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Search for compounds with radioprotective activity among synthesized *threo*-D,L-phenylserine derivatives

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Background. Research in the development of radioprotectors has focused on screening a plethora of chemicals and biological compounds. Any chemical agent that can improve the tolerance of animals to radiation is of paramount interest. The aim of the present study was to investigate the toxicity and radioprotective effect of some synthesized N-acyl-threo-DL-phenylserine derivatives (**1–14**).

Materials and methods. Three experiments were carried out on mice exposed to various doses of ^{137}Cs gamma rays (7–9 Gy). In the 1st experiment radioprotective effect of compounds **1–4** was investigated on female C57BL/6 mice. In the 2nd one, male (CBAxC57BL/6)F_1 mice were used for investigation of compounds **3, 7–12**, and in the 3rd experiment male white out bred mice were treated with compounds **5, 6, 13, 14**. For the evaluation of radioprotective capabilities of compounds, one-third of the toxic LD_{50} values were used in most cases.

Results. No radioprotective active action was revealed for the preparations **2, 13**, and only a weak effect was observed for compound **6** (26% of animals survived). Compounds **1** and **4** also showed a weak radioprotective activity. The dose reduction factor (DRF) for them was 1.09 and 1.04. Pre-treatment of male (CBAxC57BL/6)F_1 hybrids with preparations **7–12** caused a higher percent of survival in comparison with the control group after exposure to various doses of gamma irradiation. DRFs for them were showed to be in the ranges of 1.02–1.08. The most radioprotective effect was showed by compound **3** (2-amino-2-thiazolinium salt of N-formyl-*threo*-D,L-phenylserine) at a dose 250 mg/kg. The DRF for this preparation was 1.2. Pre-treatment of irradiated mice with this compound effectively reduced radiation-induced mortality of mice by protecting against the radiation-induced bone marrow damage.

Conclusions. Our results showed that 2-amino-2-thiazolinium salt of N-formyl-*threo*-D,L-phenylserine had the most radioprotective effect among the N-acyl-*threo*-D,L-phenylserine derivatives (**1–14**) investigated.

Key words: radioprotection, phenylserine derivatives, mice

INTRODUCTION

Over the past 50 years, research in the development of radioprotectors has focused on screening a plethora of chemicals and biological compounds. A radioprotector is a chemical or biological compound capable of modifying the normal response of a biological system to radiation-induced toxicity or lethality (1, 2). Any chemical agent that can improve the tolerance of normal tissue to radiation is of paramount interest (3). Several chemical agents have been tested for their radiomodifying properties (3–6). It has been reported that many substances, such as the thiol-containing compounds 2-iminothiazolidine derivatives, which are structurally similar to flavonoids are radioprotectors (1, 2, 5, 7, 8). Several synthetic compounds (lipoic acid, deoxyspergualin) (9, 10) have been tested for protection against radiation. Studies of NSAIDs have indeed shown some evidence of radioprotection (11). They have the potential to increase the survival of cells and to exert anti-cancer effects by causing cell-cycle arrest, shifting cells towards a quiescence state (G0/G1). The same mechanism of action was observed in the radioprotection of a normal tissue. NSAIDs also elevate the level of superoxide dismutase in cells. Activation of heat-shock proteins by NSAIDs increases cell survival by alteration of cytokine expression (11, 12). Several *in vivo* studies have provided evidence suggesting that NSAIDs may protect normal tissues from radiation injury (11).

Nair et al. (5) summarized a list of various categories of radioprotectors and their mechanisms of action. The radioprotectors are known to exert their action through various mechanisms, such as scavenging of free radicals, detoxification of radiation-induced species, target stabilization and enhancement of repair and recovery processes (5). Radioprotective agents generally have a role as free radical scavengers or haematopoietic stimulants.

