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Radioprotection of Irradiated Mice-Mechanisms and Synergistic Action of WR-2721 and R.L.B.*

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Zusammenfassung

Die als Strahlenschutz wirksame Verbindung WR-2721 (S-2 (3-amino-propylamino)-athyl-phosphorothioic-säure) verursacht in vitro eine Kontraktion der DNS-Ketten, aber nur wenn diese von normalen Zellen abstammen. Die Kontraktion der DNS-Ketten hat eine Abnahme der UV-Absorbtion bei 260 nm zur Folge (Hypochromizität). Es besteht eine Beziehung zwischen der durch WR-2721 erzielten Hypochromizitat und der verminderten Synthese derselben DNS, die als Matrize unter Anwesenheit dieser Strahlenschutz-Verbindung benutzt werden. Im Gegensatz dazu hat die Verbindung keine Wirkung auf die Sekundärstruktur der DNS unterschiedlicher Krebszellen oder auf die in vitro Synthese dieser DNS. In Verbindung mit R. L. B. (spezifischer RNS „Primer"), das selektiv die Replikation der DNS normaler haematopoietischer Zellen anregt, schützt WR-2721 in relativ niedrigen Dosen Mäuse vor tödlichen Dosen von Gamma-Strahlen. Man erzielt bedeutende Überlebensquoten. Der Mechanismus dieses durch WR-2721 und R.L.B bewirkten Schutzes wird noch erläutert.

Schlüsselwörter

Strahlenschutz, DNS-Kettenkontraktion, WR-2721, RNS-„Primer".

Summary

Radioprotector WR-2721 (S-2 (3-amino-propylamino)-ethyl-phosphorothioic acid) induces in vitro the contraction of DNA chains, but only when these originate from normal cells. Chain contracuon results in a decrease of UV absorbance at 260 nm (hypochromicity). A correlation exists between DNA hypochromicity induced by WR-2721 and decrease in the synthesis of the same DNAs used as templates in the presence of this radioprotector. In contrast, the compound has no effect either on secondary structure of DNAs from various cancer cells or on in vitro synthesis of these DNAs. In association with R.L.B. (specific RNA primers) which selectively prime replication of DNAs from normal haematopoietic cells, WR-2721, used at relatively low doses, protects mice against lethal doses of gamma radiation. Efficient survival rates are obtained. The mechanism of this protection by WR-2721 and R.L.B. is discussed.

Keywords

Radioprotection, DNA chain contraction, WR-2721, RNA primer (R.L.B).

Introduction

For 40 years many efforts have been devoted to a search for chemical compounds capable of protecting living organisms against radiation [16, 9, 13, 32]. Among the various compounds assayed, aminothiols and their derivatives, mainly phosphorothioates, were shown to act efficiently as radioprotectors [1, 19, 15]. More recently the possibility to use the compounds in radiotherapy or in association with chemotherapeutic drugs for the treatment of cancer has led to the search for compounds which would protect cells of normal tissues, without protecting those of cancer tissues. However, few of the chemical or biological substances tested exhibit such selective properties. So far, the best known and most efficient of these chemicals is WR-2721, which protects the cells of normal tissues but has practically no protecting effect on cancer cells [21, 32]. Unfortunately, this compounds induces rather severe side effects when administered at radioprotective doses [31, 18, 30, 11]. Commercially available yeast RNA [22] and DNA extracted from various organs of rats increased the survival rate of irradiated mice [24].

In our extensive studies we demonstrated that R.L.B. (specific RNA fragments) [6, 20, 3, 4] selectively bind in vitro to DNA from bone marrow and spleen cells and accelerate its synthesis in vitro. R.L.B. have no effect on DNA from cancer cells. When injected into animals, R.L.B. induce genesis of leukocytes and platelets even in animals treated with chemotherapeutic drugs. These properties designate R.L.B. as potential candidates for chemical radioprotection.

The object of this work was to investigate, first, the mode of action of WR-2721 at the level of DNA isolated from normal and cancer cells, then second, the possibility of synergistic effects of WR-2721 and R.L.B. on radioprotection of irradiated mice, with the aim of obtaining the highest number of surviving mice while using increasing lethal doses of gamma radiation.

