Armed Forces Radiobiology Research Institute

Chemical Protection Against X-Ray, Gamma, and Neutron Radiation

Petersburg Nuclear Physics Institute
Gatchina, Leningrad District, Russia

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Gatchina, Leningrad District, Russia

Head of Laboratory
S. A. Grachev

Leading Research Worker
A. G. Sverdlov

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Editor and NIS Initiatives Coordinator
Glen I. Reeves, M.D.

Scientific Director
E. John Ainsworth, Ph.D.

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For information about this publication, write Armed Forces Radiobiology Research Institute, 8901 Wisconsin Avenue, Bethesda, MD 20889-5603, USA, or telephone 011-301-295-0377, or send electronic mail to reeves@mx.afrri.usuhs.mil. Find more information about AFRRI on the Internet's World Wide Web at http://www.afrri.usuhs.mil.

Preface

One of the major long-term research goals of the Armed Forces Radiobiology Research Institute (AFRRI) has been the study and development of agents, either singly or in combination, that would protect personnel exposed to either photon or neutron radiation, or both. There are several scenarios, besides the obvious one of a nuclear weapon(s) detonation, where military personnel could be subjected to a single or mixed radiation field; they include cleanup operations after a reactor accident, such as that at Chernobyl, or a weapons accident or incident.

Criteria for a preventive regimen should include (1) significant dose modification factor (dose reduction factor, or DRF); (2) minimal if any side effects and no long-term toxicity; (3) oral administration, preferably no more than once daily; and (4) minimal reduction in effectiveness when administered soon after exposure rather than prior to exposure.

Some of the most effective agents to date have been aminothiols and their derivatives. Unfortunately, most of these agents have side effects such as nausea, vomiting, hypotension, weakness, and fatiguability that, while not precluding their use in clinical radiation therapy, have rendered them unsuitable for a military operations scenario. Researchers at AFRRI (Weiss et al. 1993; Landauer et al. 1993) demonstrated that administration of caffeine mitigated the neurotoxicity caused by administration of WR-3689 and WR-2721, though other authors have found that caffeine in higher doses aggravated these symptoms. Clearly, the need for a radioprotector that is both effective and safe still exists.

Dr. Joseph F. Weiss visited, on behalf of AFRRI, the authors of the present report in their laboratory at Gatchina, Russia. He was impressed by the work they were doing in this field, and how it supplemented AFRRI's research along different lines toward this same goal. Their approach, spelled out in the section "Introduction," will not be repeated here.

Briefly, the authors used a nontoxic thiol compound to block the biochemical receptors in cells of the target tissues for the side effects while not simultaneously lowering the DRF. They also tested a new compound that they synthesized for efficacy and toxicity protection. These combinations were tested against both neutron and photon irradiation using a mouse model. The authors recommended that these successful preparations be used in a large animal (canine) model, and, if successful, be followed by human toxicity studies. Realizing that the parenteral routes of administration used in their study are unsuitable for a field situation, they also outlined steps for development of oral regimens.

While this document does not reflect the opinion of AFRRI or the Department of Defense regarding the suitability of the described regimens in an operational situation, it does present a thought-provoking step toward the development of an effective yet nontoxic means of radiation protection and may stimulate further research along these or perhaps slightly different lines.

Grateful acknowledgment is given to the following scientists at AFRRI whose advice and constructive criticism were of immense value in the editing of this manuscript: Drs. E. John Ainsworth, Ramesh Bhatt, K. Sree Kumar, and Terry Pellmar and Mr. Henry Gerstenberg. Any errors in editing, however, are entirely my responsibility.

Glen I. Reeves, M.D.
NIS Initiatives Coordinator
Armed Forces Radiobiology Research Institute

Contents

Abstract

Experiments in mice showed that intraperitoneal (i.p.) injection of unithiol (sodium salt of 2,3-dimercapto-l-propanesulfonic acid) diminished toxicity of several aminothiol radioprotectors, increasing the LD_{50} of cystamine by 40% and aminoethanisothiuronium bromide hydrobromide (AET) by 64%. The optimum ratio for the doses is 0.5 molar equivalent of unithiol per radioprotective thiol. A new radioprotector (mixed disulfide of cysteamine and unithiol—MDCU) has a weak toxicity: the LD_{50} is 750 mg/kg i.p. The use of unithiol makes it possible to increase the dose of the SH-radioprotectors, enhancing the dose reduction factor (DRF) of cystamine and AET by 30% for x-ray irradiation. A somewhat lesser effect is observed with fission neutron irradiation. The DRF of MDCU is equal to 1.6 for x-ray irradiation and is 1.1 for neutron irradiation. The mechanism of antitoxic action of unithiol could not be detected in Chinese hamster fibroblasts. It may be caused by the competition of unithiol and the SH-radioprotectors for certain, as yet undetermined, biochemical structures in brain neurons. It is also possible that unithiol may decrease penetration of SH-radioprotectors into the brain.

Introduction

Extensive studies of chemical protection against ionizing radiation hazard have led to the development of efficient radioprotectors that significantly diminish radiation injury in living organisms. The most effective radioprotectors developed so far are aminothiols and their derivatives: cysteamine, cystamine, AET (aminoethanisothiuronium bromide hydrobromide), WR-638 (cystaphos), and WR-2721 (gammaphos). Some of these compounds have been successfully used to prevent complications of radiation therapy in patients with cancer and are considered as a protection against radiation hazard in space flights and in accidental radiation exposure scenarios as well as in clinical use (Bacq 1964; Mozzhukhin and Rachinskii 1979; Monig 1990; Thompson 1964). Unfortunately, all of the aminothiols have toxic side effects that limit their use in medical practice. Thus, scientists have long searched for ways to decrease their toxicity.

