a dose of 30 mg/kg was injected intravenously, Tempol (275 mg/kg) intraperitoneally, 15 min before CP administration. There were 12 and 13 mice per group.

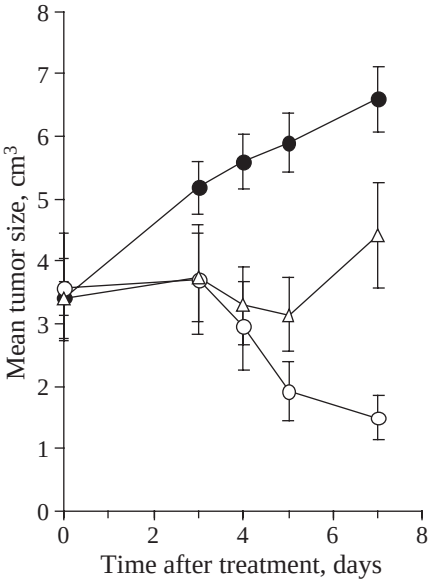

Figure 2. Inhibiting effect of Tempol on anti-tumour efficacy of CP in respect to lymphosarcoma LS. Tempol at a dose of 275 mg/kg was injected to mice with tumour transplants 24 h before CP administration at a dose of 20 mg/kg of body weight. Control (●), CP (○), Tempol + CP (Δ). There were 10 mice per group.

tumour volume grew to 3.0 cm^3). All the animals of both groups perished from the tumour on average 27.4 ± 4.3 and 24.5 ± 1.7 days after tumour transplantation (statistically insignificant differences).

Statistically significant worsening of the results of treatment of mice with LS was observed at multiple administration of Tempol prior to CP. Tempol at a single dose of 250 mg/kg was administered to mice with tumour transplants three times (72, 48 and 24 h) before CP (20 mg/kg) treatment. Mice treated with CP only as well as mice which received CP in conjunction with a single dose (250 mg/kg) of Tempol were used as controls. As can be seen in Figure 3, in mice of both control groups the tumours underwent some regression and thereafter demonstrated similar growth, whereas in mice injected with Tempol before CP the tumours regressed to a lesser extent and subsequently grew faster, so that on day 20 after tumour transplantation (11th day after treatment) the tumour volume in this group was 5.7 ± 0.41 cm^3 in comparison with 3.9 ± 0.55 cm^3 ($p < 0.05$) in the CP group and 4.2 ± 1.0 cm^3 in the CP + Tempol group. In that experiment the life span after tumour transplantation was 24.6 ± 1.54 days and 23.6 ± 1.03 days for mice treated with CP or CP in conjunction with Tempol, respectively, and 20.5 ± 0.95 days for mice treated with CP after preliminary administration of Tempol (in both cases the differences are statistically significant, $p < 0.05$).

Tempol is rapidly reduced to hydroxylamine *in vivo* and excreted from the organism, which is the explanation of its extremely low cumulative toxicity. For the same reason, at preliminary administration long before CP effect of Tempol on therapeutic action of CP is obviously caused not by its antioxidant and radical scavenging action but due to the changes occurring in the organism under the influence of Tempol.

CP is essentially a prodrug and becomes therapeutically active after metabolic conversions in the organism. Its activation pathway starts with 4-hydroxylation catalysed by specific hepatic cytochrome P450 isoforms [16]. The forming 4-hydroxy-CP upon the ring opening interconverts with its tautomer, aldophosphamide, part of which undergoes a spontaneous (non-enzymatic) elimination reaction to yield active alkylating compounds, N,N-bis(2-chloroethyl)phosphorodiamidic acid (phosphoramide mustard, PM) and acrolein, which is associated with anti-cancer [16,17] and some toxic [18,19] effects of CP. The other part of aldophosphamide is oxidized by aldehyde dehydrogenase (ADH) to the inactive 3-(amino(bis(2-chloroethyl)amino)phosphoryloxy)propanoic acid] (carboxycyclophosphamide), which is excreted from the organism [20]. The share of inactivated aldophosphomide, therefore, depends on the activity of ADH in the liver as a main organ of CP metabolism and partially in other (including tumour) tissues [21]. This share is significant and it grows under the action of ADH-inducing agents (phenobarbital a.o. [19]). In our model, phenobarbital injected to mice with LS as an

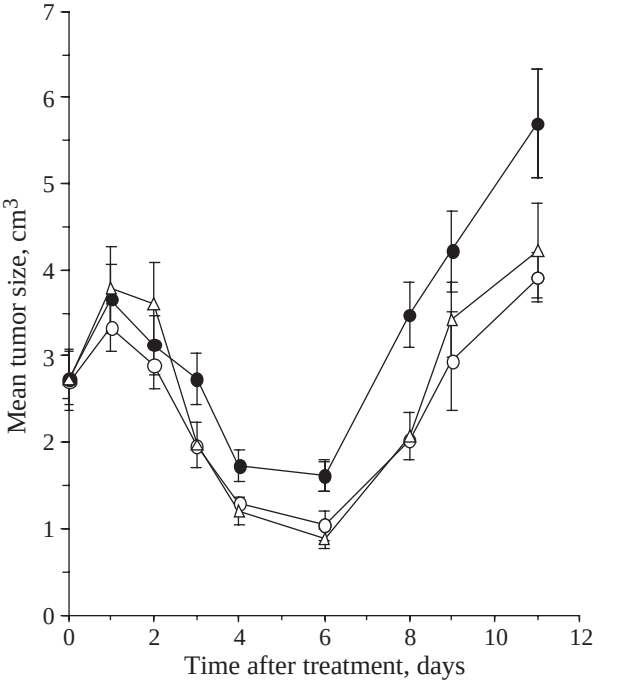

Figure 3. Effect of Tempol on anti-tumour efficacy of CP in respect to lymphosarcoma LS. (○) CP only ($n = 9$); (Δ) CP in the mixture with Tempol ($n = 9$); (●) Tempol three times, 72, 48 and 24 h before CP ($n = 10$). CP was injected at a dose of 20 mg/kg intraperitoneally; Tempol, also intraperitoneally at a one single dose of 250 mg/kg.