Material and Methods

Products

WR-2721 was synthesized by Profes-

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sor Miginiac (University of Poitiers). R.L.B.s were prepared as previously described [3]. DNA-dependent DNA polymerase I was purified from Escherichia coli extracts [3]. Deoxyribonucleoside-5'-triphosphates (dATP, dCTP, dGTP and dTTP): Miles Laboratoires U.S.A. (^{3}H)-thymidine-5'-triphosphate (sp. act. 17.5 Ci/mmol): Amersham, England.

Isolation and characterisation of DNAs

DNAs from human and animal and cancer tissues were isolated and purified as previously described [25, 5]. The RNA content of the DNA samples, determined with the orcinol reaction, was less than 10%. Protein content was less than 1%. The hyperchromic effect of incubation with O.1 N KOH was 40-45% at 260 nm for DNA samples. DNA double strandness was checked by ultracentrifugation in alkaline sucrose gradient [5].

Conditions for DNA in vitro synthesis

The incubation mixture contained (0.15 ml): Tris-HCl buffer (pH 8.0), 25 µmol; $MgCl_2$, 2 µmol; the four deoxyribonucleoside-5'-triphosphates, each 5 nmol (+^{3}H-TTP:50.000 CPM). DNA, 0,5 µg; DNA-dependent DNA polymerase I, 60 µg. WR-2721 (see legends to figures). Incubation 10 min. at 36°C. The reaction was stopped by addition of trichloroacetic acid (TCA, 5% final concentration) and cooling in an ice bath. Acid precipitable material was filtered on a GF/C glass filter, washed with TCA (5%), alcohol 95°, dried and the radioactivity was measured with a Beckman spectrometer. Results are expressed as counts per min. (C.P.M.).

UV absorbance (hyperchromicity and hypochromicity) of DNAs

UV absorbance at 260 nm of various normal and cancer cell DNAs (20 µg in 1 ml of Tris-HCl buffer 10^{-2}M pH 7.3 freshly prepared) was measured at 24°C before and after addition of WR-2721 dissolved in sterile distilled water. The blank cuvette contained an equivalent amount WR-2721 but no DNA. Contact between DNA and WR-2721 was 1 min. with gentle shaking. Absorbance at 260 nm was measured again. Results are expressed as UV absorbance decrease or increase (% of control).

Effect of WR-2721 on ^{3}H-thymidine concentration in various mouse tissues

Nine CD 1 ♂mice (22 g) from Charles River, France, were used. WR-2721, dissolved in buffered physiological NaCl solution, was administered i.p. 15 min before ^{3}H-thymidine was injected by i.p. route. Three mice received only ^{3}H-thymidine (1. 1 Ci/mouse); three received first WR-2721 (6 mg/mouse), then ^{3}H-thymidine (1. 1 Ci/mouse) and the last three received first WR-2721 (12 mg/mouse), then ^{3}H-thymidine (1. 1 Ci/mouse). Mice were sacrificed three hrs afterwards and different tissues were selected: bone marrow, spleen, liver. lung, kidney. Each tissue was incubated with TCA solution (5 % final concentration) for 30 min. with gentle shaking at 24°C. After centrifugation at 10,000 RPM, the supernatant was saved and the pellet reextracted once more with TCA. The two supernatants were mixed and radioacitivity was measured. The results are expressed as percent of radioacuvity (relative values) found in the tissues. A 100% value corresponds to mice which have received only ^{3}H-thymidine.

Effect of WR-2721 and R.L.B. on irradiated mice

CD1 ♂ mice were kept in an animal room (21 ± 1°C) for 10-15 days before start of experiments. On the day prior to irradiation, mice weighing 26-28 g were selected and separated into groups of 10. Group 1: control; group 2: receives WR-2721; group 3: receives R.L.B.; group 4: receives WR-2721 + R.L.B.

These experiments were performed every 2 months over a period of over two years (number of mice: 540 for 9.0 Gy; 60 for 100 Gy; 100 for 11.0 Gy and 150 for 12.0 Gy). WR-2721 and R.L.B. were dissolved in buffered physiologic solution (pH 7.7) and sterilized by filtration on sterile millipore disks (0.45 µm) prior to i. p. administration. WR-2721 (100 mg/kg) was injected 20-25 min and R.L.B. (160 mg/kg) 90 min respectively before irradiation. Gamma radiation was delivered by a cobalt source at doses of 9.0, 10.0, 11.0 and 12.0 Gy with a flux of 0.70 min. Survival of irradiated mice was determined daily for 30 consecutive days or even more in some cases.

