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N-Acyl dehydroalanines protect from radiation toxicity and inhibit radiation carcinogenesis in mice

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N-Acyl dehydroalanines have shown free radical scavenging activity. They react with and scavenge mainly oxygen-derived free radicals such as the superoxide anion (O_2^-) and the hydroxyl radical ($HO^\cdot$). *Ortho*-methoxyphenylacetyl dehydroalanine (AD-20) protects total-body irradiated mice against the toxicity induced by X-rays when delivered as a single dose of 700 rads in a short period of time. This degree of protection was of the same order of magnitude as that obtained with the aminothiol S-2-(3-aminopropylamino)-ethylphosphorothioic acid (WR-2721). The radioprotection of AD-20 is extended to all other doses of X-rays tested (from 600 to 800 rads). Furthermore, AD-20 inhibits the development of thymic lymphomas in C57Bl/Ka mice undergoing a leukaemogenic course of irradiation (4 × 175 rads applied at weekly intervals). We postulate that AD-20 may act as a radioprotector and anticarcinogenic agent, most probably by inactivating the oxygen-derived free radicals formed during water radiolysis.

Introduction

Membranes and DNA are critical targets for ionizing radiation and may be major sites for the development of the oxygen effect (1). Explanations of this effect have previously emphasized the involvement of reactive oxygen species such as the superoxide anion (O_2^-) and the hydroxyl radical ($HO^\cdot$) to explain some of the cytotoxic effects of ionizing radiations (2).

However, the formation of stabilized radical adducts has been proposed as a way to decrease the reactivity of free radicals (3). Such an approach has been applied to synthesize capto-dative olefins able to react with and scavenge free radicals (4). In fact, capto-dative olefins, by offering three reactive sites (Figure 1), may inactivate free radicals of different polarities. For instance, if the radical attack proceeds by radical addition to the carbon–carbon double bond (the capto-dative site), the resulting radical adduct is stabilized by the effect of both electron donating and electron withdrawing groups, which simultaneously substitute the same carbon atom, i.e. the 'capto-dative' effect (3). When the attack occurs by hydrogen atom abstraction from the methylene group (proradical site), then the radical adduct is stabilized by the capto-dative effect of both the aromatic ring and the carbonyl group. In both cases, the radical adduct formed often disappears by dimerization or by reacting with another free radical. Finally, the aromatic ring may scavenge hydroxyl radicals to give hydroxylated aromatic derivatives. Among these molecules, we have reported that the *N*-acyldehyroalanines (AD* compounds) react with and scavenge O_2^- and $HO^\cdot$ (5). Probably, in this way they inhibit both *in vitro* and *in vivo* free radical mediated processes (6–9). In particular, AD compounds inhibit lipid peroxidation when rat liver microsomes are exposed, in aerobic conditions, to a source of gamma-rays (6), suggesting that their free radical scavenging ability prevents the deleterious effects of ionizing radiation.

In vivo, ionizing radiation induces a rapid or a delayed mortality depending on both the frequency and the level of dose administered (10). Single doses of X-rays varying from 600 to 800 rads induce bone marrow toxicity with the animal dying 1 week after irradiation (10). However, in several strains of mice such as C57Bl/Ka mice, ionizing radiation when applied as fractionated doses (4 × 175 rads at weekly intervals), induces the neoplastic transformation of T lymphocytes within the thymus, and the mortality is extended from 3 to 6 months after the last irradiation (11).

It has been reported that the administration of both the antioxidant enzyme superoxide dismutase (12) and the aminothiol S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721) (13), two molecules with free radical scavenging activity, improved the 30-day survival of irradiated mice and significantly decreased the lifetime incidence of X-ray induced leukaemia (14) and radiation-induced sarcomas (15) respectively. Thus, we have hypothesized that the (*ortho*-methoxyphenylacetyl)-dehydroalanine (AD-20), by inactivating the oxygen-derived free radicals, could prevent both the toxicity induced *in vivo* by ionizing radiation delivered as a single dose in a short-term assay, and the carcinogenic effects of fractionated sub-lethal doses in a long-term experiment.

Materials and methods

Chemicals

AD-20 was synthesized by Professor H.G.Viehe and his co-workers (Laboratory of Organic Chemistry, Louvain la Neuve, Belgium). WR-2721 was a gift of Dr David E.Davidson (Walter Reed Army Medical Center, Washington, DC, USA). All other reagents were of analytical grade.

