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Anticlastogenic Effect of S-2-(3-Aminopropylamino) Ethylphosphorothioic Acid Against X-rays in Mice

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Summary

The anticlastogenic effect of the aminothiol agent S-2-(3-aminopropylamino) ethylphosphorothioic acid (WR-2721) against X-rays was assessed by *in vivo* mouse micronucleus assay. The frequency of micronuclei in the bone marrow of adult male Swiss mice treated with WR-2721, at a radioprotective dose of 200 mg/kg or 400 mg/kg body weight, 15 or 30 min before exposure to a sublethal dose of 6 Gy X-rays, was determined 24 h after X-irradiation. The protective effect of WR-2721 against X-ray-induced clastogenicity was shown in the erythropoietic system. WR-2721 administration prior to X-ray exposure was found to decrease the number of micronucleated polychromatic erythrocytes elevated by X-irradiation. The protection against the clastogenic activity of X-rays by WR-2721 was least evident when the thiol had been administered in the lower dose of 200 mg/kg body weight, 15 min before X-irradiation, and was most effective when giving WR-2721 in the higher dose, 400 mg/kg body weight, 30 min prior to exposure of mice to X-rays. Thus, the radioprotective effect of S-2-(3-aminopropylamino) ethylphosphorothioic acid against genotoxicity by X-rays appeared to depend on the dose given and the time intervals between WR-2721 administration and X-irradiation of mice. A novel clinical application of the drug could be in its use to protect against radiation-therapy-induced genotoxic damage to normal cells.

Key words
X-rays – WR-2721 – Mouse bone marrow – In vivo micronucleus test

Introduction

In recent years, the mammalian bone marrow micronucleus assay has become prominent among genotoxicity tests. It provides direct measurement of the frequency of structural and numerical chromosome aberrations. This assay is based on an increase in the number of small chromatin bodies, i.e. micronuclei in polychromatic erythrocytes by different physical and chemical agents (Schmid 1975, Heddle and Salamone 1981, Almassy *et al.* 1987, Mavournin *et al.* 1990, Krishna *et al.* 1992, Garewal *et al.* 1993, Hayashi *et al.* 1994, Salamone and Mavournin 1994).

The genetic material damage is accepted to be the primary effect of cytotoxicity induced by ionizing radiation (Saran and Bors 1990, Brooks *et al.* 1993, Natarajan 1993, Phillips and Morgan 1993, Savage 1993, Szumiel 1994). It is known that X-rays are a clastogen. The clastogenic activity of X-rays is manifested by the induction of micronuclei predominantly from acentric fragments of chromosomes (Krishna *et al.* 1992).

Chemical protectors have received considerable attention as adjuvants in cancer radiation treatment. Among radioprotective chemicals, S-2-(3-aminopropylamino) ethylphosphorothioic acid (WR-2721, Amifostine, Ethiofos, Ethyol, Gammafos, YM-08310), structurally similar to cysteamine is particularly effective in moderating radiation-induced cellular injury (Utley *et al.* 1981, Nori *et al.* 1987, Stewart 1989, Weiss *et al.* 1990, Grdina *et al.* 1991, Schuchter *et al.* 1992, Van der Vijgh and Peters 1994).

The aim of the present investigations was to assess the modulatory effect of S-2-(3-aminopropylamino) ethylphosphorothioic acid on the clastogenic activity of X-rays by determining the frequency of micronucleated erythrocytes in the mouse bone marrow.

Material and Methods

Experimental Animals

The adult male Swiss mice (Animal Breeding Unit, Cracow) employed in the experiments weighed between 27 and 30 g. All mice were acclimatized for at least one week prior to irradiation. They were maintained under constant environmental conditions with a 12/12 hour light/dark cycle. They were fed standard granulated chow and given drinking water *ad libitum*.

Chemicals and Treatment

S-2-(3-aminopropylamino) ethylphosphorothioic acid (WR-2721) obtained from the Institute of General Chemistry, Warsaw Agricultural University (Poland) was dissolved in *aqua pro injectione* (Polfa, Poland). The solutions were freshly prepared directly before treatment of the animals. The intraperitoneal route of administration was used in all experiments. The injected volume of the thiol agent analyzed was 200 μl per mouse.

Exposure to X-rays

The mice were whole-body X-irradiated using TUR Roentgen apparatus (Germany). During exposure to X-rays, the animals were placed in a well-ventilated acrylic box. Irradiation conditions: 250 kV, 10 mA, filters – 0.5 mm Cu and 0.5 mm Al, exposure rate of 1.23 Gy/min, while the target distance was 448 mm.