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Harmful effects of ionizing radiation on living organism are mainly mediated by free radical species (13). Highly active oxygen intermediates the activation of the second messenger system and elicits synthesis of three principal inducers of acute phase protein genes, namely IL-1, TNF and IL-6 (12, 14, 15). Free radical scavenging, elevation in antioxidant status and glutathione and reduction in lipid peroxidation appear to be important mechanisms of radioprotection.

Although the variety of compounds exhibit considerable radioprotecting properties in the laboratory, most of them fail in human application because of toxicity and side effects. So, the search for more effective and less toxic radioprotectants has a great spurred interest in the development of different compounds (16).

The DRF is an important aspect in radiobiology as it clearly gives an indication of quantitative capacity of the drug to enhance the tolerance of tissues to radiation and its ability to mitigate radiation-induced sickness and mortality. Since there is a continued interest and need for the identification and development of non-toxic and effective radioprotective compounds that can reduce the effect of radiation, search for new radioprotectors is an actual need.

The present study was designed to investigate the *in vivo* possible radioprotective effect of the newly synthesized non-proteinogenic amino acid *threo*-D,L-phenylserine derivatives in mice exposed to various doses of irradiation.

MATERIALS AND METHODS

Animals

Female C57BL/6, male (CBAxC57BL/6)F_1 hybrids and male white out bred mice obtained from Rapolovo breeding-ground (St Petersburg, Russia), weighting 18–21 g, were used in this study. Throughout the study the mice were cared for in accordance with the European Convention (17) and Guide for the Care and Use of Laboratory Animals (18). The animals were kept under standard conditions of temperature and humidity. A 12 h light-dark cycle was used. The animals were provided with the standard mouse chow and water *ad libitum*. All the experiments were carried out at the Institute of Biophysics (Moscow, Russia) and the Urals Research Centre of Radiation Medicine (Chelyabinsk, Russia) and followed the guidelines of the Institutional Animal Ethic Committee (IAEC). The study protocol was approved by the IAEC.

Compounds

Threo-D,L-phenylserine (tDLphs) derivatives (**1–14**) [**1** – tD,Lphs ethyl ester hydrochloride; **2** – tD,Lphs 1-tetradecyl ester p-toluenesul-fonate; **3** – N-formyl-, **4** – N-acetyl-, **5** – N-propionyl-, **6** – N-butyryl-tD,Lphs 2-amino-2-thiazolinium salts; **7** – N-propionyl-tD,Lphs 2-mercaptoethylamine salt; **8** – N-propionyl-, **9** – N-butyryl-tD,Lphs 2-amino-1-phenylethanole salts; **10, 11** – N-(N1-arylsuccinyl)-tD,Lphs ethyl esters; **12** – N-(N^1-2-fluorenyl-sulfonyl-β-alanyl)-tD,Lphs ethyl ester; **13** – N-fluorene-2-sulphonyl-tD,Lphs 1-octyl ester; **14** – N-9-fluorenone-2-sulphonyl-tD,Lphs 1-octyl ester] were synthesized by N. Dirvianskytė and co-authors (19) at the Institute of Biochemistry (Vilnius, Lithuania).

Toxicity studies

Acute toxicity of compounds was determined. The survival of mice (5 in each group) orally receiving the graduated single dose levels of each compound dissolved in sterile distilled water containing 0.1% Tween 80 was observed for 7 days (20). The LD_{50} value was determined by the accepted Litchfield and Wilcoxon (24) method.

Pre-treatment with compounds

Compounds were dissolved in sterile distilled water containing 0.1% Tween 80 and injected intraperitoneally (i.p.) to the experimental animals 15 or 30 min before gamma irradiation. The dosage of compounds in most cases approximated one-third of the toxic 50% lethal dose. Under these conditions no compound-related deaths were observed. Each pre-treatment group consisted of at least 5 mice. The control animals received the same volume of solvent without the compound.