Animals

Female NMRI mice, weighing ~25 g, were obtained from Animalerie Centrale–UCl and were used for the radioprotective study, whereas 1-month-old C57Bl/Ka male and female mice, originally obtained from the Radiobiology Department of Stanford Universtiy (USA), and bred since 1979 in the animal colony of the State University at Liège, were used for the carcinogenic study. They were housed in groups of 10 in plastic cages and received standard food and water *ad libitum*.

Animal irradiation procedures

Mice in groups of 10 were exposed to 250 kV X-rays (Phillips, RT 200/250) while restrained in perforated lucite cages placed on a rotating disc 60 cm from the target. The filtration was through 1.0 mm of copper, and the dose rate was 60 rad/min. Animals were observed over a period of 30 days, and daily mortality was recorded. When mice became lethargic, paralysed and isolated from the main group, they were killed by cervical dislocation in order to avoid unnecessary suffering. They were recorded as dead on the next day. Control and treated groups consisted of 10 female mice, and experiments were repeated at least five times.

*Abbreviations: AD-20, *ortho*-methoxyphenylacetyl dehydroalanine; DRF, dose reduction factor; mst, mean survival time; WR-2721, S-2-(3-aminopropylamino) ethylphosphorothioic acid.

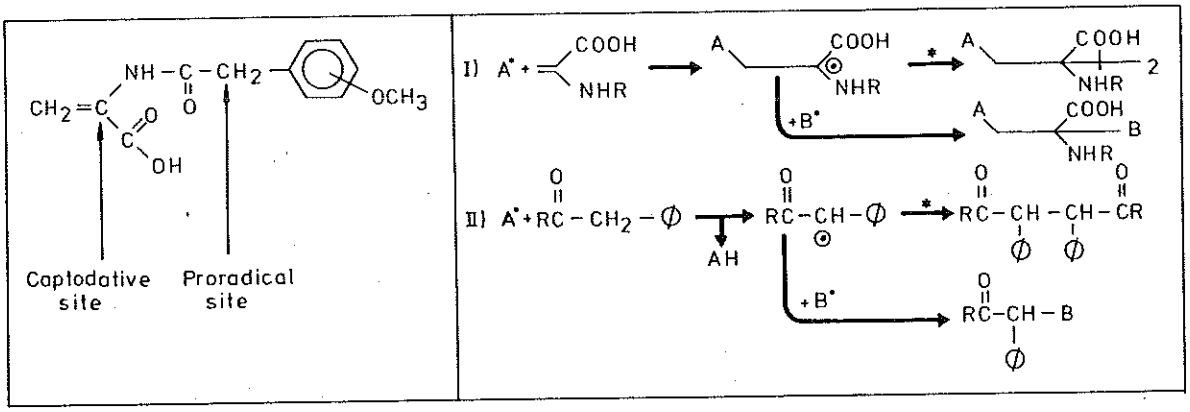

Fig. 1. Structure and possible reactions of AD compounds.