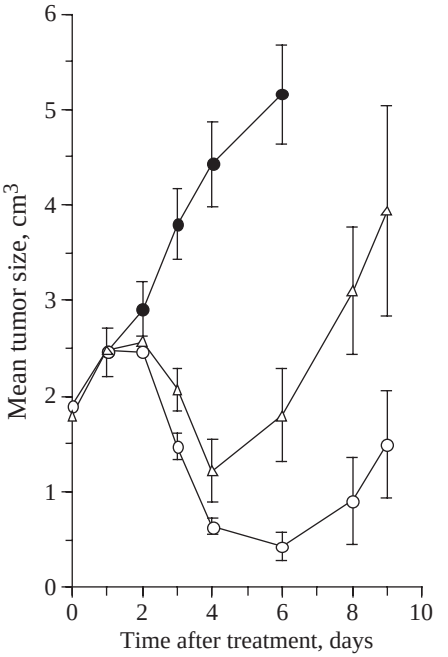

Figure 4. Effect of phenobarbital on anti-cancer efficacy of CP (20 mg/kg) in respect to lymphosarcoma LS transplants. (○) CP only; (Δ) phenobarbital at a single dose of 80 mg/kg three times, 72, 48 and 24 h before CP. All injections were intraperitoneal. (●) Control (no treatment). There were 11 mice per group.

ADH inducer 1–3 days before CP administration significantly inhibited the therapeutic effect of CP (Figure 4). On the contrary, an inhibitor of ADH activity, cyanamide, when used in combination with CP, enhanced anti-cancer activity of the latter [22]. We suggested that the body-mediated inhibiting effect of Tempol on anti-cancer activity of CP may be attributed to stimulation of aldophosphamide oxidation during CP metabolism.

To verify this assumption, in a preliminary experiment we determined ADH activity in the liver of four mice treated with Tempol in comparison to four untreated mice. Significant elevation (25%) of ADH activity in experimental animals was observed. The next experiment we conducted using an additional group of mice treated with phenobarbital as a positive control. The mice received Tempol or phenobarbital during three consecutive days according to the established procedure of hepatic enzymes induction by phenobarbital [19]. The results obtained are shown in Table I.

As can be seen, in the mice treated with Tempol, hepatic activity of ADH was slightly (by 22%), but with statistical significance ($p < 0.05$), higher than in intact animals, while in the mice treated with phenobarbital it was twice as high as in control. Tempol stimulates ADH activity less than phenobarbital, but it diminishes the therapeutic effect of cyclophosphamide to a lesser degree than phenobarbital. As can be seen in Figures 3 and 4, on day 8 after treatment, tumour volume in mice received phenobarbital + cyclophosphamide was 3-times higher than in mice received cyclophosphamide, whereas in mice treated with Tempol + cyclophosphamide it was only less by 1.8-times than in those treated with cyclophosphamide. Besides, we think that ADH activity and the extent of inactivation of certain doses of cyclophosphamide are scarcely in linear relations.

Therefore, the experimental results are in favour of the assumption that Tempol modifies the therapeutic activity of CP at least partly by altering its metabolism pathway, increasing the share of ADH-inactivated aldophosphomide, an intermediate metabolite of CP. Obviously, Tempol has no effect on microsomal hepatic enzymes, the induction of which is accompanied by a significant liver enlargement [23]—in our experiments after three times administration of Tempol the weight of mouse liver remained as in control ($5.1 \pm 0.11\%$ and $5.2 \pm 0.14\%$ of body weight, respectively). Nevertheless, it is not be excluded that Tempol may influence the activity of other enzymes participating in CP metabolism, such as glutathione-S transferase and glutathione reductase.

Conclusion

Therefore, in the model of transplanted murine tumour with high sensitivity to CP, Tempol does not manifest a significant therapeutic effect while in combined therapy with CP attenuates anti-cancer action of the drug. The attenuating effect of Tempol is less pronounced when Tempol and CP are administered concurrently and significantly increases at its preliminary multiple administration, providing evidence not in favour of the antioxidant mechanism of Tempol action in this case and suggesting an action on the enzyme systems involved in the CP activation pathway or its excretion. Experimental evidence is obtained that such an enzyme might be ADH, one of the enzymes, which inactivates aldophosphamide, an intermediate metabolite in the CP activation pathway. The correlation between the degree of ADH induction and reduction of the therapeutic efficacy of CP, which are low for Tempol and considerably higher for

Table I. Effect of Tempol and Phenobarbital on the activity of aldehyde hydrogenase in the liver of CBA male mice.

Groups of mice	Number of mice	ADH activity (nmol of NAD/min per mg of protein)	*p*
Control	5	6.3 ± 0.31	
Tempol	5	7.7 ± 0.42	<0.05
Phenobarbital	3	13.0 ± 0.44	<0.001

The drugs were injected intraperitoneally three times—72, 48 and 24 h before mice slaughter at single doses: Tempol, 275 mg/kg; Phenobarbital, 80 mg/kg.

Phenobarbital, indicates that the effect of Tempol on the therapeutic efficacy of CP is caused at least partly by induction of ADH activity.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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