Results

Action of WR-2721 on normal and cancer cells DNAs

It is known that DNAs from normal and cancer tissues do not possess the same secondary and tertiary structure and react differently in the presence of various chemical compounds [17, 3]. This may be evaluated by spectroscopic measurement in ultraviolet light. Fig. 1 and 2 illustrate the results obtained under strictly standardized conditions with WR-2721 in the course of in vitro experiments using DNAs from normal and cancer cells of different mammalian organs. The absorbance at 260 nm of double stranded DNA from bone marrow, spleen, brain and liver decreases in the presence of WR-2721 (hypochromicity) as a dose-related concentration of the compound. In contrast, the UV absorbance of DNAs from cancer tissues slightly increases in the presence of WR-2721 (hyperchromicity).

These results demonstrate that WR-2721 is able to distinguish normal cell DNA from cancer cell DNA,

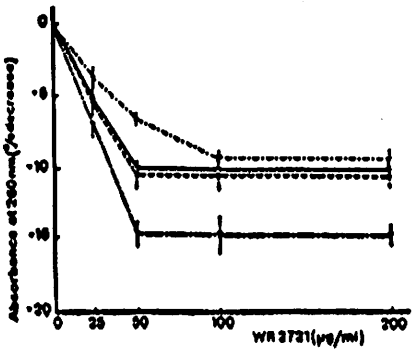

Fig. 1: UV absorbance of DNAs from normal tissues in the absence and presence of WR-2721.
Freshly prepared WR-2721 solution was used. For experimental conditions, see text. Human DNAs: –.–.–, bone marrow; ——, —— spleen, ————, liver; –.– DNA from monkey brain. UV absorbance at 260 nm was determined before and after addition of WR-2721. Data from 3 independent experiments were pooled to derive each curve shown.

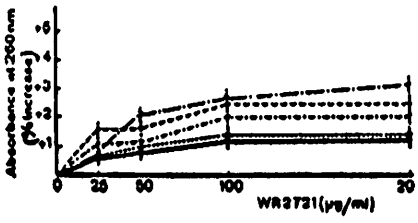

Fig. 2: UV absorbance of DNAs from cancer tissues
Experimental conditions are the same as those in the legend to fig. 1. human liver: ––––; human neurocarcinoma: –.–; mouse DNAs: –.–.– mammary carcinoma; – – –, melanoma; —— YC8 lymphoma.

which probably explains that WR-2721 does not protect cancer cells in vivo against radiation [32].
It is well established that DNA chain contraction induced by different substances [2, 3] results in a decrease of UV absorbance, while DNA chain separation (hydrogen bonds are broken) results in an increase of UV absorbance. To these changes correspond, respectively the decrease and increase of the rate of DNA synthesis in vivo [3, 17].
Fig. 3 shows that WR-2721 inhibits in vitro DNA synthesis when DNAs from cells of normal tissues are used

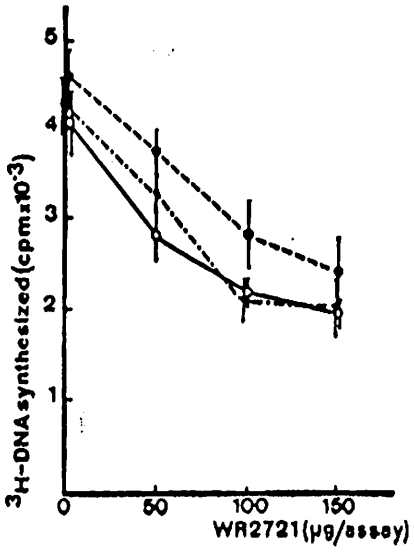

Fig. 3: The inhibitory effect of WR-2721 on in vitro synthesis of DNAs from normal tissues.
Incubation conditions (see text). Data from 4 experiments were pooled to derive each curve. Errors are standard error of the means indicated by bars. DNAs from human uterus ——; human spleen –.–; monkey brain ——. Data obtained with DNAs from cancer tissues (not presented here) showed no inhibitory effect of WR-2721.

as a template. WR-2721 administered i.p. to mice before injecting ^{3}H-thymidine, lowers the concentration of ^{3}H-thymidine (a precursor for DNA synthesis) in normal cells (fig. 4) which might involve a decrease of DNA synthesis in vivo.