Judging by their side effects (nausea, vomiting, asthenia, loss of working capacity), the toxic effects of these radioprotectors are primarily on the central nervous system (CNS). Therefore, various drugs and anticonvulsive substances have been tried to mitigate the toxic effects. For example, pentobarbital narcosis reduced hypersalivation, vomiting, and cramps caused by intravenous administration of cystamine to dogs (Mundy and Heffer 1960). Luminal (25 mg/kg), medinal (50–100 mg/kg), and librium (30 mg/kg) reduced the death rate from 80% to 20–30% in mice that were administered 300 mg/kg of cystamine (Strelnikov et al. 1969). The anticonvulsive preparation benzonal (50 mg/kg) increased the LD_{50} of cystamine by 12% (Zherebchenko et al. 1974). In their experiments, Weiss and coauthors (Landauer et al. 1993; Weiss et al. 1993) showed that caffeine (40 mg/kg) mitigated the behavioral deficits caused by administration of WR-3689 and WR-2721. Conversely, Strelnikov and associates (1969) found that caffeine (however in a higher dose: 50 mg/kg) as well as other CNS stimulators (phenamine, corasole, strychnine) intensified cramps, shortened their latent period, and increased the lethal effect of cystamine and cystaphos in mice.

However, the toxicity of aminothiol radioprotectors may be reduced not only by neurotropic preparations. The toxicity of cystamine is reduced by a concurrent administration of ACTH, cortisone, or hydrocortisone (Stern et al. 1965; Strelnikov et al. 1969). Recently, various metals (zinc, copper) and selenium were found to reduce the toxicity of WR-2721; the effect depended on the interval between administration of the metals and the radioprotectors (Weiss et al. 1987, 1990). It is important that these metals and selenium increase the radioprotective efficacy of cysteamine, AET, and WR-2721 (Brown et al. 1988; Floersheim and Floersheim 1968; Weiss et al. 1987, 1990).

One effective way of reducing the toxicity of aminothiol radioprotectors could be to use them in combination. Many authors have found that such combinations as WR-2721 and cystaphos, AET and 2-mercaptopropionylglycine, mercaptoethylguanidine and cysteamine, cystamine and AET, and cystamine and cystaphos reduce the toxicity and some side effects caused by these preparations when applied separately (Zherebchenko et al. 1974; Maisin and Mattelin 1967; Vladimirov et al. 1989). However, it is not advisable to combine preparations, each of which is toxic. The question is whether similar, if not better, radioprotective effects can be reached using a nontoxic thiol.

The protective properties of the thiol radioprotectors are associated with their effect on the stem cells of the hematopoietic system and cells of the intestinal epithelium. Their toxicity appears to be due to their effect on cells in another crucial system of the body, the CNS, and is a result of their interaction with some biochemical structures in the brain cells. The functions of these cells may be disturbed by such interaction.

A nonradioprotective and nontoxic thiol with a structure similar to the aminothiol protectors may be capable of blocking the biochemical structures in the nerve cells. This might hinder access to them by the SH-radioprotectors and thus reduce damage to the neurons' functions, thereby reducing the toxic action of the radioprotectors. If this should prove true, the toxicity of aminothiol radioprotectors could not only be diminished using such nontoxic thiols, but the dose tolerance could be increased, thus increasing the efficacy of the chemical protection against radiation hazard.

One of the most promising candidates for this role is unithiol, a sodium salt of 2,3-dimercapto-1-propanesulfonic acid. It contains two SH-groups, $CH_2(SH)CH(SH)CH_2SO_3Na$. It is a nontoxic pharmaceutical preparation that was used in medical practice in the former USSR as an antidote against Lewisite and other arsenic compounds as well as for cases of poisoning by ions of some heavy metals (Mashkovskii 1967). It was tested as a radioprotector but did not show appreciable radioprotective action.

To determine the potential antitoxic action of unithiol, we studied its effect on the toxicity of cystamine and AET in mice. We also studied the toxicity of a mixed disulfide of cysteamine and unithiol (MDCU), which we synthesized. We further examined the possibility of increasing the protective efficacy of the aminothiol radioprotectors by combining them with unithiol and testing the combinations on mice exposed to irradiation from x rays and also from fission neutrons.

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Materials and Methods

Radioprotectors

In conformity with the contract, two new radioprotective combinations were prepared and MDCU was synthesized.

Cystamine plus unithiol. Preparations of cystamine dihydrochloride and unithiol were purchased from commercial suppliers and used without further purification. The purity of unithiol was not less than 98% as shown by SH-group spectrophotometric analysis with Ellman reagent. The purity of cystamine detected by SH-group spectrophotometry after disulfide bond reduction by $NaBH_4$ was 96 ± 3%.

AET plus unithiol. AET was prepared according to a published procedure (Doherty et al. 1957). The purity of AET detected by SH-group spectrophotometry following the transguanidination reaction was 96 ± 3%.

MDCU. MDCU was synthesized by the reaction of cystamine dioxide with unithiol. This synthesis of $CH_2(SSCH_2CH_2NH_2)CH(SSCH_2CH_2NH_2)CH_2SO_3Na \cdot HCl \cdot 4H_2O$ is described below.

A solution of unithiol (1.75 g, 7.66 mmole) in 2.5 ml of 1 mM HCl was added dropwise to a suspension of cystamine dioxide (4 g, 15.5 mmole) in 3 ml of 1 mM HCl. A solution of 2.5 ml of 4 M HCl was added to the obtained solution, and the total volume of the reaction mixture was subjected to column chromatography using a 17 x 2 cm column packed with cationite Amberlite CG-120, 100–200 mesh, in H^+ form, balanced by a 1 M HCl solution. The column was washed by a 1 M HCl solution to remove the hypotaurine, then by a 2 M HCl solution at a flow rate equal to 1.3 ml/minute.