Doses and Sampling Intervals

WR-2721 was injected in a single dose of 200 mg/kg or 400 mg/kg body weight. The drug doses represent approximately one-third and two-thirds of the LD, respectively. The mice were given a single sublethal dose of 6 Gy X-rays. The thiol compound was administered alone, and 15 or 30 min prior to exposure to X-rays. The animals were treated with the aminothiol agent and/or X-irradiated always at about 10.00 a.m. The doses of the aminothiol administered in the present investigations and the time intervals between WR-2721 administration and X-irradiation coincide with those most often used in radioprotection (Yuhas 1970, Milas *et al.* 1982, Shaw *et al.* 1988, Grdina *et al.* 1991, Van der Vijgh and Peters 1994). The control material consisted of untreated mice, and those injected with *aqua pro injectione* alone. The animals were killed by cervical dislocation at about 24 h after the thiol agent treatment and X-irradiation, and bone marrow samples were withdrawn.

Bone Marrow Preparation and Staining

Both femurs were removed from each mouse. The proximal and distal ends of the femurs were cut off and the bone marrow cells were gently flushed out with foetal calf serum (Gibco Ltd., United Kingdom). The bone marrow cells were dispersed by gently pipetting and collected by centrifugation at 1000 rpm for 5 min at 4 °C. The cell pellet was resuspended in a small volume of foetal calf serum and bone marrow smears were prepared (two slides per mouse). The slides were coded to avoid observer bias. After air-drying, the smears were stained by the May-Grünwald/Giemsa combinations (Schmid 1975, Pascoe and Gatehouse 1986). With this method polychromatic erythrocytes (PCEs) stain reddish-blue and normochromatic erythrocytes (NCEs) stain orange while nuclear material is dark purple in color.

Slide Analysis

The number of micronucleated polychromatic erythrocytes (MNPCEs) among 2000 PCEs per mouse (1000 PCEs per slide) and the number of PCEs among 400 erythrocytes per animal (200 /PCEs + NCEs/ per slide) were determined. The slides were examined using a light microscope (Carl Zeiss, Germany).

Statistical Evaluation

Differences in the incidence per animal of MNPCEs per 1000 PCEs and of PCEs per 100 erythrocytes (PCEs + NCEs) were compared by an analysis of variance and Duncan's new multiple range test.

Results

MNPCEs per 1000 PCEs

The frequency of micronucleated polychromatic erythrocytes of the bone marrow of mice treated with WR-2721 and/or X-irradiated is presented in Table 1. In comparison to the controls, the number of MNPCEs was increased in all remaining groups of animals. In mice which had received an injection of WR-2721 before X-irradiation, the frequency of micronuclei was found to be decreased, in comparison to those exposed to X-rays only. The number of MNPCEs appeared to depend on the dose administered and the time intervals between WR-2721 administration and exposure of mice to X-rays. The most effective reduction in the number of micronuclei was obtained when WR-2721 was injected at the higher dose, 400 mg/kg body weight, 30 min before X-irradiation, and the least effective decrease of the frequency of MNPCEs was observed after administration of the lower dose of WR-2721, 200 mg/kg body weight, 15 min prior to exposure of animals to X-rays. In mice receiving the aminothiol alone, without subsequent X-irradiation, the frequency of micronuclei was dose-dependently increased in relation to the controls. There were no differences in the number of micronucleated polychromatic erythrocytes between the untreated control group of mice and that injected with *aqua pro injectione* only.

PCEs per 100 (PCEs + NCEs)

The number of polychromatic erythrocytes in the bone marrow of mice administered WR-2721 and/or exposed to X-rays is given in Table 1. As compared to the controls, the number of PCEs was decreased in all the experimental groups. In mice treated with the aminothiol prior to X-ray exposure, the percentage of PCEs was increased in relation to those exposed to X-rays only. The number of PCEs was dependent on the dose given and the time intervals between WR-2721 administration and X-irradiation of mice. The largest number of PCEs was found when administering the aminothiol at the higher dose, 400 mg/kg body weight, 30 min before X-irradiation, and the smallest number of polychromatic erythrocytes was observed after the WR-2721 injection in the lower dose of 200 mg/kg body weight, 15 min prior to X-ray exposure. In mice given WR-2721 alone, without subsequent X-irradiation, the percentage of PCEs was dose-dependently decreased in comparison with the controls. Differences in the number of PCEs between the untreated control group of mice and that treated with *aqua pro injectione* alone, were not found.

