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To find a suitable radioprotective agent is important to reduce side effects induced by ionizing irradiation and increasing survival rate in patients during radiotherapy.

Foundation review: Trends in the development of radioprotective agents

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People may be exposed to ionizing radiation during radiotherapy or following exposure to radionuclides in nuclear medicine. Radioprotective agents have been used to reduce morbidity or mortality produced by ionizing irradiation. Early developments of such agents focused on thiol synthetic compounds, such as amifostine. This compound reduced mortality; however, there were difficulties in administering aminothiols that led to adverse effects. Hence, the development of radioprotective agents with lower toxicity and an extended window of protection has attracted much attention. Natural compounds have been evaluated as radioprotectants and they seem to exert their effect through antioxidant and immunostimulant activities. Although recent agents have lower efficacy, they have lower toxicity, more favourable administration routes and improved pharmacokinetics compared to the older thiol compounds.

Introduction

The aim of radiotherapy is to destroy cancer cells with as little damage as possible to normal cells. Radiation causes damage to the DNA of cells; the basic principle of radiotherapy is to cause enough damage to kill cancer cells. If the cells cannot repair their DNA, they cannot grow or reproduce. However, radiation causes damage to normal cells and, hence, can result in adverse side effects. The nature and degree of such unwanted side effects depends upon the dose of ionizing radiation and the sensitivity of the organs that are irradiated. With respect to the potential application of ionizing radiation in medical practices (e.g. radiotherapy and nuclear medicine) and also potential accidental exposure to radiation (e.g. industrial nuclear accident), the development of effective radiomodifiers is of great medical importance. Radioprotective agents are synthetic compounds or natural products that are immediately administrated before irradiation to reduce injuries caused by ionizing radiation. Over the past 60 years, as a result of the great clinical need for effective radioprotectant agents, many have been prepared and tested to find more effective, less toxic, drugs. Initial attempts were focused on synthetic thiol compounds. These agents are highly effective at reducing lethality induced by irradiation. Of this class, amifostine is the only radioprotector that has been clinically approved by the Food and Drug Administration (FDA) for mitigating side effects (xerostomia) in patients undergoing radiotherapy [1,2]. This drug offers good protection, but is relatively toxic (nausea, vomiting and hypotension being some of the most common adverse effects) [3]. In view of this, the search

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continues for less toxic, more effective radioprotectors that can be easily self-administered. In recent years, radioprotective agents with a novel mode of action have been investigated; in particular, compounds that can affect haematopoietic stem cell regeneration have attracted significant interest. The aim of this strategy is to increase survival rate by stimulating the function and regeneration of the stem cell population that is decreased, due to radiation-induced damage [4,5]. Immunomodulators and cytokines represent the bulk of agents in this category. Naturally occurring compounds that function as antioxidants and immunostimulants are another strategy for the development of radioprotective agents with low toxicity. Therapeutic agents that can be administered following irradiation are another strategy for reducing side effects induced by ionizing radiation; cytokines and immunomodulators, through induction of bone marrow recovery and extrahaematological tissue regeneration can represent such a class of agent.

This review highlights the past, present and future strategies relevant to the development of radioprotectors. It also discusses the advantages as well as the disadvantages for the application of these agents.

Radiation injury

Ionizing radiation can be defined as any type of electromagnetic (such as X- or gamma rays) or particle radiation (such as neutron or alpha particles) with sufficient energy to ionize atoms or molecules; that is, to eject electrons from their outer orbitals. Ionizing radiation passing through living tissues generates reactive free radicals. These free radicals can interact with critical macromolecules, such as DNA, proteins or membranes, and can induce cell damage and, potentially, cell dysfunction and death. Damage to DNA may be the most important factor in cell death [6]. When DNA is damaged, it is followed by altered cell division, cell death, depletion of stem cell pools, organ system dysfunction and, if the radiation dose is sufficiently high, the organism will die. Although cells and tissues are equipped with endogenous enzymes (e.g. superoxide dismutase) capable of the detoxification and removal of the products of water radiolysis, when these reactive oxygen species increase in the biological system following exposure to irradiation, the endogenous system is incapable of protecting cells from the hazardous effects of free radicals. Exposure to high amounts of ionizing radiation results in damage to the haematopoietic, gastrointestinal or central nervous systems, depending on radiation dose [7].

Because the haematopoietic system has a high level of cell turnover, it is among the most radiosensitive tissues in the body. The cells are affected and suppressed at relatively low doses of acute irradiation. Exposure to ionizing radiation induces a dose-dependent decline in circulating haematopoietic cells, not only through reducing bone marrow cell production, but also by redistribution and apoptosis of mature cells [4,8,9]. In general, the decline in lymphocytes and granulocytes occurs over a period of hours or days after irradiation. Platelets also decline over a period of days, consistent with their half-life [10]. The primary cause of mortality during the early phases of radiation-induced haematopoietic syndrome is sepsis, resulting from opportunistic infection, due to low numbers of neutrophils and increased translocation of bacteria across the gastrointestinal mucosa. This is complicated by thrombocytopenia and concomitant haemorrhage and defects in the adaptive immune system resulting from apoptosis of lymphocytes and deficient lymphopoiesis [4]. Gastrointestinal syndrome is induced by a higher irradiation dose compared to haematopoietic syndrome. In this syndrome, the gastrointestinal barrier is damaged and high amounts of water and electrolytes are lost from the body, resulting in dehydration and bacteremia. Although exposure to high doses of radiation causes mortality, it has long been known that radiation can induce a broad spectrum of DNA lesions, including damage to nucleotide bases, cross-linkage, and DNA single- and double-strand breaks. It is now accepted, however, that inappropriately repaired DNA breaks are the principle lesions of importance in the induction of both chromosomal abnormalities and gene mutations and cancer [11].

Radioprotectors

Since exposure to irradiation in radiotherapy, or accidental exposure to radiation, can produce significant unwanted side effects, it is important to ameliorate such effects by the use of radioprotective drugs [12].

The ideal radioprotective agent should fulfill several criteria:

(a) It must provide significant protection against the effects of radiation.
(b) It must have a general protective effect on the majority of organs.
(c) It must have an acceptable route of administration (preferably oral, or alternatively intramuscular).
(d) It must have an acceptable toxicity profile and protective time-window effect.
(e) It must have an acceptable stability profile (both of bulk active product and formulated compound).
(f) Have compatibility with the wide range of other drugs that will be available to patients or personnel.

