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Possible Involvement of NO in the Stimulating Effect of Pifithrins on Survival of Hemopoietic Clonogenic Cells

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Abstract—Pifithrin α (PFTα), one of the first known low molecular weight modulators of activity of tumor suppressor p53, increases survival of hemopoietic clonogenic cells (evaluated by the criterion of formation of endogenous spleen CFU-C8 colonies in irradiated animals). This effect appeared when PFTα was administered either before or after irradiation. Increase in CFU-C8 was also observed after administration of two PFTα analogs, derivatives of 2-amino-4,5,6,7-tetrahydrobenzothiazole. These included a parent compound, 2-ATBT (2-amino-4,5,6,7-tetrahydrobenzothiazole), which is used for synthesis of PFTα, and a product of its intramolecular cyclization under physiological conditions, cyclo-PFT (2-(4-methylphenyl)imidazo[2,1-*b*]-5,6,7,8-tetrahydrobenzothiazole). Earlier we found that many low molecular weight compounds increasing number of CFU-C8 (e.g. isothiourea derivatives) demonstrate NO inhibitory activity. Such activity was also found in 2-ATBT and cyclo-PFT by means of EPR spectroscopy of NO. These compounds caused more than twofold inhibition of NO production *in vivo*. Thus, it has been demonstrated that PFTα and its structural analogs increase survival of hemopoietic clonogenic cells *in vivo*, and NO may play a role in the mechanism of this effect.

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Key words: pifithrin α, cyclo-pifithrin, hemopoietic clonogenic cells, endogenous spleen colonies, nitric oxide production, EPR spectroscopy of NO spin trap

Tumor suppressor p53 discovered more than 25 years ago is still subject to intensive studies. However, only recently successful attempts of screening of low molecular weight modulators of apoptotic and transcriptional properties of p53 have been undertaken. These include nutlins [1] and pifithrin-μ [2] (uncouplers of p53 interaction with its natural inactivator, MDM2), reactivators of p53 acting via inhibition of transcription factor NFκB [3], activators of other proteins exhibiting functions of p53 (e.g. p73 [4]), and also compounds restoring DNA-binding properties of mutant p53 (PRIMA-1 [5]).

Almost eight years ago screening of 10,000 chemical compounds in the model of inhibition of p53 transactivation in ConA cells and then in the model of p53-dependent apoptosis of the same cells resulted in identification of one of the first such substances. This highly active compound was named pifithrin α (protein fifty-three inhibitor, PFTα; compound (2a), Fig. 1) [6]. Hypotheses on its applied importance [7] motivated studies on synthesis of new compounds with similar properties [8-10] as well as studies of biological properties of PFTα itself. These studies not only identified additional biochemical targets of this compound, but also certain discrepancies in interpretation of mechanisms of its action. It was also found that PFTα causes potent inhibition of firefly luciferase, which is often used in evaluation of reporter gene activity, and this might also contribute to interpretation of results of experiments using this inhibitor [11].

Recent studies have found [9, 12, 13] that PFTα is a rather unstable compound, and in aqueous media at 37°C it undergoes rapid intramolecular conversion into other compounds characterized by principally different physicochemical properties (Fig. 1, compound (3)). Evidently, this phenomenon would explain many inconsistencies in

Abbreviations: 2-ATBT, 2-amino-4,5,6,7-tetrahydrobenzothiazole; CFU, colony forming unit; cyclo-PFT, 2-(4-methylphenyl)imidazo[2,1-*b*]-5,6,7,8-tetrahydrobenzothiazole; HCC, hemopoietic clonogenic cells; LPS, lipopolysaccharide; PFTα, pifithrin α; ST, spin trap.