Table 1
Results of experiments with WR-2721 and X-rays in the mouse bone marrow micronucleus assay

Group	Number of MNPCEs per 1000 PCEs	PCEs per 100 (PCEs + NCEs)	Significantly different ($p<0.05$) from groups
1. WR-2721 (200 mg/kg, −15 min) + X-rays (6 Gy)	61.83 ± 6.77	23.46 ± 3.47	*3,4,5,6,7,8,9*
2. WR-2721 (200 mg/kg, −30 min) + X-rays (6 Gy)	58.67 ± 6.19	24.54 ± 5.52	*4,5,6,7,8,9*
3. WR-2721 (400 mg/kg, −15 min) + X-rays (6 Gy)	55.75 ± 4.68	27.62 ± 5.67	*1,5,6,7,8,9*
4. WR-2721 (400 mg/kg, −30 min) + X-rays (6 Gy)	52.50 ± 4.37	29.01 ± 5.36	*1,2,5,6,7,8,9*
5. X-rays (6 Gy)	74.67 ± 11.15	14.08 ± 4.46	*1,2,3,4,6,7,8,9*
6. WR-2721 (200 mg/kg)	7.43 ± 1.40	53.21 ± 4.93	*1,2,3,4,5,7,8,9*
7. WR-2721 (400 mg/kg)	11.50 ± 2.78	46.92 ± 7.37	*1,2,3,4,5,6,8,9*
8. *Aqua pro injectione*	4.18 ± 1.25	59.25 ± 3.75	*1,2,3,4,5,6,7*
9. Untreated control	4.17 ± 1.19	39.10 ± 4.43	*1,2,3,4,5,6,7*

Data are expressed as means ± S.D. from 5–7 mice.

Discussion

In the present study, the modulatory effect of the aminothiol agent WR-2721 on the clastogenic activity of X-rays was shown. The radioprotection by WR-2721 against the clastogenicity observed in the mouse bone marrow at 24 h after X-irradiation with a sublethal dose of 6 Gy was dependent on the dose applied and the time intervals between WR-2721 administration and exposure to X-rays. The most effective anticlastogenic effect of WR-2721 was obtained when injecting the thiol drug at the higher dose of 400 mg/kg body weight, 30 min before X-irradiation, and the least one was observed after the aminothiol administration at the lower dose of 200 mg/kg body weight, 15 min prior to X-ray exposure. Moreover, the protective effect of WR-2721 against the radiation-induced suppression of mitotic activity in the erythropoietic system was stated. The number of PCEs was reduced after X-irradiation only, but in mice pre-treated with the thiol agent, the percentage of polychromatic erythrocytes was found to be higher 24 h after X-ray exposure of mice. However, when applied alone, in the protective doses against X-ray-induced radiotoxicity, WR-2721 appeared to be genotoxic and cytotoxic. It was demonstrated that in mice which had received an injection of the thiol compound, the frequency of micronuclei was increased and the number of PCEs was reduced.

Cellular endpoints following irradiation are primarily the result of the induction, processing and manifestation of DNA damage. Ionizing radiation

causes a variety of different types of damage to DNA, but DNA double-strand breaks are the most deleterious lesion induced by irradiation of mammalian cells. Moreover, DNA double-strand breaks appear to be the most important lesion responsible for the production of chromosomal alterations, such as aberrations and micronuclei (Greenstock 1986, Natarajan 1993, Phillips and Morgan 1993, Savage 1993). Ionizing radiation is a very efficient inducer of micronuclei, which are accepted to be a biological indicator of cellular damage (Almassy *et al.* 1987, Jagetia 1990, Uma Devi and Sharma 1990, Ludewig *et al.* 1991, Morales-Ramirez *et al.* 1994).

It is accepted that, under physiological conditions, S-2-(3-aminopropylamino) ethylphosphorothioic acid itself is unable to mediate radioprotection. WR-2721 may be considered as the pro-drug, because its mechanism of action requires conversion by alkaline phosphate to the active free thiol, WR-1065. This compound can be further oxidized to the symmetrical disulphide, WR-33278, or can form mixed disulphides with endogenous thiols or proteins. Thus, the degree of protection afforded by WR-2721 is dependent on its pharmacokinetics and metabolic fate of its metabolites. The cellular thiol/disulphide status at the time of irradiation appears to play an important role in the tissue resistance to ionizing radiation (Shaw *et al.* 1988, 1994, Livesey *et al.* 1990). Although the mechanisms of radioprotection by WR-2721 have not yet been completely understood, it is generally assumed that the thiol groups of its metabolites protect against damage by acting as free radical scavengers and donating hydrogen to repair target molecules, e.g. DNA molecules, the principal radiation targets (Fahey 1988, Held 1988, Murray *et al.* 1988). The thiols can inhibit indirect damage, repair both direct and indirect impairment and enhance the recovery of damaged or depleted cell populations. However, the manipulation of cellular thiol levels results in "biochemical shock" (Greenstock 1988, Shaw *et al.* 1988, Allalunis-Turner *et al.* 1989).