Unfortunately, to date, there is no radioprotector that fulfills all of these criteria. Although the initial development of radioprotective agents led to the discovery of effective, synthetic thiol compounds, as previously mentioned, the side effect profile of these agents necessitated the search for second-generation drugs that are more effective, less toxic and with more acceptable properties with respect to route and frequency of administration. Many researches appear to use the intraperitoneal (i.p.) route of administration in their studies, since it is technically easy and optimises exposure of the compound (the i.p. route having similar pharmacokinetics to i.v.). In reality, however, such drugs will never be administered i.p. to humans. The only likely acceptable routes for human dosage would be oral, subcutaneous (s.c.) and intramuscular (i.m.). In the mouse model, s.c. is often preferred because of the lack of i.m. injection sites, while in the non-human primate the i.m. route is more readily accessible. In recent years, an array of immunomodulatory agents, haemopoietic growth and stimulating factors, synthetic chelating agents and natural antioxidants have been examined for their ability to ameliorate radiation-induced damage.

Mechanisms of action of radioprotective agents

Ionizing radiation interacting with water in cells can produce reactive free radicals, such as hydroxyl radicals, hydrogen radicals and the toxic substance, hydrogen peroxide, all of which can damage critical macromolecules. The elimination of the free radical

species from the cell environment can inhibit the side effects induced by irradiation. The presence of sulfhydryl groups or other molecules capable of scavenging the radiolysis radicals arising from irradiation of water molecules can confer protection to radiation. Due to very short life of the radicals, such protective agents need to be present in the cell environment before the production of free radicals in order to neutralize their destructive properties.

Increasing the partial pressure of oxygen in the cell environment sensitizes tissues to radiation. Radioprotective agents induce hypoxia and consumption of oxygen in the cells to decrease the levels of reactive oxygen species (ROS) and hydrogen peroxide. The aminothiols can decrease oxygen levels in the cells. Thiol radioprotectors consume oxygen by forming byproducts, such as disulfide and hydrogen peroxide [13]. The mode of action (MOA) of another class of radioprotectant agents involves the stimulation, proliferation and modification of the function of haematopoietic and immunopoietic stem cells. This class of agents is commonly referred to as immunomodulators. These agents make the stimulated cells release a variety of cytokines that act on pluripotent bone marrow stem cell to stimulate their production and differentiation. These agents mitigate radiation-induced haematopoietic injury and reduce mortality [4]. Cytokines activate cellular signalling transduction pathways by binding to high-affinity membrane receptors. Activation of cytokine cascades results in the release of intracellular protein messenger. Cytokine activation affects many cell functions, including growth, proliferation, differentiation, death caused by apoptosis and growth inhibition. The consequence of these effects depends on the type of released cytokines. Primarily, cytokines affecting the proliferation and differentiation of haematopoietic cells include GM-CSF, CSF, EPO, G-CSF, TPO and interleukins [14–16].

Radioprotectors

Thiol and synthetic radioprotectors

Thiols are molecules containing free or potential sulfhydryl (SH) groups in their structure; they received a great deal of attention, from 1950 to 1985, as radioprotectants of mammalian cells. They were the first generation of radioprotectors. Aminothiols, and their phosphothioate derivatives, have been investigated as tissue radioprotectors over the past four decades [1]. Several mechanisms were proposed for this group, including free radical scavenging, hydrogen transfer, inducing hypoxia and stabilizing DNA through direct binding [2,12,13]. Approximately, 4400 compounds had been developed and tested by 1973. One of the most effective drugs developed was WR-2721 or amifostine [s-2(3-aminopropylamino)ethyl phosphorothioic acid] which is a prodrug, in which the thioester bond is cleaved by membrane-bound alkaline phosphatase, yielding a free active thiol, the active metabolite WR-1065. Amifostine has been used in clinical trials and it protects normal tissue from the acute and long-term effects of radiation and chemotherapy. It has been approved by FDA as a radioprotector and chemoprotector [2]. This drug is more effective in reducing radiation-induced cellular injury of normal tissues than in tumour cells. Alkaline phosphatase is needed to convert WR-2721 to its active metabolite which produces higher concentrations in normal cells [17–19]. WR-2721 has been reported to reduce the effect of a radiation dose by a factor of up to 2.7 in mice taking this drug intraperitoneally 30 min before exposure to gamma irradiation. This is the highest dose reduction factor (DRF) seen in a mouse 30-day lethality model [1]. The i.v. administration of amifostine 200–350 mg/m^2 for 30 min before each radiotherapy fraction is the commonly recommended schedule for radioprotection [3,18,19]. Administration by the i.v. route requires the availability of a day clinic provided with a radiotherapy unit and a specialized nurse to treat the side effects related to amifostine infusion, such as acute hypotension, sever nausea, vomiting and allergy [3]. Consequently, the problems associated with the application of amifostine by the i.v. route limits its use in patients. Alternatively, amifostine administration by the s.c. route [2,3] has been described.

Subcutaneous administration of amifostine has several potential advantages over i.v. administration, peak plasma levels associated with side effects (hypotension, nausea) may be lower after s.c. administration because of slower release into the blood stream, and s.c. administration is easier to perform routinely in the clinic [2,3,20]. Subcutaneous administration of amifostine is well tolerated and to a great extent prevents the early toxicity associated with therapy and prevents delays in radiotherapy. The s.c. route is much simpler and saves time, compared with the intravenous route, and can be safely and effectively applied within the busy activities of daily radiotherapy practice [3,21–23]. The major adverse effect of the s.c. route was nausea and cutaneous reactions [23,24].