Fractions containing the mixed disulfide were combined and concentrated via rotary evaporation to a volume of 5 ml. The obtained viscous yellowish solution with 2 ml water was transferred to a small glass and then lyophilized; 2.4 g of a powdered compound were obtained.

HPLC analysis showed the presence of a single peak. Analysis for Cl^- ions showed that the product obtained by lyophilization represents a mixture of the monohydrochloride and dihydrochloride forms. Aqueous solutions of this preparation were shown to be acidic and therefore have to be neutralized prior to their administration to animals. Thus, it is advisable to transform this compound to the monohydrochloride. For this purpose, dry resin Amberlite CG-400, 200–400 mesh in OH^- form was added to 2.2 g of the compound in 20 ml of water, resulting in a final pH of 6.2. The obtained solution was filtered from the resin, the resin was washed by 10 ml of water, and the united filtrate was evaporated to 5 ml via rotary evaporator, then lyophilized; 1.8 g of a powdered compound were obtained. HPLC analysis showed the presence of a single peak.

Disulfide groups in the compound were analyzed by SH-group spectrophotometry after reduction of the disulfide bond by $NaBH_4$, as described in Habeeb (1973). Analysis showed 102 ± 3% content of -S-S-group against the calculated value for the MDCU molecule.

Calculated values for the elemental analysis of $C_7H_8N_2O_3S_5 \cdot HCl \cdot 4\ H_2O$ (M.W. 447) were:

	C	H	S	Cl.
	18.81%	6.09%	35.85%	7.93%
We found:	18.71%	6.15%	35.50%	7.90%

The aqueous solution of the MDCU monohydrochloride was found to be near neutral; pH for the 0.01 M solution was 6.8.

The absorption spectrum of the aqueous solution of the mixed disulfide has a maximum absorption of 245 nm, characteristic of any organic disulfide. The

calculated MDCU extinction coefficient was 850 l/mole cm—two times higher than that of a compound with one disulfide bond (E = 300–400 l/mole cm). These data show that a molecule of MDCU contains two disulfide groups.

The doses of the radioprotectors were calculated from their salts. Toxicity was evaluated by the LD_{50} value, and their protective effect was derived from the percentage of the animals that survived 30 days after irradiation.

Subjects

The studies were carried out in mice, isolated cultured fibroblasts (V79 cells) of Chinese hamsters, and neurons in slices of rat hippocampi.

Mice. Male (CBAC57BLF1) F1 mice age 2.5–3 months and weighing 18–22 g were used. The protectors were administered prior to irradiation: intraperitoneally, 15 minutes before irradiation, or orally by buffered feeding needles, 40 minutes before. Mice were housed in cages holding five each. They were carefully observed for signs of fighting. Bedding was wood chips. Illumination was consistent with the season. The standard laboratory chow mixture used in the former USSR was used; components included oats, sunflower seeds, white bread, milk, carrots, green grass, meadow haw, fish oil, fish flower, yeast, and salt.

For the toxicity studies, six mice were used for each dose level, and each determination was repeated six times. Results were combined and the mean arithmetic value and its error calculated. For the irradiated groups, 20 animals were used in each group, and irradiation was repeated three times.

Chinese hamster cells. To study the influence of cysteamine, unithiol, and their combination, we used an asynchronous culture of Chinese hamster V79 cells in the exponential phase of growth. Cysteamine and unithiol were dissolved in the culture medium, and the cells were incubated for 20 minutes at room temperature. Then the cells were washed, and a fresh medium was added. The settled cells were incubated for 6–7 days thereafter, and the number of colonies was counted. Survival was determined relative to untreated cells**.** Different concentrations of cysteamine and unithiol were used.

Rat hippocampal neurons. Hippocampal slices were prepared by the standard method (Teyler 1980). Two slices from each of 10 rats were used. Cystamine was studied in 10 slices, unithiol in the other 10. Cystamine was not rinsed off the slices. The methods used in our experiments are described elsewhere (Peimer et al. 1986). Recording of the population spike was conducted from the layer of pyramidal cells of the CA 1-2 sector—using the sectors of the hippocampal formation described by Lorente de No (1934)—by the usual method, using metallic or glass microelectrodes with resistance 2–5 milliohm. For stimulation of the Schaffer's collaterals, we used bipolar platinum electrodes with total diameter up to 100 µ in a glass or lac insulation. The electric pulse duration was 100 µ seconds, with amplitude up to 10V. The results were analyzed by computer.

Irradiation

X rays. Irradiation conditions were as follows: RUM-17 x-ray machine, 220 kV, 15 mA, filtration 0.5 mm Cu + 1.0 mm Al. The dose rate was 1.8 Gy/minute. A VA-J-18 dosimeter was used. During x irradiation the mice were placed in separate cells of Plexiglas cages. Ten mice were irradiated simultaneously.

Fission neutrons. Irradiation by fission neutrons was carried out in a vertical biochannel of the Petersburg Nuclear Physics Institute WWR-M reactor (Sverdlov 1974). The neutron mean energy was 0.85 MeV; the contribution of gamma quanta to the total dose was 25%, dose rate was 14 cGy/min, and irradiation was circular. Dosimetry was carried out using tissue equivalent graphite and polyethylene ionization chambers filled with ethylene and CO_2. A threshold detector was used to determine the energy spectrum. During neutron irradiation the mice were placed in duralumin (alloy) cages in separate cells. There were 10 mice per cage, and 5 cages in the irradiation chamber. A rotary platform was used to give uniform exposure. The temperature inside the chamber was 20 °C; ambient air was provided through a ventilation system. Dose exposure levels were determined for each cage. After exposure, the mice were returned to the same conditions and cages as before irradiation.