In conclusion, the results of the present study clearly demonstrate a dependence of the anticlastogenic effect of S-2-(3-aminopropylamino) ethylphosphorothioic acid against X-rays assayed by the mouse bone marrow micronucleus test, on the doses of WR-2721 and the time of administration in relation to radiation exposure. Taking into consideration the clinical aspects of the present findings, attempts to obtain the best pharmacological prescription for radioprotection to reduce the geno- and cytotoxicity of ionizing radiation to normal tissues, including the haemopoietic system, should be taken into account in radiotherapy.

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References

ALLALUNIS-TURNER M.J., WALDEN T.L., SAWICH CH.: Induction of marrow hypoxia by radioprotective agents. *Radiat. Res.* **118:** 581–586, 1989.

ALMASSY Z., KREPINSKY A.B., BIANCO A., KOTELES, G.J.: The present state and perspectives of micronucleus assay in radiation protection. A review. *Appl. Radiat. Isotope* **38:** 241–249, 1987.

BROOKS A.L., KHAN M.A., JOSTES R.F., CROSS F.T.: Metaphase chromosome aberrations as markers of radiation exposure and dose. *J. Toxicol. Environ. Health* **40:** 277–288, 1993.

FAHEY C.R.: Protection of DNA by thiols. *Pharmacol. Ther.* **39:** 101–108, 1988.

GAREWAL H.S., RAMSEY L., KAUGARS G., BOYLE J.: Clinical experience with the micronucleus assay. *J. Cell. Biochem.* Suppl. 17F: 206–212, 1993.

GRDINA D.J., CARNES B.A., GRAHN D., SIGDESTAD C.P.: Protection against late effects of radiation by S-2-(3–aminopropylamino) ethylphosphorothioic acid. *Cancer Res.* **51:** 4125–4130, 1991.

GREENSTOCK C.L.: Radiation-induced aging and induction and promotion of biological damage. In: *Free Radicals in Aging and Degenerative Disease.* Alan R. Liss, New York, 1986, pp. 197–219.

GREENSTOCK C.L.: Radiation chemical consideration for the radioprotection of cellular targets. *Pharmacol. Ther.* **39:** 139–145, 1988.

HAYASHI M., TICE R.R., MACGREGOR J.T., ANDERSON D., BLAKEY D.H., KIRSH-VOLDERS M., OLESON JR. F.B., PACCHIEROTTI F., ROMAGNA F., SHIMADA H., SUTOU S., VANNIER B.: In vivo rodent erythrocyte micronucleus assay. *Mutat. Res.* **312:** 293–304, 1994.

HEDDLE J.A., SALAMONE M.F.: Chromosomal aberrations and bone marrow toxicity. *Environ. Health Perspect.* **39:** 23–27, 1981.

HELD K.D.: Models for thiol protection of DNA in cells. *Pharmacol. Ther.* **39:** 123–131, 1988.

JAGETIA G.C.: Frequency of micronucleated cells in the mouse bone marrow after exposure to various doses of γ-radiation. *Mutat. Res.* **230:** 45–48, 1990.

KRISHNA G., FRIEDLER R., THEISS J.C.: Simultaneous evaluation of clastogenicity, aneugenicity and toxicities in the mouse micronucleus assay using immunofluorescence. *Mutat. Res.* **282:** 159–167, 1992.

LIVESEY J.C., GRUNBAUM Z., KROHN K.A.: Binding of aminoalkylphosphorothioate radioprotective drugs to rodent tissue proteins. *Biochem. Pharmacol.* **39:** 1807–1812, 1990.

LUDEWIG E., KOCH F., KAMPRAD F., MELZER R.: The micronucleus test in pigs: induction of micronuclei in polychromatic erythrocytes by various doses of X-rays. *Mutat. Res.* **249:** 1–6, 1991.

MAVOURNIN K.H., BLAKEY D.H., CIMINO M.C., SALAMONE M.F., HEDDLE J.A.: The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat. Res.* **239:** 29–80, 1990.

MILAS L., HUNTER N., REID B.O., THAMES D.: Protective effects of S-2-(3–aminopropylamino) ethylphosphorothioic acid against radiation damage of normal tissues and a fibrosarcoma in mice. *Cancer Res.* **42:** 1838–1897, 1982.