Radioprotective effect studies

Whole-body irradiation was performed on the plant IGUR with 4 sources of ^{137}Cs, at a dose rate 58–60 cGy/min and 5.5 cGy/min, dose range 7–9 Gy. The mice pre-treated with compounds **1–4** were irradiated with doses 7–8 Gy, the mice pre-treated with compounds **7–12** with doses 7.5–9 Gy, and the mice receiving compounds **5, 6, 13, 14** were irradiated with the dose 8.5 Gy. Parameters of the curves "dose-effect" were determined by Litchfield's (21) method. The protective capacity of many agents was exposed as the dose reduction factor (DRF) which was computed as the ratio of the radiation $LD_{50/30}$ value with the compound to that without the compound (22). 5 mice were used for one dose to determine $LD_{50/30}$. Irradiation with 4 doses using this number of animals gives an opportunity to calculate DRF, which allows proposing the radioprotective effect in the most reliable way.

Mice were placed in ventilated Plexiglas cages and irradiated in groups of 5 animals simultaneously with a dose rate 60 cGy/min. Radioprotective activity of compounds was estimated according to 30 days' survival of animals. At least 35 mice were used for the investigation of one compound.

Colony-forming unit (CFU) assay and collection of bone marrow cells

CFU-spleen(s) assay was performed as described by Sugiura et al. (23). The amount of endogenic colonies on the spleen of mice was ascertained on day 9 after the irradiation at doses 6.5 Gy with a dose rate 58 cGy/min and 7 Gy with a dose rate 5.5 cGy/min. The fixation of material and calculation of macrocolonies were performed as described by Pereverzev (24).

Bone marrow cells were collected by flushing them out from the femurs of mice using 23-gauge needles.

Statistical analysis

The results were expressed as mean values ±S.E.M. Differences between the control and treated groups were statistically analysed by Student's t test with $p < 0.05$ considered as significant.

The percent of mortality experienced by animals given compounds prior to irradiation with that of animals exposed only to radiation was compared.

RESULTS

Toxic and radioprotective properties of compounds are summarized in Table 1. In primary toxicity studies LD_{50} for compounds was revealed. Acute toxicity tests showed the most compounds to be of low toxicity. For compounds **1, 10–12** LD_{50} was 1500 mg/kg or higher. The highest toxicity among the investigated compounds was revealed for preparations **2, 6**, and **14**.

Threo-D,L-phenyl-serine derivatives **1–4** were evaluated *in vivo* for their radioprotective effects against ^{137}Cs gamma rays using the female C57BL/6 mice. The radioprotective activity of preparations was examined by measuring the survival of mice, receiving these compounds 15 min before the exposure to radiation. The results were recorded for 30 days and compared with the results obtained for the controls. The most widely used and preferred procedure for comparing the efficacy of protective agents in experimental animals was to determine the DRF. If a compound is radioprotective, the value of DRF of the compound becomes larger than 1.0. Irradiation with 4 doses by using at least 5 animals in groups provides an opportunity to calculate DRF at $LD_{50/30}$ which allows proposing the radioprotective effect more reliably.

No radioprotective action was revealed for preparation **2**. Compounds **1** and **4** showed an insignificant radioprotective activity with the DRF for these compounds being 1.09 and 1.04, correspondingly. Only administration of compound **3** (dose 250 mg/kg) prior to radiation exposure resulted in positive benefits and significantly protected from mortality. The 30 days' survival was 100% at the dose of 7 Gy, and 60% at the doses 7.5 and 8 Gy. The percent of survivors in the control group was 40% lower than in the treated mice exposed to radiation by 7 and 7.5 Gy, and 100% lethality was observed by using 8 Gy dose. The $LD_{50/30}$ was about 8.6 Gy for compound 3-injected animals and 7.16 Gy for the control mice, yielding a DRF to this compound of 1.2. However, it should be emphasized that the radioprotective effect of the preparation decreased markedly by lowering the injected dose (100 mg/kg or 50 mg/kg).