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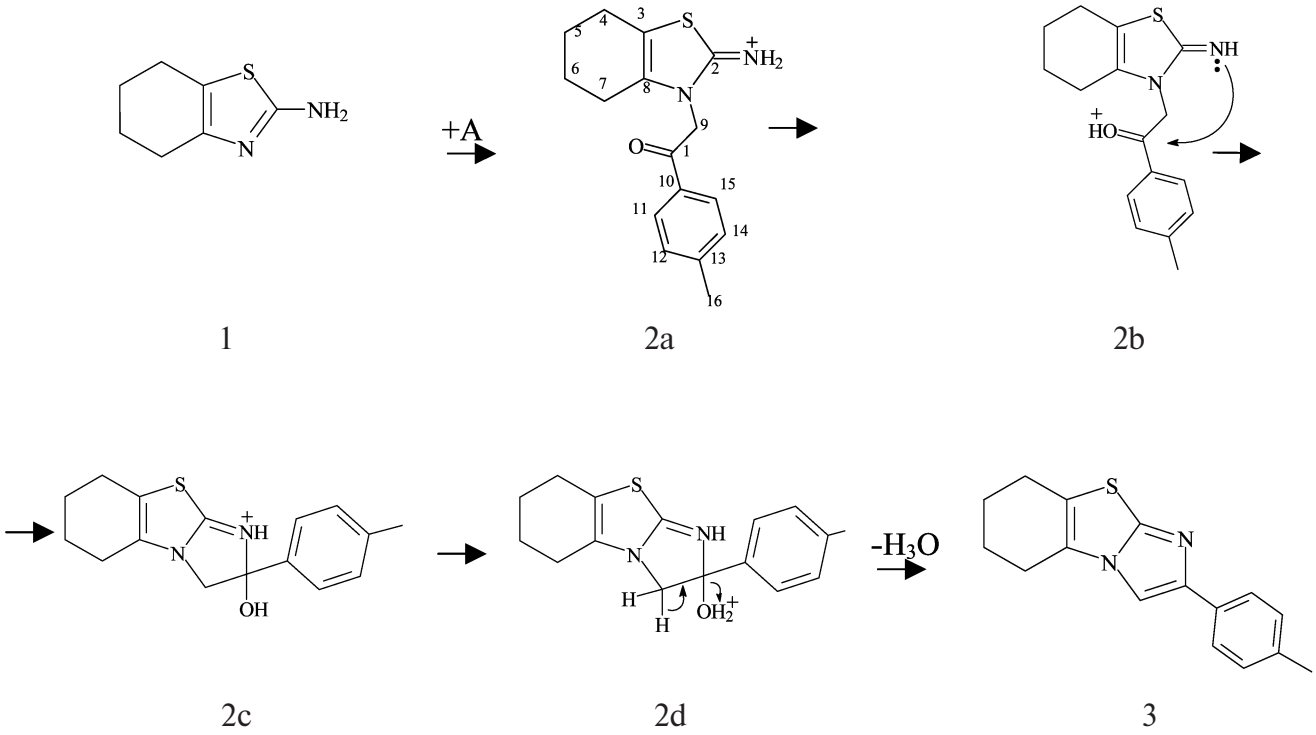

Fig. 1. Scheme of synthesis and intramolecular regrouping of pifithrin-α. Compound (A) is 2-bromo-4′-methylacetophenone; (1) is 2-amino-4,5,6,7-tetrahydrobenzothiazole (2-ATBT); (2a) is 2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-(4′-methylphenyl)ethanone (PFTα); (3) is 2-(4-methylphenyl)imidazo[2,1,-*b*]-5,6,7,8-tetrahydrobenzothiazole (cyclo-PFT).

interpretations of results of molecular biological studies on the properties of PFTα. We have focused our attention on PFTα-induced attenuation of the lethal effect of ionizing radiation (6 and 8 Gy) on mice (this irradiation effect is usually associated mainly with impairments in hematopoiesis); the authors of that observation suggested that the effect of PFTα may be attributed to the inhibitory effect of PFTα on p53 [6].

To explore the reason(s) responsible for increase in survival of irradiated animals by PFTα, we compared its effect on survival of mouse HCC (hemopoietic clonogenic cells) *in vivo* with the effect of two other compounds. One of these compounds is a precursor of PFTα used for its chemical synthesis (Fig. 1, compound (1)), and the other is a product of intramolecular conversion of PFTα known as cyclo-PFT (Fig. 1, compound (3)).

Results of our study indicate that in spite of differences in chemical structure and especially the physicochemical properties of the compounds used, all of them demonstrate similar efficiency in promoting HCC survival of sub-lethally irradiated mice. On one hand, this suggests that involvement of only p53 cannot account for the radioprotective effect of PFT *in vivo*, and on the other hand, there is promise in a search for new modulators of survival of stem cells (for example, HCC) among compounds containing structural elements typical for the PFT analogs tested in this study.