Results

Effect of Unithiol on Toxicity of Radioprotectors

The influence of unithiol on the toxicity of cystamine and AET was investigated, and the toxicity of MDCU, in which the radioprotector is chemically bound to unithiol, was determined. The amount of unithiol in combination with cystamine and AET was estimated from the thiol equivalents of these compounds. Molar thiol equivalents of cysteamine and AET are equal to their molecular weights since they contain one free or potential SH-group, whereas those of cystamine and unithiol are equal to half of their molecular weights since they contain two free or potential SH-groups. Thus, every dose of an aminothiol protector has a corresponding dose of unithiol. For example, using 0.5 molar equivalent of unithiol, 300 mg of AET requires 61 mg of unithiol, while for 400 mg, 82 mg of unithiol would be used, and so on.

As shown in table 1, the toxicity (LD_{50}) of the radioprotectors in our experiments was closely related to that described by others (Mozzhukhin and Rachinskii 1979; Thompson 1964). At the same time, toxicity was sharply decreased by simultaneous i.p. administration of an 0.5 equivalent dose of unithiol. The LD_{50} of cystamine rose by 40% and that of AET by 64%. This effect of unithiol far exceeded the antitoxic action of other agents (thiol and nonthiol) tested by other authors (Zherebchenko et al. 1974; Jacobus 1959; Kalistratov et al. 1972; Pugacheva et al. 1972; Suvorov and Shashkov 1975; Takagy et al. 1971).

A decrease in toxicity was not observed by simultaneous oral administration of unithiol and either cystamine or AET (table 2). Perhaps this is related to the way in which unithiol is absorbed from the stomach. When unithiol is used as a drug, it is given as pellets instead of a solution (Mashkovskii 1967). However, when oral administration of cystamine was combined with i.p. administration of unithiol, then all the mice were able to survive a universally lethal dose of protector; even a 1600 mg/kg (LD_{100}) dose was no longer lethal.

Table 1. Toxicity of radioprotectors and its change under the influence of unithiol (intraperitoneal administration)

Protector	Dose of unithiol	LD_{50}, mg/kg	Change (%)
Cystamine	–	392	
	0.5 equivalent	550	40*
	0.75 equivalent	533	36*
AET	–	358	
	0.5 equivalent	588	64*
	0.75 equivalent	496	38*
MDCU		750	

*$p < 0.05$

Toxicity of the orally administered AET was markedly unaffected by unithiol, regardless of the method of administration. The reason for this

Table 2. Toxicity of radioprotectors and its change under the influence of unithiol (oral administration)

Protector	Dose of unithiol	LD_{50}, mg/kg	Change (%)
Cystamine	–	1392	
	0.5 equivalent	1396	< 0.3 (N.S.)
	0.5 equivalent (i.p.)	1600	All mice survived*
AET	–	1300	
	0.5 equivalent	1350	4 (N.S.)
	0.5 equivalent (i.p.)	1450	11 (N.S.)
MDCU		1900	All mice survived*

*This dose is normally 100% lethal, so further increasing the dose was of no practical importance.

remains a mystery. However, it should be remembered that the radioprotective effect of orally administered AET is highly conjectural. It has been observed by only a few researchers and as a rule, quite some time (a few hours) after administration. The weak efficacy of oral administration of AET and i.p. injection of unithiol may be explained by the different rates of absorption and a discrepancy between the times of resorptive action of the two preparations.

As a whole, our experiments showed a high antitoxic effectiveness of unithiol with thiol protectors and demonstrated the most effective relationships between doses of radioprotective compounds and unithiol. As judged from our data, the best radioprotective combination uses approximately 0.5 molar equivalent of unithiol. Higher proportions (0.75 equivalent and more) were less effective, perhaps because of the additional toxic influence of unithiol itself when administered in such large amounts.

Effect of Unithiol on the Effectiveness of Chemical Protection in Mice Against X Rays

Since combining unithiol with aminothiol radioprotectors reduced their toxicity, it may be possible to increase the radioprotective effect by increasing the dose. It is known that the protective effect of the thiol radioprotectors depends on the dose; increasing the dose increases the protection (Mozzhukhin and Rachinskii 1979; Thompson 1964). In earlier studies, unithiol showed no radioprotective effect. It is nontoxic at 168 mg/1000 g, or 1.5 equivalent. Its effect appears to be solely in decreasing the toxicity of aminothiol radioprotectors.

Efficacy of intraperitoneal administration of protectors. Table 3 shows that the radioprotective effect of cystamine and AET given in their usual doses (150 mg/kg) and administered i.p. was clearly exhibited in our experiments. In both magnitude of effect and dependence on dose, our data agree with

Table 3. Survival percentage **(***M* ± *SD*) of x-irradiated mice protected by cystamine, AET, their combination with unithiol, and MDCU (intraperitoneal administration)