The protective capability of compound 3 (dose 250 mg/kg) was also evaluated on male (CBAxC57BL/6)F1 hybrids, and 80% survival and significant ($p < 0.05$) DRF equal to 1.27 was obtained (the data is not shown in Table 1).

The state of bone marrow haematopoiesis of C57BL/6 mice pre-treated with compound **3** on day 9 after irradiation with doses 6.5 Gy (a dose rate 50 cGy/min) and 7.5 Gy (a dose rate 5.5 cGy/min) is showed in Table 2. The pre-treatment of irradiated animals with compound **3** had the stimulating action on the process of endogenously forming colonies in the spleens of mice. The number of spleen colony-forming units (CFUs) significantly increased in the test group of animals irradiated with 6.5 Gy. Stimulation action of this compound on the cellularity of bone marrow was also obvious in both test groups ($p < 0.05–0.02$).

Table 1. **Dose LD_{50} and radioprotective activity of *threo*-D,L-phenylserine derivatives following intraperitoneal injection before exposure to an acute whole-body irradiation with various doses of gamma rays**

	LD_{50} (mg/kg)	Dose injected (mg/kg)	n	Time (min)[a]	% survival of mice at 30 days					DRF[b] ($LD_{50/30}$)
					Dose of irradiation (Gy)					
					7	7.5	8	8.5	9	
1	1640		5	15	80	60	40			
2	400	550	5	15	40	0	0			1.09
3	750	130	5	15	100*	60*	60*			
4	690	250	5	15	80	60	0			1.20*
Control (solvent [c])		230	5	15	60	20	0			1.04
7	600		5	15		80	80	80	60	
8	550	200	5	15		80	60	60	40	1.06
9	700	185	5	15		60	60	60	0	1.05
10	1500	230	5	15		80	60	60	0	1.04
11	>1500	500	5	15		80	80	20		1.02
12	1500	500	5	15		80	80	60	40	1.05
Control (solvent [c])		500	5	15		50	30	10	0	1.08
5	840	300	15	30				60		
		100						40		
6	450	250	15	30				26		
		100						26		
13	>800	300	15	30				0		
		100						0		
14	400	250	15	30				40		
		100						20		
Control (solvent [c])			15					0		

Note. [a] Time interval between injection of compound and irradiation; [b] dose reduction factor (DRF); [c] sterile distilled water having 0.1% Tween 80; * significantly different compared to control.

Table 2. **The state of bone marrow haematopoiesis of C57BL/6 mice pre-treated with compound 3 on day 9 after irradiation**

Dose of irradiation	Groups	Cellularity of bone marrow (mln / femur)	Spleen weight	CFUs / spleen
6.5 Gy	Compound + Irradiation	7.57 ± 1.20*	32.30 ± 4.90	4.60 ± 0.80*
	Control + Irradiation	4.10 ± 1.10	25.30 ± 1.10	1.60 ± 0.80
	Biological control	20.80 ± 1.80	57.90 ± 3.70	
7.5 Gy	Compound + Irradiation	9.40 ± 0.90**	42.40 ± 6.60	12.00 ± 3.50
	Control + Irradiation	6.10 ± 0.80	44.60 ± 2.90	11.60 ± 1.60
	Biological control	24.10 ± 0.90	70.40 ± 5.20	

Note. CFUs – haematopoietic spleenic colony-forming units on day 9. Differences are significant in comparison with the irradiated control. * $p < 0.05$; ** $p < 0.02$. The number of animals in groups is 7.

Pre-treatment of male (CBAxC57BL/6)F1 hybrids with preparations **7–12** caused a higher percent of survival in comparison with the control group after exposure to various doses of gamma radiation (Table 1). The preparations showed some radioprotective effects and their DRFs were in the range 1.02–1.08.