The subcutaneous implantation of a biodegradable pellet containing a slow-release formulation of amifostine was evaluated as a drug delivery system for radioprotection in mice (Table 1). The amifostine pellet group had a sustained blood level of WR-1065

TABLE 1

Characteristics of radioprotective agents studied for protection against lethality induced by gamma irradiation

Group	Agent	LD50 or MTD[a] (mg/kg)	Dosage[b] (mg/kg)	Route[c]	Window[d]	Duration administration[e]	Dose[f] IR (Gy)	DRF[g]	% Survival (treat)[h]	% Survival (control)[i]	Reference
Synthetic	WR-2721	920	365	i.p.	0.5	Single	–	2.29	–	–	[1]
	WR-2721 pellets	na	3× 18 mg	s.c. implant	2	Single	–	1.79			[22]
	WR-3689	1120	450	i.p.	0.5	Single	–	2.22	–	–	[1]
	WR-151327	630 (MTD)	200	i.p.	0.5	Single	–	1.46	–	–	[26,1]
	Kojic acid	>1400	142	s.c.	24	Single	8.2	–	63	0	[43]
	Cystein	2000	1000	i.p.	0.16	Single	–	1.4	–	–	[25]
	Mercaptopropionylglycine (MPG)	na	20	i.p.	0.25	Single	–	1.1	–	–	[25]

TABLE 1 (*Continued*)

Group	Agent	LD50 or MTD[a] (mg/kg)	Dosage[b] (mg/kg)	Route[c]	Window[d]	Duration administration[e]	Dose[f] IR (Gy)	DRF[g]	% Survival (treat)[h]	% Survival (control)[i]	Reference
	5-HT (5-hydroxytryptamine)	160 (i.v.)	40	i.p.	0.08	Single	–	1.7	–	–	[25,92]
	Tempol-H (nitroxide)	325 (MTD)	325	i.p.	0.16	Single	–	1.3	–	–	[28]
	Oltipraz	na	100	p.o.	0.5	Two days	8	–	91	48	[93,94]
	Edaravone	na	480	i.p.	0.5	Single	–	1.3	–	–	[95]
	Chromone-thiazolidine	790	395	i.p.	0.5	Single	–	1.48	–	–	[96]
	Germathiazolidine	300	150	i.p.	1.5	Single	8.1	–	70	0	[97]
	Diltiazem	na	100	i.p.	na	na	8	1.25	82.5	0	[98]
	Nimodipine	na	10	i.p.	0.5	Single	–	1.19	–	–	[26]
	Dihydroxychromone	>1500	222	s.c.	24	Single	8.2	–	52	0	[99]
	WR-151327	na	450	p.o.	na	Single	–	1.2	–	–	[100]
Immunomodulator	Ginsan	na	100	i.p.	24	Single	–	1.45	–	–	[55]
	Palmitoylated peptide IL-1β	na	80 μg/kg	s.c.	24	Single	–	1.07	–	–	[51]
	5-AED	>4000	160	s.c.	24	Single	–	1.26	–	–	[16,4]
	5-AED	>4800	1600	p.o.	24	Single	11	–	60	5	[16]
	Progenipoietin	na	100 μg/ mice	s.c.	24	Single	2× 5.5	–	100	0	[52]
	Oxymetholone	>5000	640	p.o.	24	Single	8	1.14	75	15	[8]
	genistein	na	200	s.c.	24	Single	9.5	1.16	91	15	[5]
	Glucan	na	20	i.v.	0.16	Single	–	1.4	–	–	[25]
Natural	α-TMG	1120	600	i.p.	0.16	Single	–	1.09	–	–	[62,63]
	Vitamin E	na	400 IU/kg	s.c.	24	Single	10.5	1.23	80	4	[60,61]
	Menthe arvensis	>1000	10	i.p.	1	Five days	–	1.2	–	–	[82]
	Ginger rhizome	500	10	i.p.	1	Five days	–	1.15	–	–	[80]
	Triphala	240	10	i.p.	1	Five days	10	–	58.3	4	[89]
	Hippophae rhamnoides	na	30	i.p.	0.5	Single	10	–	82	0	[86]
	Mangifera indica	400	2	i.p.	1	Single	10		50	0	[91]
	Pilea microphylla	1661	900	i.p.	0.5	Single	–	1.2	–	–	[101]
	Abana	1800	20	i.p.	1	Five days	10		62	3	[102]
	Abana	na	20	i.p.	1	Five days	–	1.2	–	–	[103]
	Panax ginseng	1200	10	i.p.	0.5	Four days	8		80	30	[83]
	Mentha piperita	na	40 μoil/mice	p.o.	0.5	Three days	8		83	0	[84]
	Tinospora cordifolia	na	10	p.o.	1	Seven days	8	–	33	0	[85]
	Aegle marmelos	1750	15	i.p.	1	Five days	–	1.15	–	–	[87]
	Amaranthus paniculatus	na	800	p.o.	24	Fifteen days	–	1.36	–	–	[88]
	Ageratum conyzoides	3000	75	i.p.	0.5	Single	–	1.3	–	–	[90]
	Liv 52 (herbal)	na	500	p.o.	1	Seven days	–	1.2	–	–	[104]
	Vicenin	>100	50 (μg/kg)	i.p.	0.5	Single	–	1.37	–	–	[105]
	Myristica fragrans	na	10	p.o.	0.5	Three days	–	1.3	–	–	[106]
	Emblica officinalis	na	100	p.o.	0.5	Seven days	9	–	87.5	0	[107]
	Septilin	1250	100	i.p.	1	Five days	10	–	58.3	0	[108]
	Tinospora cordifolia	500 (MTD)	200	i.p.	1	Single	10	–	76	0	[109]

na: not reported.

[a] MTD: maximum tolerated dose is lethal dose for 10% of mice in 30 days [1].
[b] Amount of drug administrated in mg/kg or other units to animal.
[c] The route of drug delivered to animal: s.c. = subcutaneous injection, p.o. = oral gavage, i.v. = intravenous injection, s.c.-implant = slow-release amifostine implanted subcutaneously.
[d] Pre-irradiation period (in hours) in which the applied agent (– = pre-irradiation).
[e] Frequency of time that agent administrated.
[f] Dose of gamma irradiation exposed to animal.
[g] DRF = dose reduction factor, as estimated by the ratios of LD50/30 values for the agent treated versus vehicle-treated animals.
[h] Percentage of surviving animals within treated group.
[i] Percentage of surviving animals within control group.

two hours after implantation, in contrast to the sharp peak of concentration seen at 30 min for a group receiving amifostine by the s.c. route. Locomotory activity was significantly reduced in the amifostine pellet group; however, the onset of the locomotor deficit was delayed, compared with groups receiving amifostine by the s.c. route. Such a slow-release drug delivery formulation had the advantages of reduced toxicity and an extended time-window for protection [22]. This drug delivery system may be suitable for protecting people from imminent nuclear attack. Additional work in this area is still needed to improve formulation design and to evaluate the pharmacokinetics of this route in patients.