	Irradiation dose, Gy						
Experimental conditions	6	7	8	9	10	11	12
Control (irradiation without protectors)	100	55 ± 3	25 ± 4	0	0	0	0
Irradiation + cystamine (150 mg/kg)	100	95 ± 5	84 ± 3	85 ± 2	63 ± 3	10 ± 3	4
Protection efficacy*	0	40†	59†	85†	63†	10†	4
Irradiation + cystamine (300 mg/kg) + unithiol (152 mg/kg)	100	100	100	100	88 ± 5	65 ± 5	55 ± 5
Protection efficacy*	0	45†	75†	100†	88†	65†	55†
Irradiation + AET (150 mg/kg)	100	95 ± 4	70 ± 6	66 ± 5	40 ± 7	20 ± 4	10 ± 6
Protection efficacy*	0	40†	45†	66†	40†	20†	10†
Irradiation + AET (300 mg/kg) + unithiol (64 mg/kg)	100	95 ± 5	100	100	95 ± 5	85 ± 5	55 ± 7
Protection efficacy*	0	40†	75†	100†	95†	85†	55†
Irradiation + MDCU (350 mg/kg)	95 ± 5	94 ± 5	85 ± 5	80 ± 3	60 ± 4	40 ± 3	10 ± 4
Protection efficacy*	5	39†	60†	80†	60†	40†	10†

*Difference between survival of irradiated animals unprotected and protected by radioprotectors
† Difference significant at the $p < 0.05$ level

Table 4. $LD_{50/30}$ (cGy, midline dose) of x-irradiated mice and DRF of radioprotectors (intraperitoneal administration)

Experimental conditions	$LD_{50/30}$	DRF
Control (irradiation without protectors)	710	–
Irradiation + cystamine (150 mg/kg)	1025	1.4
Irradiation + cystamine (300 mg/kg) + unithiol (152 mg/kg)	1268	1.8
Irradiation + AET (150 mg/kg)	937	1.3
Irradiation + AET (300 mg/kg) + unithiol (64 mg/kg)	1192	1.7
Irradiation + MDCU (350 mg/kg)	1122	1.6

numerous published data (Bacq 1964; Mozzhukhin and Rachinskii 1979; Thompson 1964).

Because the addition of unithiol reduced the toxicity of the radioprotector, doses twice as high as usual were tried. This considerably enhanced the radioprotective effect at every radiation dose given. The differences in protective action between protectors alone and their combinations with unithiol are best shown at the higher radiation doses. At 11 and 12 Gy, the protective effect of cystamine and AET given in their usual doses is very small (10 and 4.5%, respectively, for cystamine and 20 and 10% for AET), whereas for the combination, where double doses of the protectors were used, cystamine increased survival by 65 and 55%, respectively, and AET by 85 and 55%. MDCU (350 mg/kg) was also more effective than cystamine or AET at 150 mg/kg; survival was 60 and 40% higher with irradiation doses of 10 and 11 Gy, and 10% higher at 12 Gy.

The differences are particularly striking when the radioprotective effect is expressed as the dose reduction factor (DRF). As shown in table 4, doubling the dose raises the DRF of cystamine from 1.4 to 1.8 and of AET from 1.3 to 1.7, that is, the DRF of both protectors is increased by 30%. The DRF of MDCU is high, too, reaching 1.6. The pronounced protective action of these combinations and MDCU on radiation-induced, acute intestinal distress must be emphasized, as the problem is very real and difficult to solve.

Efficacy of oral administration of protectors. Analogous results were obtained in the experiments with oral administration of the protectors to mice (table 5).

Table 5. Survival percentage ($M \pm SD$) of x-irradiated mice using cystamine, AET, their combination with unithiol, and MDCU (oral administration)

	Irradiation dose, Gy				
Experimental conditions	8	9	10	11	DRF
Control (irradiation without protectors)	0	0	0	0	
Irradiation + cystamine (300 mg/kg)	50 ± 4	20 ± 4	0	0	0
Protection efficacy*	50	20	0	0	1.1
Irradiation + cystamine (1300 mg/kg) + unithiol (152 mg/kg)	100	52 ± 3	31± 4	10 ± 5	
Protection efficacy*	100	52	31	10	1.3
Irradiation + AET (300 mg/kg)	80 ± 2	40 ± 3	20 ± 3	30 ± 4	
Protection efficacy*	80	40	20	30	1.2
Irradiation + AET (800 mg/kg) + unithiol (81 mg/kg)	90 ± 2	50 ± 3	30 ± 2	0	
Protection efficacy*	90†	50†	30	0	1.2
Irradiation + MDCU (1600 mg/kg)	45 ± 5	50 ± 2	0	0	
Protection efficacy*	15†	50†	0	0	1.2

*Difference between survival of irradiated animals unprotected and protected by radioprotectors. All nonzero values are significant at the $p < 0.05$ level.
† Differences between protective effects at 8 and 9 Gy are not significant.

Cystamine alone (300 mg/kg) protects from death only 50% of mice subjected to 8 Gy of x irradiation, whereas the combination with a fourfold (1300 mg/kg) amount of cystamine permitted 100% survival. At 9 Gy, cystamine given in the usual oral dose (300 mg/kg) enhanced survival by 20%, but a fourfold dose raised it to 52%. Cystamine at this higher dose affords protection to 31% of mice even at 10 Gy. To a lesser degree, unithiol exerts some effect on the protection afforded by AET.

MDCU has a protective effect with oral administration as well as i.p., whereas cysteamine itself is known to be ineffective when given this way. As for cystamine, adding unithiol makes it possible to increase the dose of cystamine sufficiently to enhance chemical protection with oral administration. However, unithiol alone appeared to be ineffective in our previous experiments when given orally and must be administered intraperitoneally when oral doses of cystamine or AET are used. Perhaps this is because the free SH-groups of unithiol decrease the absorption of the radioprotector from the stomach.

Looking at this part of the study as a whole, it can be said with confidence that chemical protection against ionizing radiation can be substantially enhanced by taking advantage of the antitoxic action of unithiol to increase the dose of the thiol protectors. This possibility is particularly important in situations with high radiation levels where protectors in their usual doses are ineffective.