The survival results of the radioprotection studies of compounds **5, 6** and **13, 14** investigated on male white inbred mice showed some radioprotective activity of preparations **5** and **14**. The percentage of survival of mice for 30 days irradiated with 8.5 Gy of gamma rays was 60% and 40%, respectively, by using higher doses of preparations (300 mg/kg and 250 mg/kg), and 40% and 20%, accordingly, at lower doses of compounds (100 mg/kg) injected 30 min prior to irradiation. No radioprotecting effect was revealed for compound **13** and only a weak radioprotective effect was observed for compound **6** (26% of the irradiated animals survived by using both 100 mg/kg and 250 mg/kg doses of preparation).

In the present study we demonstrated that compound **3** was the strongest radioprotective agent in mice with respect to 30 days' survival after gamma irradiation. When this compound (250 mg/kg) was administered 15 min prior to irradiation, the $LD_{50/30}$ value increased to about 8.6 Gy (control 7.16 Gy), and DRF value was calculated to be about 1.2. It is known that the higher the DRF value, the greater is the radioprotection. The DRF for compounds **1, 4** and **7** were shown to be 1.09, 1.04 and 1.06, respectively, so they were less effective, however, compound **7** (N-propionyl-phenylserine 2-mercaptoethylmine salt) injected i.p. (200 mg/kg) 15 min prior to irradiation induced 80% survival of male (CBAxC57BL/6)F1 hybrids irradiated with dose $LD_{95–99}$.

DISCUSSION

Our earlier studies (25–27) have shown a broad spectrum of biological activity of threo-phenylserine derivatives. It has been revealed that they can regulate inflammatory and autoimmune processes in the animal models of arthritis and can be useful for the development of new NSAIDs that possess anti-inflammatory effect. However, no study has reported on their radioprotective activity and the dose reduction factor (DRF) yet.

It is known that a single whole-body exposure of mice to ionizing radiation results in a complex set of syndromes, whose onset, nature and severity are a function of both total radiation dose and radiation quality. In the present study, we have demonstrated that among the threo-phenylserine derivatives investigated the strongest radioprotective agent in mice with respect to 30 days' survival after gamma irradiation was compound **3**. Pre-treatment with this compound with the dose of 250 mg/kg prior to radiation exposure results in positive benefits and significantly protects from mortality as well as the radiation-induced bone marrow damage. It is known that death after irradiation is mainly attributed to gastrointestinal and haematopoietic syndrome because irradiation inhibits the proliferation of stem cells. The mortality after irradiation could also be due to immunosuppression that increases the chances of infection (28). There exists information that in mice death with 10 days' post-irradiation is due to gastrointestinal damage, while death occurring between 11 and 30 days' post-irradiation is due to haematopoietic damage inflicted by radiation (29). Proliferating cells are highly sensitive to irradiation; therefore, the effect of whole body irradiation is mainly felt by the highly proliferating germinal epithelium, gastrointestinal epithelium and the bone marrow progenitor cells. Any damage of bone marrow cells will impair the normal physiological process drastically, causing an adverse impact on survival (29).

Since in our experiments pre-treatment of mice with compound **3** also protects bone marrow cells from the radiation-induced damages, we suggest that the action mechanism of this active compound is realised through the defence of the bone marrow haematopoiesis, because in the range of the doses investigated "bone marrow" form of death is dominating, and the data of the end test allow to suggest the idea concerning the protection of stem haematopoietic cells. The pre-treatment of irradiated animals with compound **3** had the stimulating action on the process of endogenously formed colonies in the spleens of mice. The nature of macroscopic nodules on the spleen of irradiated mice was revealed by Till and McCulloch (30). Subsequently it was shown, that each colony arose from one progenitor cell (31, 32), and a colony-forming unit in the spleen (CFU) became a synonym of haematopoietic stem cell.

Radioprotection by chemical agents is usually dependent on the timing of the administration (33), thus, the dose and timing of the administration of compounds are very important for studying radioprotection (34). Since radioprotection of compounds **5** and **14** was observed when compounds were administered 30 min prior to the irradiation, the primary action of these compounds might be the quenching of free radicals generated by irradiation. However, two different doses showed different radioprotective effect.