Radioprotectant combination therapy has been tested in an attempt to increase protection against radiation damage and/or to decrease the toxicity through the use of mixtures of chemical and biological protectants. The combination most frequently studied was immunomodulators and thiol compounds [25,17]. An additive effect was obtained upon combination of 16,16-dimethyl PGE2 and WR-2721. When PGE2 was combined with a dose of WR-2721 (200 mg/kg), the protection increased synergistically to a maximal DRF of 2.5 [25]. The addition of nimodipine (10 mg/kg), as a calcium antagonist, to WR-151327 (200 mg/kg) produced additive radioprotective effects from 1.46 to 1.67, and increased the magnitude and duration of the locomotory deficit, compared with WR-151327 alone [26]. Recently, phosphonol (WR-3689) has been tested for its ability to act as radioprotectants. Phosphonol is similar to amifostine in mechanism effects and capacities, but suffers from a similar spectrum of toxicities; however, it seems to be slightly less toxic and have marginally better bioavailability when delivered orally [27].

Despite amifostine's current clinical applications, it has not been approved for use in any clinical nuclear/radiological exposure setting. The disadvantages of amifostine are as follows: toxicity; limited routes of administration; narrow time windows; cost and limited protection of the central nervous system.

Nitroxides

A series of free, stable nitroxides have been prepared and tested as radioprotectors. The main mechanisms are thought to be a free radical scavenging, superoxide dismutase-like activity. Tempol [4-hydroxy-2,3,6,6-tetramethyl piperidine-1-oxyl] is the lead compound in this group. Nitroxide had a differential protection for normal tissue (bone marrow) compared to tumour tissue [28]. The reduced form of tempol, tempol-H (the hydroxylamine), did not demonstrate aerobic radioprotection. Tempol-H provided protection against the lethality of whole-body radiation in mice with a dose modification factor of 1.3, which was similar to tempol [28]. Administration of tempol, before irradiation, significantly reduced radiation-induced salivary hypofunction in mice [29,30]. Tempol had a significant effect on producing hypotension and increasing heart rate at the doses required to produce radioprotection. It also has a short time-window of effect [28]. These negative attributes of nitroxides clearly limit their usefulness for clinical applications. Recently, there have been some studies carried out on tempol-H to find whether it is well tolerated and less toxic: should this goal be achieved, this compound may be considered a potential candidate for clinical applications to protect patients undergoing radiotherapy. Tempol-H is not expected to lead to a significant drop in blood pressure and seems to be associated with lower haemodynamic toxicity [28].

Bis-benzimidazol

The bis-benzimidazol family has two benzimidazole groups and one phenol group, conferring minor DNA groove-binding properties. These compounds fluoresce strongly upon binding to dsDNA and have been marketed by Hoechst as reagents for the *in vitro* estimation of DNA concentration and for histological applications. Hoechst 33342 is the most well-known compound from this family; it binds strongly and selectively to double-stranded DNA but not to double-stranded RNA [31]. The mechanism of radioprotective activity of Hoechst 33342 is to donate an electron from the ligand to damaged DNA [31,32]. Intravenous administration of Hoechst 33342 (70 mg/kg) 30 min before irradiation results in a significant radioprotective effect with DMF of 1.2 in a mouse lung model [33].

The methylproamine analogue of Hoechst 33342 showed *in vitro* radioprotective properties and was up to 100 times as potent as WR-1065 in V79 cells [31]. The DNA-bound methylproamine ligand acts as a reducing agent, through an electron transfer mechanism, repairing transient radiation-induced oxidizing species on DNA. Topical application may provide a means of delivery with minimal systemic uptake. Martin *et al.* proposed that methylproamine has a potential use as a topical preparation of a radioprotector [31], but additional studies need to evaluate the efficacy and toxicity of methylproamine in animals.

Superoxide dismutase and metal complexes

Superoxide dismutase (SOD) enzymes are naturally occurring intracellular enzymes which scavenge O_2^- by catalyzing its conversion to hydrogen peroxide and oxygen. It has become clear that these enzymes provide an essential defence against the superoxide radical. The copper-, zinc- and manganese-containing SODs (Cu, Zn, Mn and SOD) are the most common type of SOD [34,35]. A pharmaceutical version of a copper–zinc-containing SOD has been marketed under the name of Orgotein, which has been used for ameliorating radiation side effects in patients [36–38]. Intraoral administration of a manganese superoxide dismutase-plasmid/liposome (Mn SOD-PL) 24 hours before a single-fraction 30 Gy irradiation prevented oral cavity mucusitis in mice [36]. The study showed that Mn SOD gene therapy did not protect orthotopic tumours from radiation damage. Also intraoesophageal injection of SOD-PL, before irradiation, mediated a significant decrease in apoptosis [36]. Intratracheal injection of Mn SOD-PL protected normal lung, but not orthotopic tumours, from irradiation. Mice receiving a Mn SOD-PL complex, followed by irradiation with 18 Gy, showed prolonged survival [34]. Thus, Mn SOD-PL administration significantly improved tolerance to the oral side effects induced by radiation [38–41]. More studies are needed to evaluate the efficacy and toxicity of SOD-PL in animal by systemic administration. In addition, more studies will be required to find SOD-related therapies with a greater half-life of action. The limitations for these agents are their short half-life, large molecular weight and potential immunogenicity [35,41]. To overcome these limitations, there has been a considerable interest in developing synthetic SOD mimics that have long half-life, low molecular weight, reduced toxicity and cost-effectiveness.