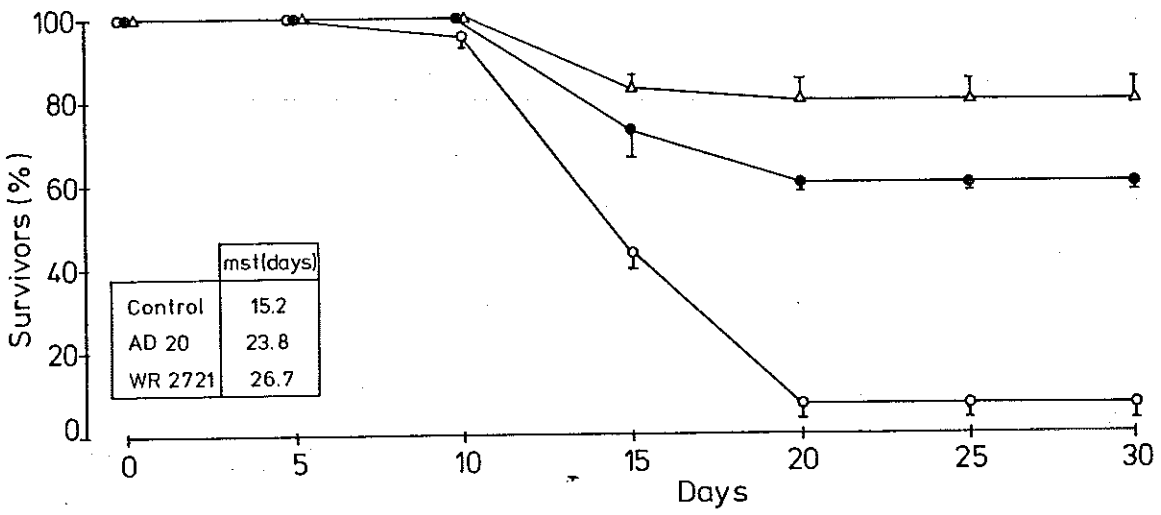

Fig. 2. Effects of AD-20 and WR-2721 on the survival of irradiated mice (700 rads). Survival rate expressed as percentage (%) is plotted against time after exposure to 700 rads of X-rays. At 15 min before irradiation NMRI mice were given an i.p. injection of either gum arabic (open circles) or 500 mg/kg body weight of AD-20 (closed circles), or 500 mg/kg of WR-2721 (open triangles). Each experimental group consisted of 10 mice. Results are means ± SD from seven separate experiments.

Animal survival data were pooled from the separate experiments.

When X-rays were applied as fractionated doses, the animal irradiation conditions were the same as described above, except that the distance from the target to the source was 40 cm, and the dose rate was 130 rad/min. The animals were killed when moribund and dissected to ascertain the presence of a thymic lymphoma.

Administration of chemicals

AD-20 was administered to the animals as a water suspension by using 2% gum arabic as vehicle. It was injected i.p. at the indicated concentration 15 min before irradiation. WR-2721 was dissolved in sterile deionized water and injected i.p. 15 min prior to irradiation.

The parameters observed were the number of survivors at the end of the experiment (day 30) and the mean survival time (mst) as described by Geran *et al.* (16).

Results

Effects of AD-20 and WR-2721 on survival of irradiated mice

Both AD-20 and WR-2721, administered i.p. at a single dose of 500 mg/kg 15 min prior to irradiation, protect mice against the toxicity of 700 rads of X-rays (Figure 2). Control mice show a typical pattern of mortality extending from 10 to 20 days, whereas treatment with either AD-20 or WR-2721 significantly enhanced the radioresistance of animals. The survival time for AD-20 and WR-2721 treated mice was 23.8 and 26.7 days, respectively, as compared to 15.2 days for non-treated mice. In addition, the number of survivors 30 days after irradiation was 60 and 80% in the AD-20 and WR-2721 groups, respectively, as compared to 6% in the control group.

Effect of AD-20 on the survival of mice irradiated with an increasing dose of X-rays

To verify the efficacy of the radioprotective activity of AD-20, its effect was tested against different doses of X-rays. Table I shows that at all the doses of X-rays used (varying from 600 to 800 rads), the number of survivors at day 30 was greater in the groups of mice treated with AD-20 than in the control mice. Treatment with AD-20 not only decreased the mortality but also increased the survival time of irradiated mice. The mean survival time for each dose of X-rays utilized was superior in animals treated with AD-20 as compared to control non-treated mice.

Effect of AD-20 on the kinetics of lymphoma appearance in irradiated mice

When C57Bl/Ka mice are exposed to fractionated doses of X-rays (4 × 175 rads at weekly intervals), thymic lymphomas appear after a latency period of 3–6 months (11). Figure 3 shows the evolution of the lymphoma formation (per cent of tumour incidence). AD-20 was given at a total dose of 400 mg/kg divided

Table I. Radioprotective effect of AD-20 on whole-body X-ray irradiated mice (600–800 rads)

Dose (rads)	mst[a] (days)		Survival[b]	
	Control	AD-20	Control	AD-20
600	24.9 ± 1.7	29.5 ± 0.3	33/50	48/50
650	18.4 ± 1.7	27.4 ± 1.2	14/50	42/50
700	13.6 ± 1.7	25.1 ± 1.8	4/50	33/50
750	11.9 ± 0.6	19.3 ± 2.3	0/50	21/50
800	11.6 ± 0.7	14.6 ± 1.5	1/50	7/50

AD-20 was administered i.p. at 500 mg/kg for NMRI female mice 15 min before irradiation.
[a]Values are expressed as days, and represent the means ± SD from five separate experiments.
[b]Number of survivors at day 30 in relation to the number of mice exposed to X-rays varying from 600 to 800 rads.