MATERIALS AND METHODS

Synthesis of substances and their characteristics. Derivatives of 4,5,6,7-tetrahydrobenzothiazole were synthesized by conventional methods. Taking into consideration instability of PFTα, which might account for differences in physicochemical characteristics of this compound as well as the product of its cyclization, cyclo-PFT [12], Tables 1 and 2 show comparative characteristics of these substances.

Structure and purity of these compounds were confirmed by thin-layer chromatography, elemental analysis (Table 1), and ^{1}H-NMR spectroscopy (Table 2). Thin layer chromatography was carried out using Silufol UV-254 plates. Data of NMR spectra (Bruker DRx500) showed the following: signal multiplicity (s, singlet; d, duplet; t, triplet; q, quadruplet; m, multiplet; b, broad) and number of protons at numerated carbon atoms (Fig. 1, compound (2a)). Chemical shifts (δ) are expressed as parts per million, ppm, versus tetramethylsilane as the internal standard.

Pifithrin-α (PFTα) was synthesized in a two-step reaction [13] (Fig. 1). In the first step, 2-amino-4,5,6,7-tetrahydrobenzothiazole hydrochloride (2-ATBT) was synthesized from cyclohexanone, iodine, and thiourea [14].

PFTα was synthesized by N-alkylation of this compound with 2-bromo-4′-methylacetophenone; the yield of

Table 1. Composition of PFTα and cyclo-PFT

Compound	T_{melt}, °C	Molecular mass	Brutto formula	Elemental analysis, calculated/found, %			Reference
				C	H	N	
PFTα	185-187 (182 [17])	367.3	$C_{16}H_{18}N_2OS \cdot HBr$	52.32/51.98	5.21/5.28	7.62/7.53	our data
		367.3	$C_{16}H_{18}N_2OS \cdot HBr$	52.32/52.33	5.21/5.48	7.63/7.91	[23]
Cyclo-PFT	256-262	358.25	$C_{16}H_{16}N_2S \cdot HBr \cdot 1/2H_2O$	53.64/53.33	5.06/5.59	7.82/7.78	our data
	185 [17]	268.4	$C_{16}H_{16}N_2S$	71.60/71.75	6.01/5.82	10.44/10.45	[23]

Table 2. ^{1}H-NMR spectrum of PFTα and cyclo-PFT, δ, ppm

Reference	PFTα		Cyclo-PFT	
	our data	[12]	our data	[12]
Frequency, MHz	500	250	500	250
Solvent	DMSO-d_6	DMSO-d_6	DMSO-d_6	DMSO-d_6
s (1H) NH	9.50	9.50		
s (1H) C9			8.50	8.47
d (2H) C11, 15	7.95	7.95	7.70	7.70
d (2H) C12, 14	7.45	7.44	7.32	7.30
s (2H) C9	5.70	5.70		
m (4H) C4, 7	2.50	2.54	2.75	2.75
s (3H) C16	2.40	2.42	2.35	2.33
m (4H) C5, 6	1.70	1.72	1.9	1.88

the product, 2-(2-imino-4,5,6,7-tetrahydrobenzothiazol-3-yl)-1-(4′-methylphenyl)ethanone, was 60% [10, 15].

Cyclo-PFT, 2-(4-methylphenyl)imidazo[2,1-*b*]-5,6,7,8-tetrahydrobenzothiazole, was obtained by boiling PFTα for 1 h in a methanol–water mixture (2 : 1 v/v) as a semi-hydrated bromide salt with yield of 72% [12, 16, 17].

Animals and determination of hemopoietic clonogenic cell survival. HCC survival was investigated by the method of endogenous spleen colonies [18]. Experiments were carried out using 5-month-old male mice, F1 hybrids (CBA×C57Bl/6) of 30-34 g; the animals were kept and fed under standard conditions of a laboratory vivarium. The mice were subjected to general γ-irradiation (using a Lutch instrument and dose power of 0.3 Gy/min) at the dose of 6.5 Gy.