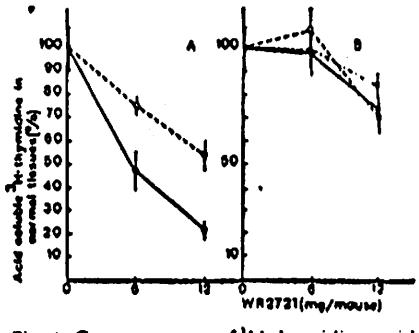

Fig. 4: Concentration of ^{3}H-thymidine (acid soluble) in tissues from mice in the absence and presence of WR-2721.
Experimental conditions (see text). The relative values are expressed in comparison to those observed in the absence (100%) of WR-2721. Each point on the curve represents pooled data from 3 mice.
Panel A: —— spleen; ——, bone marrow
Panel B: –––– liver; —— kidney; –.–.– lung

Effect of WR-2721 and R.L.B. on spleen of irradiated mice

At 9.0 Gy, gamma irradiation of mice causes death of practically all unprotected mice in 10-15 days. 24 h after irradiation spleens taken from these mice weigh 70-80% less than those of non irradiated animals.
When mice are treated with either WR-2721 or R.L.B. or both before irradiation, spleen weight decreases by about 40-50% of normal weight; this could allow spleen to remain functional in many irradiated mice, since mouse survival is 70-80% when WR-2721 and R.L.B. are administered together.

Protection of mice irradiated with lethal doses of gamma radiation

As described in Materials and Methods, mice were protected or not against lethal doses of gamma radiation (9.0; 10.0; 11.0 and 12.0 Gy). Protected mice received either WR-2721, R.L.B. or both, administered at a given time before irradiation. At 9.0 Gy, mortality of unprotected mice reaches 95-100%. No survivors were obtained with higher doses of irradiation. Fig. 5 illustrates survival of unprotected and protected (WR-2721 + R.L.B.) mice irradiated at 9.0 Gy. Unprotected mice do not survive more than 10-12 days after irradiation while 73 percent of treated mice survive. Under the same experimental conditions WR-2721 or R.L.B., each used alone has some protective effect in mice survival (tab. 1). In spite of the relatively low dose of WR-2721 (100 mg/kg), this compound exhibits, when associated with R.L.B. a very significant radioprotective effect. In fact, if this low concentration of WR-2721 is used in association with R.L.B. for protecting mice irradiated at 10.0, 11.0 and 12.0 Gy, a significant number of survivors are observed at 10.0 Gy (fig. 6) while at 11.0 and 12.0 Gy only about 45 ± 16 percent of the animals survive for 30 days. At

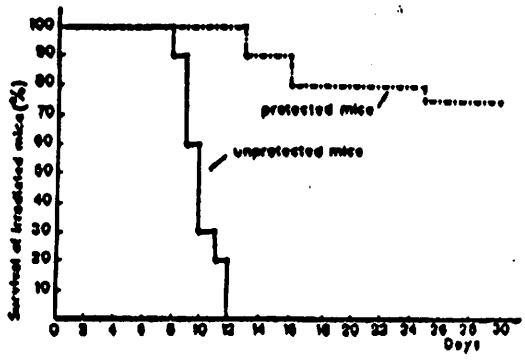

Fig. 5: Percent survival of unprotected and protected mice irradiated at 9.0 Gy.
Experimental conditions (see text). Protected mice received WR-2721 (100 mg/kg) and R.L.B. (160 mg/kg) by i. p. route 15-20 and 90 min respectively before irradiation. Each group represents 180 mice (mean values). Data are expressed as percent survival at indicated intervals.