A group of synthetic SOD mimetic compounds has been developed to be used for ameliorating radiation-induced tissue injury. These agents have a metal ion (Cu, Fe, Mn and Zn) at their active centres to form chelates, which behave like the metal centre of native SOD. Manganese complexes of kojic acid and 7-hydroxy flavone showed potent SOD activity *in vitro*. The SOD activity of the Mn complex of kojic acid was 10,000 times as potent as kojic acid itself [42]. In studies performed in our laboratory, we treated mice with Mn and Zn complexes of kojic acid and amifostine (1 mM/kg) before the lethal dose of gamma irradiation. The complexes of kojic acid significantly reduced mortality induced by radiation. The percentage survival was as follows: vehicle 0%; kojic acid–Mn complex 50%; kojic acid–Zn complex 38% and amifostine 81%. The complexes had higher LD_{50} values compared to amifostine and they were effective when administrated 24 hours before gamma irradiation. An extended window effect has been one of the major advantages of these complexes [43].

Administration of Cu(II)2 (3,5-diisopropyl salicylate)4 (3,5-DIPS) complex to mice, at a dose of 80 μmol/kg 24 hours before irradiation, produced 58% survival rat at a lethal whole-body dose of gamma radiation [44]. Sorenson *et al.* showed that Cu(II)2 (3,5-DIPS) i.p. at a dose of 10 μmol/kg, either before or after LD50/30 irradiation, produced survival rates of 60 and 75%, respectively, in rats versus the survival rate of 40% for vehicle-treated rats [44]. Daily administration of the SOD mimic, AEOL 10113 [manganese(III) mesotetrakis (*N*-ethyl pyridinium-2-yl)] porphyrin for five days at a dose of 6 mg/kg, which began 15 min before irradiation, increased the tolerance of lung to ionizing radiation and it significantly reduced the severity of radiation-induced lung injury [35]. Because of the short half-life of protection of the aminothiols and native biological compounds, these complexes have the advantage of an extended time-window of radioprotection (~24 hours). It is clear that further research is needed in order to assess the toxicity and pharmacokinetics of these agents in animals.

Cytokines

Ionizing radiation affects haematopoietic tissues and reduces the neutrophil and platelet numbers. Reduction in these circulating blood cells can result in septicaemia, haemorrhage, anaemia and death. One of the strategies for novel radioprotective agents is the stimulation, maintenance and proliferation of progenitor cells from bone marrow. Cytokines can stimulate haematopoietic stem cells. Combination treatment with stem cell factor (SCF) and thrombopoietin (TPO) synergistically protected $CD34^+$ CFU-megakaryocytes against X-ray-induced death [45]. We showed that SCF upregulated ERK-dependent expression in cells and induced radioresistance [46]. It is essential that, in order for cytokines to exert their effects, stem cell populations must not die completely. Haematopoietic recovery depends on the percentage of residual haematopoietic stem cells. The higher the radiation dose is, the weaker the efficacy of haematopoietic growth factors is. Thus, the use of haematopoietic growth factors should be restricted to the range of intermediate radiation doses [47].

Granulocytes, lymphocytes and platelets are the most important cells to be reconstituted, because these cells have a short half-life in circulating blood and are reduced rapidly after exposure to irradiation. As a result of this, there will be a rapid onset of infection and thrombocytopenia following exposure to radiation [47]. Treatment of human peripheral mononuclear cells with IL-3 and SCF prevents apoptosis induced by gamma irradiation *in vitro* [48]. Administration of a single dose of FLT-3 ligand (SCF) resulted in a significant survival rate (65%) in irradiated mice [49].

Administration of IL-1 (100 ng), IL-6 (200 ng) or IL-1 + IL-6 to mice 20 hours before exposure to 9.5 Gy resulted in survival rates of 76.9, 71.4 and 84.6%, respectively, compared to 13.3% in control animals [50]. Progenipoietin-1 (ProGP), a dual agonist of Flt-3 and G-CSF receptors, has the capacity to mobilize large numbers of haematopoietic stem cells into the peripheral blood. When mice received a single subcutaneous injection (100 μg) of ProGP, 24 hours before a split dose of 11 Gy, 100% of ProGp-treated mice survived for up to nine months. By contrast, all mice in the control group died within 23 days of exposure to irradiation. Within three days of irradiation, neutrophil and total mononuclear cell numbers markedly decreased in treatment and control group, however, in ProGP-treated mice, these cells' populations recovered rapidly, approaching near normal levels by day 17 post-irradiation. The number of platelets and haemoglobin levels were significantly increased those receiving ProGP [51]. Administration of IL-1 (0.1 μg per mouse) i.p. 20 hours before exposure to 9.5 Gy of gamma irradiation, resulted in an 80% survival rate versus 0% in the control group, with a DRF of 1.25 [52]. It is considered as a particularly important protection agent, because only very small doses were required. The effectiveness of cytokines as regeneration agents is increased when combined with other cytokines. While IL-1 had a radioprotective effect, it produced inflammatory responses which restricted its clinical application as a radioprotector. Proteolytic cleavage of IL-1 produces a nonapeptide. The IL-1 nonapeptide was unable to illicit inflammation-related effects, but it could mimic the radioprotective capacity of IL-1, although with a lower potency [53]. Treatment of mice with the palmitoylated nonapeptide of IL-6 (80 μg/kg), at 24 hours before gamma irradiation, showed a DRF of 1.07. The protective effects of palmitoylated peptide were not observed when administrated after irradiation. This agent increased red blood cells and platelets, but did not improve the recovery of neutrophils, monocytes and lymphocytes. Since this molecule did not produce severe side effect, it may be a useful starting point for the design of new peptides with more efficacy [53]. Combination protocols with different cytokines have been shown to enhance neutrophil and platelet recovery after irradiation and enhance the survival rate in irradiated animals [14,16]. Unfortunately, some cytokines have disadvantages that limit their use in clinical practice. Also, some of them have adverse side effects, such as proinflammatory activity or immunogenicity. These agents have been proved to be infective when administrated systemically.

Immunomodulators

Some agents are able to induce haematopoietic cytokines; they are referred to as immunomodulators. Immunomodulators are non-cytokine drugs that have been proposed as an alternative to stimulate haematopoietic stem cells. The release of cytokines through the effect of immunomodulators can stimulate growth, differentiation and proliferation of haematopoietic progenitor and stem cells. In this way, this agent may protect and repair through enhanced production of bone marrow cells, circulating granulocytes, lymphocytes and platelets [12].