MORALES-RAMIREZ P., VALLARINO-KELLY T., MERCADER-MARTINEZ J., RODRIGUEZ-REYES R.: Induction of micronuclei by acute and chronic exposure in vivo to gamma rays in murine polychromatic erythrocytes. *Mutat. Res.* **341:** 47–55, 1994.

MURRAY D., VANANKEREN S.C., MILAS L., MEYN R.E.: Radioprotective action of aminothiols in vitro and in vivo: comparison between effects on DNA damage and cell survival. *Pharmacol. Ther.* **39:** 151–153, 1988.

NATARAJAN A.T.: Mechanisms for induction of mutations and chromosome alterations. *Environ. Health Perspect.* **101** (Suppl. 3): 225–229, 1993.

NORI D., KIM J.H., HILARIS B., CHU F.C.: Radiosensitizers and protectors. In: *Radiation Therapy of Gynecological Cancer.* Alan R. Liss, New York, 1987, pp. 319–329.

PASCOE S,. GATEHOUSE D.: The use of a simple haematoxylin and eosin staining procedure to demonstrate micronuclei within rodent bone marrow. *Mutat. Res.* **164:** 237–243, 1986.

PHILLIPS J.W., MORGAN W.F.: DNA double-strand breaks in mutagenesis. *Environ. Mol. Mutagen.* **22:** 214–217, 1993.

SALAMONE M.F., MAVOURNIN K.H.: Bone marrow micronucleus assay: A review of the mouse stocks used and their published mean spontaneous micronucleus frequencies. *Environ. Mol. Mutagen.* **23:** 239–273, 1994.

SARAN M., BORS W.: Radical reactions in vivo – an overview. *Radiat. Environ. Biophys.* **29:** 249–262, 1990.

SAVAGE J.R.K.: Update on target theory as applied to chromosomal aberrations. *Environ. Mol. Mutagen.* **22:** 198–207, 1993.

SCHMID W.: The micronucleus test. *Mutat. Res.* **31:** 9–15, 1975.

SCHUCHTER L.M., LUGINBUHL W.E., MEROPOL N.J.: The current status of toxicology protectants in cancer therapy. *Semin. Oncol.* **19:** 742–751, 1992.

SHAW L.M., GLOVER D., TURRISI A., BROWN D.Q., BONNER H.S., NORFLEET A.L., WEILER C., GLICK J.H., KLIGERMAN M.M.: Pharmacokinetics of WR-2721. *Pharmacol. Ther.* **39:** 195–201, 1988.

SHAW L.M., BONNER H.S., BROWN D.Q.: Metabolic pathways of WR-2721 (Ethyol, Amifostine) in the Balb/c mouse. *Drug Metab. Dispos.* **22:** 895–902, 1994.

STEWART F.A.: Modification of normal tissue response to radiotherapy and chemotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* **16:** 1195–1200, 1989.

SZUMIEL I.: Ionizing radiation-induced cell death. *Int. J. Radiat. Biol.* **66:** 329–341, 1994.

UMA DEVI P., SHARMA A.S.K.V.S.: Mouse bone-marrow response to low doses of whole–body gamma irradiation: induction of micronuclei. *Int. J. Radiat. Biol.* **57:** 97–101, 1990.

UTLEY J.F., QUINN C.A., WHITE F.C., SEAVER N.A., BLOOR C.M.: Protection of normal tissue against late radiation injury by WR-2721. *Radiat. Res.* **85:** 408–415, 1981.

VAN DER VIJGH W.J.F., PETERS G.J.: Protection of normal tissues from the cytotoxic effects of chemotherapy and radiation by Amifostine (Ethyol): Preclinical aspects. In: *Amifostine (Ethyol): A New Cytoprotective Agent.* Y.W. YARBRO, R.S. BORNSTEIN, M.J. MASTRANGELO (eds), W.B. Saunders, Philadelphia, *Semin. Oncol.* **21** (Suppl. 11): 2–7, 1994.

WEISS J.F., KUMAR K.S., WALDEN T.L., NETA R., LANDAUER, M.R., CLARE P.: Advances in radioprotection through the use of combined agent regimens. *Int. J. Radiat. Biol.* **57:** 709–722, 1990.

YUHAS J.M.: Biological factors affecting the radioprotective efficiency of S-2-(3aminopropylamino) ethylphosphorothioic acid (WR-2721). $LD_{50/30}$ doses. *Radiat. Res.* **44:** 621–628, 1970.

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