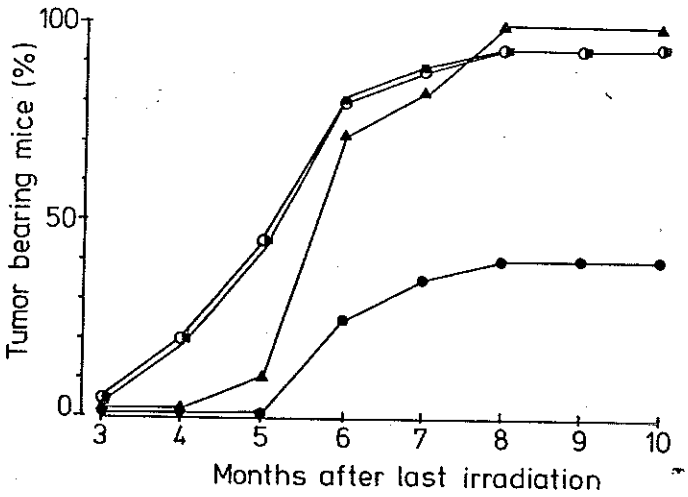

Fig. 3 Effect of AD-20 on the kinetics of radiolymphomas formation. Mice were exposed once a week to a total-body irradiation (175 rads of X-rays) during 4 weeks. AD-20 was injected 15 min before the first irradiation at 1 × 400 mg/kg (closed triangles), before the second and fourth irradiation at 2 × 200 mg/k (closed circles) and before all irradiations at 4 × 100 kg/kg (closed squares). Control mice received i.p. gum arabic (open circles). Each experimental group consisted of 10 female and 10 male C57Bl/Ka mice, except the control group which consisted of 42 mice.

as 1 × 400 (closed triangles), 2 × 200 (closed circles) and 4 × 100 (closed squares) mg/kg. At 5 months, 45% of irradiated non-treated mice (open circles) had developed thymic lymphomas. The same percentage of radio-lymphomas appeared in animals receiving AD-20 at 4 × 100 mg/kg, whereas only 10 and 0% of lymphomas was recorded in groups treated with AD-20 at 1 × 400 and 2 × 200 mg/kg, respectively. At 6 months ~80% of irradiated non-treated mice developed a tumour. After 8 months, the tumour incidence reached 93% and remained at that level. The administration of AD-20 either as a single dose of 400 mg/kg (before the first irradiation) or as 4 × 100 kg/kg (before each irradiation), did not prevent the appearance of tumours; as in the case of non-treated mice, >90% of the animals developed a tumour 8 months after irradiation. However, AD-20 administered as a fractionated dose (2 × 200 mg/kg, before the second and fourth irradiation) induced protection against the formation of lymphomas; after 6 months, only 25% of the treated mice developed tumours. This percentage had increased to only 40% after 8 months and remained constant thereafter (up to 14 months).

Discussion

Free radicals are reactive substances which interact with every type of cellular macromolecule, leading to metabolic disturbances, cell injury and even to cell death (17–19). The toxicity of ionizing radiation has been proposed to be free radical-mediated (1,10,20,21). For instance, water radiolysis produces mainly $H^{\cdot}$, hydrated electrons and $HO^{\cdot}$. These primary free radicals may interact with neighbouring macromolecules, thus impairing the integrity of their structure or functions. Reactive oxygen species may play important roles as mediators of this pleiotropic responses (10,21).

When animals are total-body exposed to ~600–800 rads of X-rays, delivered as a single dose in a short time, a bone marrow toxicity appears between 1 and 2 weeks after irradiation and, depending on age, sex, strain and nutritional state, the mortality induced is extended from 8 to 20 days after irradiation (10). Confirming our previous results, irradiated mice were protected from this lethality when receiving the capto-dative olefin AD-20 prior to irradiation (Table I). The degree of protection was of the same order of magnitude as that obtained with WR-2721 (Figure 2), even though a great difference was observed when comparing their dose reduction factor values (DRF). Indeed, for AD-20 the DRF was calculated to be 1.20 (P.Buc-Calderon and M.Roberfroid, in preparation), whereas for WR-2721, depending on the strain of mice utilized, this value varied from 2.4 to 2.7 (13). One possible explanation for this difference might be the fact that aminothiols not only act as radioprotectors through their free radical scavenging properties, but also by inducing a modification in the tissue oxygen concentration, e.g. a local hypoxia (10,33).