β-Glucans are water-soluble polysaccharides that are purported to have immunopharmacological activity, they seem to act particularly as biological response modifiers, regulating host immune response. Glucan administration increased the number of endogenous pluripotent haematopoietic stem cells that had been depleted by irradiation in mice [54]. Administration of glucan (20 mg/kg) 10 min before gamma irradiation resulted in a DRF of 1.4, compared to a DRF of 2.2 for WR-2721 (400 mg/kg). Combination of glucan (10 mg/kg) and WR-2721 (100 mg/kg) produced a DRF of 1.7 [25]. Polysaccharide-induced stimulation of macrophage and granulocyte production would appear to be the main mechanism of glucan action [25].

Ginsan is a polysaccharide extracted from the roots of panax ginseng that stimulates the endogenous production of cytokines, such as IL-1 and IL-6. When mice received ginsan at a dose of 100 mg/kg up to 24 hours before gamma irradiation, the survival rate significantly increased, with a DRF of 1.45. Ginsan increased the number of bone marrow cells, spleen cells, GM-CFC, circulating neutrophils, lymphocytes and platelets in irradiated mice [55]. More studies are needed to evaluate the systemic toxicity, such as inflammatory response and efficacy of ginsan with oral delivery. It has been noted that aminothiols with low dose and less toxicity have more efficacy compared to immunomodulators.

Good protection with oxymetholone, as an anabolic-androgenic steroid, was observed when it was administrated orally 24 hours before 8 Gy gamma irradiation in mice. The survival rate, 30 days after irradiation, in the group treated with 640 mg/kg of oxymetholone was 75%, with a DRF of 1.14, versus 15% in the control group (Table 1). Oral administration of oxymetholone ameliorated the radiation-induced decrease in circulating platelets and erythrocytes, but had less of an effect on the number of white blood cells (WBC). This drug has advantages, such as: it can be administered orally; it has an extended window of effect and is less toxic [8]. Further studies are needed to establish the exact mode of action of oxymetholone.

Recently Whitnall *et al.* developed 5-androstendiol (AED) as a new radioprotector [56,57]. AED is a natural hormone (a dehydroepiandrosterone derivative) produced in the reticularis of the adrenal cortex. It stimulates cytokines, such as IL-1, IL-3, IL-6 and facilitates recovery from radiation-induced haemopoietic injury. Administration of AED stimulated myelopoiesis and enhanced circulating neutrophil and platelet numbers, but not red blood cells (RBC). The number of granulocyte–monocyte progenitors in bone marrow increased with AED treatment. Subcutaneous injection of AED (160 mg/kg), 24 hours before gamma irradiation, significantly improved survival rate, with a DRF of 1.26 [4]. Oral administration of AED (1600 mg/kg), 24 hours before irradiation (11 Gy), enhanced the survival rate up to 60% versus 10% in the control group (Table 1) [16]. AED had low toxicity with respect to histopathology and clinical chemistry [16]. AED affects myelopoietic tissue and increases the number and function of neutrophils, monocytes and NK cells and indirectly acts as an antimicrobial agent. It also enhances resistance to potentially fatal infection, which can occur following exposure to ionizing radiation [4]. Different schedules were studied for pharmacological and preventive effects of AED for severe neutropenia, thrombocytopenia and anaemia induced by irradiation in rhesus monkeys. AED was administered three to four hours after irradiation with a 4 Gy dose. Administration (i.m.) on five consecutive days, of a 15 mg/kg micro-particle preparation, provided the greatest radiation protection [56]. Injection site reactions were observed over 48 hours with s.c. administration, but not for the intramuscular injection [56]. The survival rate of rhesus macaques receiving a lethal dose of irradiation was evaluated by administration of AED. AED was administrated intramuscularly two to four hours after a lethal dose of irradiation and was continued for five days consecutively at a dose of 15 mg/kg. This protocol significantly reduced the number of deaths in treated monkeys. Five of the 40 (12.5%) treated animals died, compared to 13 of 40 (32.5%) of the animals in the control group. This study showed that the primary cause of death in the majority of irradiated primates was thrombocytopenia, and that the secondary complication was sepsis following opportunistic infection [57]. Although AED has low toxicity and potent radioprotective effects, making this compound an interesting candidate for a radioprotective agent, it has some problems, which include low oral efficacy and local inflammatory responses at the site of injection. It is noticed that s.c. injection is not suitable as a safe and simple route for routine use in the public. Oral delivery and intramuscular injection are more suitable routes than i.v. and s.c. injection. Dehydroepiandrosterone, and its derivatives, are potent inhibitors of glucose-6-phosphate dehydrogenase (G6PD). Inhibition of G6PD activity by these agents induced growth arrest, favism disease and decreased DNA synthesis [58].

Natural antioxidants

Natural compounds in the diet provide functional antioxidants, such as vitamins, minerals and enzymes. Reduction of oxidation damage by such natural antioxidants provides a degree of protection against ionizing radiation injury.

Although thiol synthetic compounds such as WR-2721 showed good radioprotection, their toxicity at optimum protective doses promoted the search for alternatives to synthetic compounds that would be less toxic and highly effective. In general, natural radioprotectors have a lower degree of protection compared to synthetic thiol agents. Generally, DRFs lower than 1.3 for 30-day survival are reported [59]. Vitamin E (alpha tocopherol) and related analogues are nutraceuticals that can scavenge singlet oxygen and superoxide-anion radicals. Vitamin E, administrated at a dose of 400 IU/kg s.c. before irradiation in mice, showed an increase in survival rate of up to 79% versus 4% in the vehicle-treated control group [60]. Vitamin E significantly enhanced 30-day survival of treated mice at a dose of 400 IU/kg with a DRF of 1.23 [61]. Oral administration of vitamin E did not increase the survival rate in mice treated with gamma irradiation [60]. A water-soluble derivative of vitamin E called tocopherol monoglucoside (TMG) showed radioprotective activity; the LD_{50} for 24 and 72 hours at 30-day survival were found to be 1120 and 1000 mg/kg, respectively [62]. An intraperitoneal injection of TMG (600 mg/kg), within 10 min of lethal irradiation, increased survival; the protection was not great, with a DRF of 1.09 [63]. Although reduction of oxidative damage by vitamin E and related analogues is generally thought to be the mechanism of these compounds, other mechanisms may play a significant role in their overall biological effect. For example, it was noted that α-tocopheral succinate induced the production of two cytokines: G-CSF and IL-6. These cytokines cause the proliferation of committed progenitor cells in the neutrophil lineage and

stimulate the production of thrombocytes [64]. Initial induction of G-CSF levels and its gradual decline over 48 hours indicated that α-tocopherol succinate may have a wide window for protection against radiation. Alpha-tocopherol succinate was found to be the best of all tocopherols tested for induction of cytokines [64].