Protection Against Neutron Irradiation

The chemical protection of an organism against neutron exposure is especially difficult in view of the high damage potential of these particles (Sigdestadt et al. 1976; Sverdlov et al. 1969; Sverdlov 1974). The opportunity to enhance such protection in some way or other is all-important. Thus, a major objective of our study was to evaluate antiradiation activity of a combination of the thiol radioprotectors with unithiol in animals subjected to neutron irradiation. This is especially important because chemical protection against neutrons, as a rule, requires high doses of SH-radioprotectors (Sverdlov 1974).

The effect of the radioprotectors under study and their combinations with unithiol in mice irradiated by fission neutrons is shown in table 6. Cystamine and AET given in their usual doses increased the

Table 6. Survival percentage ($M \pm SD$) of fission-neutron irradiated mice using radioprotectors alone and combined with unithiol

	Total irradiation dose, cGy				
Experimental conditions	200	250	300	350	400
Control (irradiation without protectors)	94 ± 3	87 ± 2	31 ± 2	0	0
Irradiation + cystamine (150 mg/kg)	100	87 ± 2	60 ± 2*	0	0
Irradiation + cystamine (300 mg/kg) + unithiol (152 mg/kg)	100	94 ± 2*	71 ± 2*	31 ± 2*	0
Irradiation + AET (150 mg/kg)	100	100*	75 ± 2*	27 ± 3*	12 ± 2*
Irradiation + AET (300 mg/kg) + unithiol (64 mg/kg)	100	100*	87 ± 2*	74 ± 3*	19 ± 3*
Irradiation + MDCU (350 mg/kg)	100	100*	60 ± 4*	21 ± 2	0

*Difference from control significant at $p < 0.05$

survival rate from a lethal dose of 300 cGy of gamma-neutron radiation[1] (225 cGy from neutrons)—cystamine by about 30% and AET by 40%, which corresponds to our previous data (Sverdlov et al. 1969; Sverdlov 1974). The radioprotective effect of cystamine disappeared and that of AET was decreased to 30% as the radiation dose was increased to 350 cGy (260 cGy of neutrons). On further increasing the gamma-neutron dose to 400 cGy, the effect of AET was decreased to 12%. The addition of unithiol to cystamine and AET, which makes it possible to increase the radioprotector dose, measurably increased the efficacy of chemical protection: the combination of unithiol with cystamine defended mice not only at 300 cGy but at 350 cGy as well, when cystamine by itself is ineffective.

The combination of AET with unithiol was even more effective. It protected 75% of irradiated mice at 350 cGy in contrast to 27% for AET alone, and even at the supralethal dose of 400 cGy, it enabled survival of nearly 20% of animals.

The role of unithiol is clearly demonstrated by comparing the DRF of the protectors taken in their usual doses and in the enhanced doses made possible by the use of unithiol (table 7). The DRFs of the combinations were higher than the DRFs of the radioprotectors alone, taken in their usual dose. The combination of cystamine with unithiol increased the DRF by nearly 10%, and the combination of AET with unithiol raised it by 20%.

These protectors and their combinations with unithiol are less effective for neutron irradiation than radiation with low linear-energy transfer (LET). This aspect of chemical protection against the hazards of neutron radiation is well known (Bacq 1964; Mozzhukhin and Rachinskii 1979; Sigdestadt et al. 1976; Sverdlov et al. 1969). However, it should be pointed out that the essential protective effect against neutron irradiation is accomplished by increasing the radioprotector dose and by reducing its toxicity by using unithiol. This protection is higher than that afforded by other combinations, for example, combinations of sulfur-containing protectors with each other or with 5-methoxytryptamine (5-MOT) (Bogatyrev et al. 1983).

Table 7. $LD_{50/30}$ (cGy, midline dose) for mice irradiated by neutrons and gamma rays (approximately 75% of dose due to neutrons) and DRF for radioprotectors and their combinations with unithiol

Experimental conditions	$LD_{50/30}$	DRF*
Control (irradiation without protectors)	275	–
Irradiation + cystamine (150 mg/kg)	312	1.1
Irradiation + cystamine (300 mg/kg) + unithiol (152 mg/kg)	330	1.2
Irradiation + AET (150 mg/kg)	325	1.2
Irradiation + AET (300 mg/kg) + unithiol (64 mg/kg)	375	1.4
Irradiation + MDCU (350 mg/kg)	312	1.1

*Differences significant from control at $p < 0.05$

The combination of an enhanced dose of AET with unithiol in our experiments provided protection against neutron radiation with a DRF of 1.4, that is, the same as AET or cystamine in their usual dose against much less damaging low LET radiation. Thus, this combination increases the efficacy of chemical protection not only against x and gamma rays, but also against fission neutrons. Animals irradiated by neutrons withstand the combination and MDCU as well as x-irradiated animals.

Mechanism of Action of Unithiol

Our next step was to try to determine the mechanism by which unithiol diminishes the toxic action of the aminothiol radioprotectors cystamine and AET. To analyze the phenomenon, we needed to consider more than one circumstance. First, it was necessary to provide fixed ratios of unithiol with the aminothiols and then to increase the unithiol dose as the radioprotector dose was increased. This would show that the described effect of unithiol was not due to the effect of this dithiol on any regulator systems but to its interaction at this concentration with the radioprotectors at the cellular level. In

[1]Technical note: It is impossible, using a reactor, to give a pure neutron dose. Some gamma and x rays are always mixed in. Hence the compound word, gamma-neutron.

connection with this, we studied the influence of unithiol on the toxic effects of cystamine and cysteamine *in vitro* with V79 cells of Chinese hamster in culture and with slices of rat hippocampal neurons.[1] The results of the experiments with cysteamine on the V79 cells are summarized in table 8.[2]

For the first time, in these experiments (as opposed to the experiments in animals), unithiol by itself demonstrated a toxic effect, although it was present in fairly large concentrations (25 mM and more). The reason for this is a valid avenue for special investigation. However, one fact stands out: in spite of its toxicity, unithiol did not strengthen the damaging action of cysteamine on the cells in any of the experimental variations.