Genomic modification leading to mutation and cancer represents one of the most cited examples of delayed toxicity of ionizing radiation (22,23). The involvement of free radicals in such a carcinogenic process correlates well with the protective effects of free radical scavengers, as seen by the inhibition in the development of radiation-induced sarcomas by WR-2721 (15), and the significant decrease in the life-time incidence of X-ray induced leukaemia obtained by intravenous doses of superoxide dismutase (14). Nevertheless, no information about the eventual role of free radical production in the mechanism of radio-induced lymphomas has yet been published. Previous data showed that a leukaemogenic dose of irradiation induces the appearance of preneoplastic cells, called 'preleukaemic cells' within the thymus (24), and that these cells depended upon the thymic micro-environment for their progression to autonomous frank neoplastic cells, called 'leukaemic cells' (25). However, this environment itself was modified during the preleukaemic period (25). A bone marrow graft performed immediately after the last dose of X-rays inhibited the development of thymic lymphomas (11): it induced a restoration of the properties of the thymic microenvironment and simultaneously the disappearance of preleukaemic cells (26). Thus it has been proposed that modifications of the environmental conditions within the thymus are necessary for the transformation of preleukaemic cells into leukaemic cells. AD-20, when applied before the second and fourth exposure to X-rays, not only delayed the appearance but also decreased the formation of thymic lymphomas (Figure 3). Nevertheless, administered at a single dose of 400 mg/kg before the first irradiation, it delayed the formation of lymphomas but at the end of the experiment the percentage of mice bearing lymphomas was identical to that of the control group. A dose of 100 mg/kg, even if administered four times, was without effect (Figure 3). We do not have explanations concerning the effect of AD-20 as a function of the

schedule protocol applied, since other different combinations are still lacking in order to advance a more elaborate hypothesis. It seems that four doses of 100 mg/kg each of AD-20 were not enough to achieve a 'suitable local concentration' in order to inactivate the free radicals formed within the thymus and bone marrow. AD-20 at 1 × 400 mg/kg appeared to protect mice against radiation damage, but this protection was not maintained so as to hinder the effects of the other three irradiations. In that sense, the dose of AD-20 at 2 × 200 mg/kg appears to be in the middle of both situations, thus suggesting one possible explanation for its protective effect.

In other protocols of carcinogenesis, such as the two-stage model for skin cancer (27–29), ionizing radiation, in addition to its weak initiating activity, was reported to be active in malignant progression (30). Since in one experimental skin carcinogenesis protocol AD compounds were shown to decrease the appearance of skin carcinomas, but not early papillomas (T.K. Vo, personal communication), an hypothesis can be proposed to explain the mechanism of formation of radio-induced lymphomas and the protective role of AD-20 in this model. Free radicals, by modifying the DNA, could be involved in the process of preleukaemic cells induction (initiators?) and by inducing a modification in cell homeostasis (oxidative stress, lipid peroxidation, etc.) could generate the environmental conditions leading to tumour formation (promoting effect?). Thus, AD-20 by its scavenging ability, could reduce the intensity of the initiation of the malignant process and/or modify the environmental conditions necessary for tumor formation. This aspect is at present under investigation.

Ionizing radiation might impair the oxygen metabolism leading to a metabolic amplification of the initial physicochemical damage, possibly via increased lipid peroxidation (31), thus suggesting that radiation damage progresses during the post-irradiation period, long after the primary and secondary radiation chemical species have disappeared (32). Since AD compounds were reported to scavenge oxygen-derived free radicals (5), to inhibit *in vitro* rat liver microsomal lipid peroxidation initiated by gamma rays (6), and AD-20 in particular, to increase the $LD_{50(30)}$ of X-rays from 610 to 730 rads (P.Buc-Calderon and M.Roberfroid, in preparation), we postulate that AD-20 interferes with the radical chain reactions such as lipid peroxidation by inactivating the oxygen-derived free radicals, and in this way it may act as a radioprotective and anticarcinogenic agent.

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