Melatonin (*N*-acetyl-5-methoxy tryptamine) is a hormone produced by the pineal gland that scavenges hydroxyl and peroxyl radicals and peroxynitrite anions [65]. Several studies have shown that it has radioprotective effects. Vijayalaxmi *et al*. observed that exposure of mice to 8.15 Gy of ionizing radiation treated with melatonin (250 mg/kg) resulted in a survival rate of 85% compared with 50% in a control group [66]. Melatonin administration (50 mg/kg i.p.), before irradiation, prevented radiation-induced liver oxidative damage [67]. Administration of a single oral dose (300 mg) of melatonin to healthy human volunteers reduced the frequency of chromosomal aberrations and micronuclei induced by irradiation in cultured lymphocytes [68]. Melatonin is found in highest concentrations in cell nuclei, which contain the most sensitive target molecule for ionizing radiation, namely DNA [6]. Melatonin at a whole-body dose of 300 mg p.o. was well tolerated in healthy humans and adverse side effects have not been observed [68]. Also, it was tested at doses up to 800 mg/kg in animals and unfavourable toxicity was not observed [6]. It should be noted that melatonin plays an important role in the regulation of a number of physiological processes [6] and is widely used, mainly as a self-administered, sleep-inducing medication, at doses ranging from 0.3 to 8 mg [69]. Administration of melatonin at a whole-body dose of 75 mg significantly increased the total sleep time in human subjects [69]. Intake of melatonin by young, healthy subjects induced a mild hypotensive effect over a 24-hour period, which could interfere with the management of hypertensive patients being treated with nifedipine [70]. Since inducing sleep and hypotension effect are unwanted effects of radioprotectors, particularly for use in the public, future studies need to evaluate the behavioural effects of melatonin at an optimal dose for protection in human.

Flavonoids are a family of polyphenolic compounds found in fruits and vegetables; as such, flavonoids exhibit strong antioxidant activities. The radioprotective effects of some flavonoids have been investigated, mainly through the use of the micronucleus test for anticlastogenic and cell proliferation activity. The micronuclei (MN) assay has been widely used for the detection of carcinogenic and genotoxic potential of various ionizing radiations and chemicals. Damage to chromosomes manifests as DNA breaks and fragments, which appear as micronuclei in the rapidly proliferating cells. It can be detected in the cytoplasm as small nucleus-like particles. It has been shown that a single i.p., dose of hesperidin, a flavonone glucoside, at a dose of 80 mg/kg, 45 min before gamma irradiation (2 Gy) reduced the frequency of micronucleated polychromatic erythrocytes (MNPCEs). The total MNPCE values were reduced 2.85-fold in the hesperidin group, compared with the respective irradiated control [71]. The effects of other flavonoids, orientin, vicenin, naringin, quercetin and rutin were investigated against the genotoxicity induced by irradiation in mice bone marrow cells [72–75]. These flavonoids significantly protected mice bone marrow cells, when administrated at low doses before exposure to radiation. The protective effect of flavonoids in mice may be attributed to their direct hydroxyl radical scavenging potency and thus behave like an endogenous enzyme [74]. Although flavonoids have been shown to reduce the damage induced by irradiation and, in some cases, at a level comparable with thiol synthetic compounds, the test used to determine efficacy was the DNA damage assay. Values obtained from these, or related, tests cannot determine the likelihood of protection from the acute side effects induced by gamma irradiation, such as haematopoietic and gastrointestinal syndromes. Genistein (4,5,7-trihydroxyflavone) is a natural isoflavone found in soybeans, which may be potentially useful for reducing acute radiation injury when injected s.c. 24 hours before irradiation at doses from 100 to 400 mg/kg. This protocol produced significant enhancement in 30-day survival for mice. The maximum survival rate was observed by genistein treatment at a dose of 200 mg/kg was 91% versus 15% in a vehicle control group. Administration of genistein in this protocol had a DRF of 1.16. In contrast, mice given genistein 1 hour before gamma irradiation failed to show improvement in 30-day survival. Genistein had no overt adverse effects, was well tolerated and could be easily administrated [5]. Although genistein has good antioxidant activity, it is eliminated rapidly from the systemic circulation in mice. Also, it was effective when administrated 24 hours (not 1 hour) before irradiation. This might suggest that the protective effects of genistein were due to other actions than its antioxidant properties [5]. Improved survival was related to enhanced regeneration of haematopoietic stem cells and accelerated neutrophil and platelet recovery [76]. In the meantime, genistein showed antimicrobial activities *in vitro* [77].

Herbal medicine

Plant products have various pharmacological properties and have been used for the treatment of various diseases long ago. Therefore, screening herbal drugs offers a major focus for new drug discovery. In this way, attention over the past 15 years has shifted towards the evaluation of plant products as radioprotectors, due to their efficacy and low toxicity. The proposed radioprotective efficacy of plant extracts is as a result of their containing a large number of active constituents, such as antioxidants, immunostimulants and compounds with antimicrobial activity. Most efficacy studies on plants have been on total extracts for their ability to protect against radiation-induced chromosomal aberrations and micronuclei formation; they were assessed by genotoxic tests, such as micronucleus and metaphase analysis. Citrus extract, at a dose 250 mg/kg, was shown to mitigate genotoxicity induced by gamma irradiation, when administrated 1 hour before γ-irradiation. Citrus extract protected mice bone marrow 2.2-fold, compared to the non-drug-treated irradiated control [78]. Hawthorn fruit has been widely used for the treatment of cardiovascular disease. Administration of hawthorn extract at a dose of 100 mg/kg, 1 hour before gamma irradiation (2 Gy), reduced the frequency of MnPCES and its efficacy was comparable with amifostine at a dose of 100 mg/kg [79]. The radioprotective effect seems to be largely due to the high levels of phenolic and flavonoid compounds with strong antioxidant activities [78,79]. Table 2 shows the efficacy of some of the plant preparations in genotoxicity assays. A number of plants have been found to be effective in providing protection against radiation-induced lethality in mice.