Tested preparations were administrated to mice intraperitoneally (i.p.) in sterile saline (0.2 ml) 5 min before irradiation. Water insoluble cyclo-PFT and indralin (the latter was kindly supplied by E. Yu. Kovtun, SPC Farmzaschita, Moscow) were administered intragastrically as a suspension in 1% starch solution 20 min before irradiation. Spleen taken from mice eight days after irradiation was fixed in ethanol–acetic acid mixture (4 : 1) and the number of colonies (0.2 mm in diameter or more) was calculated on the spleen surface. These colonies formed by surviving HCC are usually defined as CFU-C8 (CFU, colony forming unit). Each group of animals used in these experiments contained 12 mice. Statistical treatment of mean value of spleen colonies formed by surviving HCC was carried out according to the methodical recommendations described in [19].

Determination of nitric oxide production. The NO-modulating activity of the synthesized compounds was studied using 5-month-old albino non-inbred male mice (initial genotype of Swiss line) of 27-30 g; animals were held under standard conditions of a laboratory vivarium

with free access to food and water. Experiments were repeated three times with total number of animals in each group from 12 to 19.

Four hours before sacrifice with ether and fixation of liver samples in liquid nitrogen, animals received injection of lipopolysaccharide (LPS) dissolved in saline (i.p., 1.5 mg/kg, 0.5 ml per mouse; Sigma, USA). The tested compounds were injected into animals 10 min before or 3 h after administration of LPS solution. A spin trap (ST) was injected 30 min before specimen preparation. The ST contained the following components dissolved in 0.9% NaCl: 300 mg/kg sodium diethyldithiocarbamate trihydrate (DDC; Baum-Lux, Russia), 30 mg/kg iron sulfate heptahydrate (Baum-Lux), and 150 mg/kg sodium citrate dihydrate (Merck, Khimmed, Russia).

Production of NO radical was determined using EPR spectroscopy spin trap by the method of Vanin et al. [20]. The super fine spectrum (SFS) of the iron nitrosyl complex ST–NO formed by NO and the spin trap (ST) was registered in livers of the experimental animals. The data represent values of amplitudes of the first low-field STS component corrected for sample weight, amplitude of the second line of simultaneously recorded reference (Mn^{2+}/MgO), and for amplitude of the spectrum of Cu^{2+}–DDC complex, which also formed in the analyzed samples and partially overlapped with the ST–NO spectrum [21].

Statistical analysis. Data represent mean ± standard deviation. Statistical differences of the mean values were evaluated by means of dispersion analysis and also Dannet and Newman–Keuls criteria [22].

RESULTS AND DISCUSSION

Tables 1 and 2 show that the synthesized compounds (PFTα and cyclo-PFT) are characterized by high purity and principal physicochemical differences (including differences in melting point and elemental composition). The NMR spectra reflect the following characteristic features of these compounds (Fig. 1, compounds (2a) and (3)): cyclo-PFT lacks a proton signal with δ of 9.5 because it lacks an imino group, but there is a marked signal with δ of 8.5 corresponding to one proton at C9 (in contrast to the two-proton signal at PFTα C9).

Data on the radioprotective effect of PFTα in mice [6] subjected to irradiation with doses causing death of irradiated animals associated with depression of hematopoiesis seem to support the biological activity of PFTα as a p53 inhibitor. So it was important to evaluate the radioprotective effect of this compound on HCC survival, which mainly determines survival of irradiated animals [18].

Differences in physicochemical and toxicological properties of the synthesized compounds influenced selection of doses for each preparation and time interval between treatment of animals and subsequent irradiation. In the case of 2-ATBT LD_{50}, a dose causing death of 50% of animals, was >100 mg/kg, whereas in the case of PFTα it was about 40 mg/kg. In accordance with the authors who discovered the p53 inhibitor [6], readily water-soluble preparation of PFTα was injected into animals 5 min before irradiation (1 mg/kg, 2.7 μmol/kg). 2-ATBT was administered at the same time interval (27 mg/kg, 142 μmol/kg). Since solubility of cyclo-PFT in aqueous medium is about 0.2 μM [13], this compound was administered intragastrically (47 mg/kg, 131 μmol/kg) as a suspension in starch solution 20 min before irradiation.