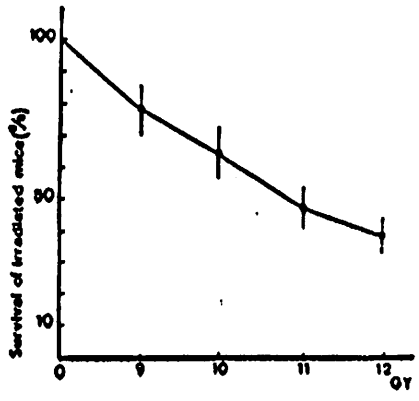

Fig. 6: Percent of protected mice irradiated with lethal doses of γ-irradiation.
Experimental conditions (see text). WR-2721 (100 mg/kg) and R.L.B. (160 mg/kg) were given i.p. 15-20 and 90 min respectively before irradiation. In each experiment 10 mice were used for each dose. Total number of mice: 180 for 9.0 Gy; for 10 Gy; 100 for 11.0 Gy and 150 for 12.0 Gy. Errors are standard error of the means represented by bars.

these two latter doses of radiation there are practically no survivors when either WR-2721 or R.L.B. is used alone.

Discussion

In order to avoid the well-known cytotoxic effect of WR-2721, we chose to use a low dose (100 mg/kg) for radioprotection of mice [32]. Under these conditions, efficient radioprotection of a large number of mice is achieved only if WR-2721 is used together with R.L.B. (RNA primers for DNA replication.) This protection extends into supra-lethal doses of gamma radiation. The synergistic effect of both substances is required. The mechanisms by which WR-2721 and R.L.B. protect mice are demonstrated by in vitro and in vivo experiments.

In vitro, WR-2721 inhibits the synthesis of DNA isolated from normal tissues. This is in accordance with the in vitro decrease of DNA UV absorbance (hypochromicity). Under the same experimental conditions, WR-2721 induces a slight UV absorbance increase (hyperchromicity) in DNAs from cancer tissues. These observations correlate with the observed decrease of DNA in vitro synthesis in the presence of WR-2721 when DNAs from normal tissues are used as templates. They also correlate with descriptions found in the literature of a selective radioprotective effect of WR-2721 on normal cells [31]. The differential behavior of normal and cancer DNAs in the presence of WR-2721 can be accounted for by differences in their secondary structures, that of cancer DNAs being always destabilized, with hydrogen bonds broken in several areas [3].

WR-2721 induced hypochromicity of DNA means that its secondary structure is tighter than it normally is. This is further evidenced by the observation, in vitro, that WR-2721 increases DNA melting temperature [28].

It is conceivable that during radiation in the presence of WR-2721 an endonuclease that causes single-strand break in irradiated DNA [27] would then have its activity reduced and, on the contrary, repair of DNA dammage would be favoured.

WR-2721 differs profoundly from carcinogens, which tend to relax DNA secondary structure, especially when it is already destabilized, i. e. in cancer cells [3, 4]. It should be mentioned that WR-2721, by binding to normal DNA strands, may prevent fixation of these carcinogens, which is hindered by DNA contraction. This could explain the results reported by many authors showing that WR-2721 protects normal tissues against the toxicity of alkylating agents and the early effects of irradiation [12, 8, 14, 26, 23, 29].

R.L.B. also plays an important part in radioprotection. These RNA primers also bind to DNA from normal tissues only, and very specifically to DNAs from bone marrow and spleen, the in vitro synthesis of which they have been shown to accelerate. In vivo their activity is evidenced by a high acceleration of leukocyte and platelet genesis [20, 3].

When injected into mice prior to irradiation, WR-2721 and R.L.B. allow survival of a large number of animals at lethal radiation doses (9.0 and 10.0 Gy). Even at 12.0 Gy, we still find a significant number of survivors (fig. 6). But if used alone at the same radiation dose, WR-2721 does not allow the survival of irradiated mice.

Tab. 1: Percent survival of unprotected and protected mice irradiated at 9.0 Gy. Expenmental conditions (see text). WR-2721 (100 mg/kg) and R.L.B. (160 mg/kg) were given i. p. 15-20 and 90 min respectively before-irradiation S. D.: standart deviation

	Number of experiments	Number of mice	% of survivors at 30 days	S. D.
Control	18	180	6.5	± 7.7
WR-2721	18	180	48	± 15
R.L.B.	18	180	45	± 12

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