Pre-irradiation administration of an extract of *Zingiber officinale* at a dose of 10 mg/kg once daily for five consecutive days has a DRF of 1.15. Its free radical scavenging, elevation in antioxidant status

TABLE 2

Characteristics of radioprotective agents studied for protection against genotoxicity induced by gamma irradiation

Group	Agent	Dosage[a]	Route[b]	Window[c]	Duration administration[d]	Dose IR (Gy)[e]	Efficacy (%)[f]	Test[g]	Subject[h]	Reference
Synthetic	WR-2721	400	i.p.	0.5	Single	6	32	Mn	Mice	[19]
	WR-2721	200	i.p.	0.5	Single	1.5	25	Mn	Mice	[110]
	WR-2721	100	i.p.	1	Single	2	78	Mn	Mice	[79]
	Captopril	25	i.p.	1	Single	2	57	Mn	Mice	[111]
	Cimetidine	15	i.p.	2	Single	2	38	Mn	Mice	[112]
	Ranitidine	5	i.p.	2	Single	2	47	Mn	Mice	[112]
	Famotidine	1.5	i.p.	2	Single	2	45	Mn	Mice	[112]
Natural	Glutathione	400	i.p.	1	Single	6	20	Mn	Mice	[19]
	Rutin	5 μmol/kg	p.o.	6	Single	1.5	38	Mn	Mice	[74]
	Epicatechin	5 μmol/kg	p.o.	6	Single	1.5	35	Mn	Mice	[74]
	Naringin	2	i.p.	0.75	Single	2	46	Mn	Mice	[72]
	Hesperidin	80	i.p.	0.75	Single	2	65	Mn	Mice	[71]
	Melatonin	300	p.o.	1.2	Single	1.5	56–62.7	Mn/*in vivo*/*in vitro*	Human	[6,68,113]
	Genistein	5 μmol/kg	p.o.	6	Single	1.5	40	Mn	Mice	[74]
Herbal	*Hippophae rhamnoides*	40	i.p.	0.5	Single	2	52	Mn	Mice	[114]
	Abana	20	i.p.		Five days	2	76	Mn	Mice	[115]
	Citrus aurantium	250	i.p.	1	Single	1.5	52	Mn	Mice	[78]
	Hawthorn	200	i.p.	1	Single	2	82	Mn	Mice	[79]
	Liv 52	500	p.o.	1	Seven days	1.5	42	Mn	Mice	[104]
	Tinospora cordifolia	200	i.p.	1	Single	2	81	Mn	Mice	[109]
	Panax ginseng	200	i.p.	0.5	Single	1.5	43	Mn	Mice	[110]

[a] Amount of drug administrated in mg/kg or other units to animal.
[b] The route of drug delivered to animal: s.c. = subcutaneous injection, p.o. = oral gavage.
[c] Pre-irradiation period (in hours) in which the applied agent.
[d] Frequency of time that agent administrated.
[e] Dose of gamma irradiation exposed to animal.
[f] Efficacy (%) = [MnPCE (irradiated group) − MnPCE (treated group)]/MnPCE (irradiated group) × 100.
[g] Micronucleus test was used for determining MnPCE in mice bone marrow cells or cultured human lymphocyte. Mn = micronucleus.
[h] Type of subject was used for micronucleus test.

and superoxide-anion scavenging ability are the proposed main mechanisms of radioprotective action by *Zingiber* [80,81]. Treatment of mice with *Mentha arvensis* extract (10 mg/kg, i.p.) for five consecutive days had a DRF of 1.2 [82]. Administration of mice with different herbal preparations (*abana*, *Triphala*, *Hippophae rhamnoides*, *Mangifera indica*, *Panax ginseng*, *Mentha piperita*, *Tinospora cordifolia*, *Aegle marmelos*, *Amaranthus paniculatus* and *Ageratum conyzoides*) before irradiation, resulted in reduced mortality and symptoms of radiation sickness compared to the irradiated controls [83–91]. The results for some plant origin extract are shown in Table 1.

The doses of herbal preparations that were effective in radioprotection was significantly lower than the toxic dose and this is one of the major advantages of these preparations, compared to synthetic compounds. There are disadvantages to using plants as radioprotective agents, such as: low to mild efficacy (with a DRF <1.3) and a short protective time-window (in most cases 30 min to 2 hours before irradiation).

Further studies are necessary to identify the bioactive compounds responsible for radioprotective efficacy and to extend time-window (e.g. 24 hours prior irradiation). Although there have been many plants evaluated for their ability to reduce radiation-induced sickness in animals, their is insufficient evidence at present to support their potential use in patients during radiotherapy.

Conclusions

The development of radioprotective agents is important for protecting patients from the side effects of radiotherapy, as well as the public from unwanted irradiation. Despite the extensive research performed with respect to the development of radioprotective agents, there is no approved drug to prevent acute radiation syndrome. Synthetic thiol compounds provided good protection and, at that time, this strategy encouraged investigators and governments to search for related agents with greater efficacy. Amifostine is the only drug that has been approved by FDA for prevention of xerostomia induced by irradiation. Toxicity, and the unfavourable routes of administration, have prevented the widespread use of this drug in practice. It is necessary to design other suitable modes of delivery of this agent in order to reduce side effects. Although initially, the aim was to find a radioprotector with high DRF, this was not practically possible. More recent

studies focused on developing drugs with moderate efficacy, low toxicity and that can be administered easily.

Today, there are two strategies to find an approved radioprotective agent: immunomodulators and natural herbal medicine. Although cytokines directly act on the production and proliferation of haematopoietic system, they have limitations, such as inflammatory response and unfavourable administration route. A new approach is to find a safe chemical or biological compound capable of binding cytokine receptors and hence, stimulating the release of two or more cytokines and indirectly regenerating haematopoietic stem cells. Another strategy is to find natural products with actions as free radical scavengers and capable of inducing bone marrow recovery.

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