Preliminary experiments on the hippocampal slices showed that, with the addition of cystamine in concentrations near 10 µM into the incubation medium, the population spike resulting from stimulation of the Schaffer' s collaterals increased in amplitude to 100–150%.[3] This reaction came immediately after addition of the radioprotector and lasted about 20 minutes. The reaction of the hippocampal neuron slices to unithiol, added to the incubation medium in the same concentration, was the same. Thus, we found some variation in the function of the nerve cells under the effect of cystamine and unithiol taken in concentrations approaching those in an organism at a protective dose of radioprotectors and an antitoxic dose of unithiol (taking into account the blood-brain barrier).

Unfortunately, these experiments do not fully explain the influence of the cystamine and unithiol combination on neurons. However, they appear to be interesting in themselves because they testify to the influence of the radioprotector and unithiol on nerve cells and exhibit one type of such influences.

Table 8. Survival percentage of V79 cells after adding cysteamine, unithiol, or their combination

Preparation	Concentration (mM)	Survival *M*	*SD*
Cysteamine	0	100	
	50	74	7
	100	28	12
	150	16	6
Unithiol	15	100	
	25	66	7
	50	35	8
	75	28	6
Cysteamine + Unithiol	100 + 50	38	11
Cysteamine + Unithiol	100 + 15	26	8
Cysteamine + Unithiol	100 + 25	38	15
Cysteamine + Unithiol	150 + 75	25	5

Note: Differences between the effects of cysteamine alone and a combination of cysteamine and unithiol are not statistically significant at the p = 0.05 level.

[1]Cystamine only was used in animals and in hippocampal slices. As cystamine has no protective effect *in vitro*, we used cysteamine in our *in vitro* experiments. We also used "cysteamine" when we referred to a mixed disulfide between cysteamine and unithiol. In this case, cysteamine is not a separate compound but part of the molecule MDCU which consists of two parts: cysteamine and unithiol bound by a chemical bond.

[2]These experiments were carried out by L.I. Kotlovanova.

[3]These experiments were carried out by G.T. Bozhko.

Discussion

Our study showed that the toxic action of the aminothiol protectors in mice can be decreased by combining it with unithiol. This effect is significant enough to be of practical interest. The LD_{50} of cystamine and AET when administered i.p. increase by 40 and 64%, respectively, which shows an antitoxic action much stronger than that of other agents (Zherebchenko et al. 1974; Jacobus 1959; Kalistratov et al. 1972; Pugacheva et al. 1972; Suvorov and Shashkov 1975; Sverdlov et al. 1969; Takagy et al. 1971). Unithiol diminishes the toxicity of cystamine with either i.p. or oral administration of the radioprotector. When AET is administered orally, the effect of unithiol is modest, which is possibly attributable to AET absorption from the digestive tract and unithiol absorption from the abdominal cavity. It may be that the two compounds are not supplied to nervous tissue concurrently and, consequently, do not work together.

The antitoxic action of unithiol was only observed with i.p. injection; oral administration had no such impact. This point requires special study. However, it is necessary to stress that the radioprotective action of such thiols as cysteamine and AET with oral administration is either not detected or weakly expressed. Among the aminothiol radioprotectors, cystamine, which has no free SH-groups, is the only one that is efficient not only with i.p. injection but with oral administration as well. It is felt that the presence of free thiol groups in the unithiol molecule (as in the cysteamine molecule) creates the condition that degrades its effectiveness at oral administration. The use of special medicinal form (tablets) for oral administration of unithiol may indirectly support this fact (Mashkovskii 1967).

A new preparation with radioprotective properties—mixed disulfide of cysteamine with unithiol in which aminothiol and dithiol are chemically bound—was studied in our experiments. Reduction of the aminothiol toxicity was detected in this case, too. There is reason to believe that the disulfide linkage may be broken in an organism and that unithiol may diminish the toxic effect of the released radioprotector. It is worth noting from a practical standpoint that the preparation has little toxicity with oral administration. It does not require i.p. injection of unithiol to reduce the radioprotector toxicity, which is necessary when cystamine is administered orally.

We also found that in order to achieve the antitoxic action of unithiol vis-a-vis the aminothiol radioprotectors, a specific quantitative ratio between the two substances must be maintained. The optimum ratio for doses is 0.5 molar equivalent of unithiol per radioprotective thiol. These data are liable to be of interest in the elaboration of the corresponding prescription. They are equally important for understanding the mechanism of the unithiol's antitoxic action as regards the thiol radioprotectors.

As may be seen from our experiments, unithiol's ability to enhance tolerance to aminothiol permits larger doses and thus a better radioprotective effect of the SH-compounds. The combination with unithiol under x-ray irradiation increased the DRF of cystamine and AET by 30% with i.p. administration and by 18% with oral cystamine administration. The DRF of MDCU was 1.6 at i.p. injection. Thus, the use of unithiol substantially enhances the effectiveness of protection provided by the aminothiol radioprotectors. The survival rate is increased not only in animals irradiated with a minimum universally lethal dose but also in animals subjected to supralethal doses.