Table 3 shows results of calculation of spleen colonies reflecting the effect of tetrahydrobenzothiazole derivatives on HCC survival in γ-irradiated mice. All three compounds caused basically the same (3-4-fold) increase in CFU-C8 number. The well-known radioprotector indralin [24], used as a positive control, caused 8-

Table 3. Effect of 2-amino-4,5,6,7-tetrahydrobenzothiazole derivatives on survival of hemopoietic clonogenic cells

Group	Treatment	CFU-C8	Mass of spleen, mg
1	6.5 Gy	1.2 ± 1.3	35 ± 4
2	PFTα + 5 min + 6.5 Gy	4.7 ± 2.0*	38 ± 6
3	2-ATBT + 5 min + 6.5 Gy	3.5 ± 1.7*	36 ± 3
4	cyclo-PFT + 20 min + 6.5 Gy	5.0 ± 2.2*	39 ± 5
5	6.5 Gy + PFTα	4.8 ± 1.4*	41 ± 5
6	cyclo-PFT + 20 min + 6.5 Gy + INDO	2.6 ± 0.8*,**	41 ± 3
7	6.5 Gy + INDO	3.9 ± 0.6*	41 ± 3
8	Indralin + 15 min + 6.5 Gy	8.4 ± 2.5*	49 ± 2*

Note: PFTα – 1 mg/kg, i.p.; 2-ATBT – 27 mg/kg, i.p.; cyclo-PFT – 47 mg/kg, per os; indomethacin (INDO) – 10 mg/kg, i.p., immediately after irradiation and then 1 and 2 days after irradiation; indralin – 100 mg/kg, per os.

* Statistically significant difference ($p < 0.05$) versus control group 1.

** Statistically significant difference ($p < 0.05$) versus group 4.

fold increase in CFU-C8 number and significant increase in mass of the spleen. The tested compounds also caused some increase in mass of spleen (10-15%) compared with control data; although this increase is not statistically significant, it is typical for compounds increasing CFU-C8.

Treatment of animals with PFTα right after irradiation also positively influenced HCC survival, the 4-fold increase in CFU-C8 number being basically the same as in animals of group 2 treated with PFTα before irradiation.

Indomethacin, a known modulator of survival of epithelial crypt stem cells [25], caused almost 2-fold decrease in survival of CFU-C8 in mice pretreated with cyclo-PFT before irradiation (group 6), whereas treatment of irradiated mice with indomethacin alone exhibited a stimulating effect on CFU-C8 survival (group 7), consistent with previous observation [26].

There is increasing evidence on interaction between transcription factors p53 and NFκB, which is essential for cell survival [3]. Since NFκB is one of the major regulators of expression of inducible nitric oxide synthase (iNOS), we investigated the effect of 2-amino-4,5,6,7-tetrahydrobenzothiazole derivatives on LPS-induced NO production. Figure 2 shows that 2-ATBT (100 μmol/kg), a parent compound for synthesis of PFTα, and a cyclic product, cyclo-PFT (100 μmol/kg), demonstrated marked NO-inhibitory activity; these compounds caused 2.5-3-fold decrease in NO content in livers of LPS-pretreated mice. Under this scheme of experimental administration of a potential inhibitor (1 h before preparation of liver samples), PFTα (1.1 μmol/kg) did not influence NO production, possibly due to lower dose compared with the other substances. Selection of this dose was determined by manifestations of PFTα toxicity in the presence of LPS and ST components. However, when PFTα was administrated to mice together with an iNOS inducer, lipopolysaccharide, there was statistically significant (almost 3-fold) decrease in NO production. This effect of suppression of iNOS expression was also observed for other low molecular weight compounds [27, 28].