So, among the investigated threo-phenylserine derivatives the compound **3** with DRF of 1.2 exhibited the highest radioprotective effect. This value is close to the value 1.3 of antioxidant radioprotectors tempol and edaravole (33, 35). It is known that the destructive action of ionizing radiation is predominantly due to reactive oxygen species (ROS), including superoxide radical, hydroxyl radical and hydrogen peroxide generated by the decomposition of water (36, 37). These free radicals interact with biomolecules and bring about the changes in their structure and function leading to damage and/or cell death (3). Free radicals scavenging may be one of the important actions of the compound **3** in addition to the protection of bone marrow cells from the radiation-induced damages.

CONCLUSIONS

The data obtained are rather reliable and show the positive properties of some of *threo*-D,L-phenylserine derivatives which can serve as the basis for more comprehensive studies.

Pre-treatment with compound **3** reduced radiation-induced mortality in mice most effectively by protecting against the radiation-induced bone marrow damage. Further investigations are needed to ascertain the optimal conditions for the possible use of compound **3** as a radioprotector.

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JUNGINIŲ, PASIŽYMINČIŲ RADIOPROTEKCINIU AKTYVUMU, PAIEŠKA TARP SINTEZUOTŲ *treo*-D,L-FENILSERINO DERIVATŲ

S a n t r a u k a

Tikslas. Ištirti kai kurių sintezuotų N-acil-*treo*-D,L-fenilserino derivatų (**1–14**) toksinį ir radioprotekcinį poveikį.

Medžiaga ir metodai. Buvo atlikti trys eksperimentai su pelėmis. Pirmuoju eksperimentu su C57BL/6 pelių patelėmis ištirtas **1–4** junginių radioprotekcinis poveikis. Antrame **3, 7–12** junginių tyrimams buvo naudoti $(CBAxC57BL/6)F_1$ patinai, o trečiame eksperimente tiriant **5, 6, 13** ir **14** junginius – nelinijinių pelių patinai. Įvertinant junginio radioprotekcines galimybes daugeliu atvejų buvo naudota viena trečioji toksinės LD_{50} junginio dozės. Junginiai suspenduoti steriliame vandenyje, turinčiame 0,1% Tween 80, ir 0,2 ml tirpalo suleidžiama į pilvo ertmę 15 ar 30 min. prieš apšvitinimą. Pelės buvo švitinamos įvairiomis ^{137}Cs gama spindulių dozėmis (7–9 Gy). Gyvūnų išgyvenamumas buvo stebimas 30 dienų po apšvitinimo.

Rezultatai. **2, 13** preparatai nepasižymėjo radioprotekciniu poveikiu ir tik silpnas efektas stebėtas tiriant **6** junginį (išgyveno 26% gyvūnų). **1** ir **4** junginiai taip pat buvo silpni radioprotektoriai. Dozės mažinimo veiksnys (*dose reduction factor* – DRF), apskaičiuojamas kaip radiacinės $LD_{50/30}$ reikšmės santykis su naudojamu junginiu ir be jo (kontrolė), šių tirtų junginių svyravo nuo 1,02 iki 1,08. Ryškiausiu radioprotekciniu poveikiu pasižymėjo **3** junginio (N-formil-*treo*-D,L-fenilserino 2-amino-2-tiazolino druska) 250 mg/kg dozė. Jo DMF buvo 1,2. Jis efektyviai sumažino apšvitintų pelių žūtį, apsaugodamas kaulų čiulpus nuo radiacinio pažeidimo.

Išvados. Mūsų gauti rezultatai rodo, jog tarp tirtų N-acil-*treo*-D,L-fenilserino derivatų (**1–14**) ryškiausiu radioprotekciniu poveikiu pasižymėjo N-formil-*treo*-D,L-fenilserino 2-amino-2-tiazolino druska.

Raktažodžiai: radioprotekcija, fenilserino junginiai, pelės