A similar effect was found with fission-neutron action. The DRF of cystamine and AET when combined with unithiol increased in comparison with the DRF of each individual radioprotector: the cystamine DRF increased by 10% and the AET DRF by 16%. Along with that, the efficiency of AET-based protection in mice was higher than the effectiveness observed by us previously in experiments

using the aminothiol radioprotectors and their cross-combination or combination with indolylalkylamine (Bogatyrev et al. 1983). Considering that modification of a neutron injury is a difficult problem to resolve, the enhanced effectiveness of chemical protection against fission neutron hazard using unithiol is worthy of notice. However, the effect of unithiol in irradiation of animals by neutrons is of a lesser degree than its protection against low LET radiation.

Consequently, unithiol extended the radioprophylactic capabilities of traditional aminothiol radioprotectors used in conditions of both low and high LET radiation, although to a lesser degree in the latter case. This effect of unithiol does not appear to be specific to mice. We have detected the effect in other biological species, for example, in rats (Grachev et al. 1994), that indicates the feasibility of studying it in animals more closely related to human beings, such as dogs and monkeys, and, in the future, in humans.

The study of the antitoxic mechanism of unithiol vis-a-vis aminothiol protectors is a complex issue that may be no less complicated than the problem of chemical protection itself. The results of our study of unithiol's influence on toxicity of radioprotectors of other types (5-methoxytryptamine (indolylalkylamine) and di-ammonium salt amidothiphosphoric acid), carried out in parallel, may be useful for analyzing the problem, as we found that unithiol has no effect on their toxicity. These data show that the antitoxic action of unithiol is unlikely to be associated with a nonspecific increase of an organism's resistance to chemical agents. We are apparently dealing here with a specific interaction of thiol-containing compounds, SH-radioprotectors, and unithiol, at the cellular level.

The results obtained in this study on the correlation between unithiol's antitoxic effect and its optimum ratio to the SH-containing radioprotectors provide more evidence for this assumption. Such factors are characteristic of a cell concentration relationship rather than an organism's reactions, which are typically threshold reactions. Although our attempt to detect the antitoxic effect of unithiol in Chinese hamster V79 cells was indeed unsuccessful, this does not disprove our assumption. It is believed that unithiol only weakens those toxic effects of the thiol radioprotector that are related to cell excitation and has no connection with the structures responsible for cell growth.

This assumption is supported by the fact that the toxic effects of the SH-radioprotectors on an organism reveal themselves as a disturbance in the function of the nervous system rather than by damage to certain cells, tissues, or organs or by dysfunction of specific organs. It should be added that in our earlier experiments on the same Chinese hamster V79 fibroblasts, although using another technique (cultivation of cells on glass), the antitoxic action of unithiol in relation to cystamine was observed. Under the radioprotector's effect (10^{-2} mg/ml), the cells changed their shape, became rounded, and slipped off the glass**.** The addition of unithiol (1:2 in relation to cystamine) prevented this effect (Grachev et al. 1994). It is therefore worthwhile to continue studying isolated cells in a culture.

However, the results of our tentative experiments on rat hippocampal slices are of a greater interest in relation to the problem of unithiol's mechanism of action. The stimulating effect of cystamine as well as of unithiol in inducing activity in these neurons was found in these experiments. Further neurophysiological and neurochemical studies are required to identify the processes underlying the antitoxic effect of unithiol. In addition, we should not forget that these results were obtained in *in vitro* experiments. Another way of influencing toxicity of the aminothiol radioprotectors is possible *in vivo* by limiting their penetration to the brain through the blood-brain barrier. In general, the information on mechanisms of unithiol's antitoxic action presented in this report should be considered as tentative and facilitating development of approaches to this complex issue.

Suggestions for Further Research

- Study the effect of unithiol alone and in combination with cystamine or AET or MDCU on behavioral toxicity.

- Study unithiol's antitoxic effect with respect to SH-radioprotectors in dogs and humans. If such studies have a positive outcome, wider use of aminothiol radioprotective compounds will be possible in medical practice and other fields.

- Study the possibilities of reducing the thiol radioprotectors' toxic action using unithiol administered not only i.p. but also orally (the use of tablets, capsules, and so on). Establish an optimum timing of unithiol and cystamine administration per os.

- Study the mechanism of unithiol antitoxic action *in vitro* (neurophysiological and neurochemical studies of neurons) and *in vivo* (study the influence of unithiol on aminothiol protectors' penetration to the brain and certain neuronal structures using radionuclides).

- Study the possibility of using unithiol analogues, other thiol compounds (including other dithiols), and similar compounds as antitoxic agents.

- Conduct systematic studies of the new radioprotector MDCU: its absorption, distribution, elimination, and efficacy in protecting against irradiation in other biological species.

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13. ABSTRACT *(Maximum 200 words)*

Experiments in mice showed that intraperitoneal (i.p.) injection of unithiol (sodium salt of 2,3-dimercapto-l-propanesulfonic acid) diminished toxicity of several aminothiol radioprotectors, increasing the LD_{50} of cystamine by 40% and aminoethanisothiuronium bromide hydrobromide (AET) by 64%. The optimum ratio for the doses is 0.5 molar equivalent of unithiol per radioprotective thiol. A new radioprotector (mixed disulfide of cysteamine and unithiol—MDCU) has a weak toxicity: the LD_{50} is 750 mg/kg i.p. The use of unithiol makes it possible to increase the dose of the SH-radioprotectors, enhancing the dose reduction factor (DRF) of cystamine and AET by 30% for x-ray irradiation. A somewhat lesser effect is observed with fission neutron irradiation. The DRF of MDCU is equal to 1.6 for x-ray irradiation and is 1.1 for neutron irradiation. The mechanism of antitoxic action of unithiol could not be detected in Chinese hamster fibroblasts. It may be caused by the competition of unithiol and the SH-radioprotectors for certain, as yet undetermined, biochemical structures in brain neurons. It is also possible that unithiol may decrease penetration of SH-radioprotectors into the brain.

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