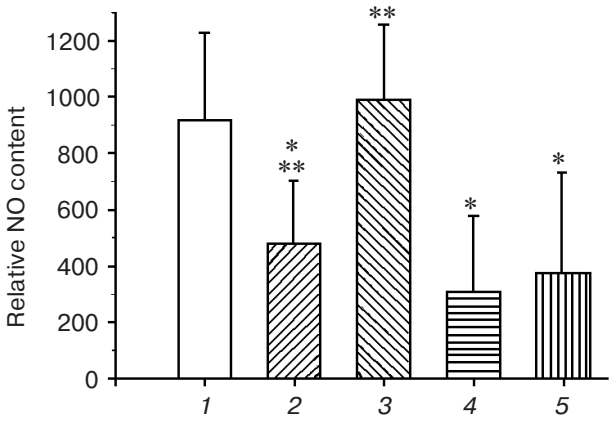

Fig. 2. Effect of 2-amino-4,5,6,7-tetrahydrobenzothiazole derivatives on LPS-induced NO production *in vivo*: *1*) LPS (1.5 mg/kg), 4 h^A; *2*) PFTα (0.4 mg/kg), 4 h 10 min; *3*) PFTα, 1 h; *4*) cyclo-PFT (35 mg/kg), 1 h; *5*) 2-ATBT (19 mg/kg), 1 h. Letter "A" in superscript is used for designation of a time interval between administration of a particular compounds and liver sample fixation; * statistically significant difference ($p < 0.05$) versus control group 1; ** statistically significant difference ($p < 0.05$) versus groups 2 and 3.

DISCUSSION

Thus, taking into consideration previously published data from other laboratories, results of the present study give a certain basis for new conclusions.

It seems unlikely that p53 is a primary target for PFTα critical for survival of animals and HCC *in vivo*. There is evidence that PFTα inhibits p53-independent apoptosis of thymocytes induced by dexamethasone, heat shock-induced expression of proteins [29], as well as expression of proapoptotic CD95 receptor [30]. However, in JB6 cells PFTα stimulated increase in both p53-dependent and p53-independent apoptosis [31]. In cultures of A2780 and HCT116 cells, PFTα did influence expression of p53, p21, and MDM-2 induced by γ-irradiation [12]. PFTα did not influence apoptosis of cortical astrocytes treated with H_2O_2 [32]. In addition, cell reactions associated with altered gene activity are usually developed within hour time intervals. Pretreatment of animals 5 min before irradiation [6] does not provide such time interval. We suggest that reactions important for initiation of survival programs of cells and whole animals are also developed after irradiation, and our experiments confirm this viewpoint (Table 3, group 5). However, this period does not exceed 60 min because PFTα is unstable and is rapidly decomposed; at 37°C its half-life period is about 1 h [12].

However, we should emphasize the following: intramolecular regrouping of PFTα into cyclo-PFT is accompanied by formation of some intermediates (Fig. 1, (2b)-(2d)) that may also influence HCC survival. Our suggestion that specific structure of these compounds may be promising for design for new biologically active preparations has some basis. In addition, it appears that under certain experimental conditions PFTα can behave as a prodrug and cyclo-PFT formed from PFTα is more lipophilic than its precursor; the octanol/water partition coefficient (logP) is 2.2 and 4.3 for PFTα and cyclo-PFT, respectively [13]. This property may determine significant accumulation of cyclo-PFT in membranes. Such possibility is also confirmed by recent data on significant anti-angiogenic activity of cyclo-PFT *in vivo* [33].

All three compounds share a common structural fragment (4,5,6,7-tetrahydrobenzothiazole) with some differences in the extracyclic nitrogen atom (amino-, imino groups, and tertiary nitrogen atom in cyclo-PFT). It is possible that this element of chemical structure is

related to biological activity, for example, to HCC survival. We demonstrated earlier that many compounds inhibiting *in vivo* NO production increased HCC survival in sub-lethally irradiated animals [34, 35]. All these compounds share a common structural element, the thioamidine fragment (-HN–C(=NH)–S-), which is an isostere of the guanidine group of L-arginine, the substrate for NO synthase [36]. Indeed, treatment of animals with both 2-ATBT and cyclo-PFT 1 h before liver sample fixation for EPR analysis caused a decrease in LPS-induced NO content to 41 and 33%, respectively, compared with NO content in control mice treated with LPS only. It should also be noted that like pifithrins, 2-aminobenzothiazoles are considered as promising anti-inflammatory preparations [37, 38]. This may also indicate their effect on NFκB activity and consequently on cell survival programs including stem cell survival. There is some structural similarity between PFTα and ALT-711, breaking glycoside cross-linking bonds between proteins [39].

Data on indomethacin inhibition of the stimulating effect of cyclo-PFT on HCC survival suggests involvement of products of catalytic activity of COX (possibly prostaglandins) in the mechanism of the radioprotective effect of cyclo-PFT [40, 41].

Thus, the search of biologically active preparations in the above considered class of chemical compounds acquires a "second breath" and more definitive direction.

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