FULLERENE NANOPARTICLES AND THEIR ANTI-OXIDATIVE EFFECTS –
A COMPARISON TO OTHER RADIOPROTECTIVE AGENTS.

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Summary

Radiation therapy occupies an important position in the treatment of malignant diseases in spite of the existence of radiation side effects on normal tissues. Thus, substances reducing both acute and late radiation effects on healthy tissues are being developed. Currently a sulphur-containing compound amifostine (WR2721, ethyol) is used in clinical practice as a radioprotectant. However, it has considerable side effects including hypotension (found in 62% of patients), hypocalcaemia, diarrhoea, nausea, and vomiting. Carbon nanospheres known as fullerenes and their water soluble derivatives (e.g. $C_{60}(OH)_{24}$, dendrofullerene DF-1) exert anti-oxidative properties and reduce damage to the DNA in irradiated cells. Water soluble fullerenes are low-toxic substances and thus, they are attractive in terms of their use as radioprotectants.

KEY WORDS: ionizing radiation, fullerenes, antioxidant, radioprotection, nanoparticles

1. Introduction

Radiotherapy is one of the major treatment modalities in the managements of human cancer. However, it can lead to a number of side effects in the human body as a consequence of a series of events over different time periods: from those shorter than 10^{-12} s to many weeks. The energy transfer from a photon and/or a particle to atoms and molecules results in a direct change, i.e. a chemical conversion of a macromolecule, which could be of importance for the biological function. The critical event is damage to the DNA in the nucleus and formation of DNA double-strand breaks (DSB). This initial event can be caused by two mechanisms: either by a direct damage to the DNA by the radiation energy or indirect damage mediated through radicals, peroxides and superoxides produced during the water radiolysis. In the case of radiation with low LET (Linear Energetic Transfer) including gamma radiation and X-rays, a prevalent proportion of the radiation damage results from the indirect mechanism. In this review an effect of classical radioprotectants is compared to an effect of water-soluble C60 fullerenes.

2. Mechanism of radioprotection

The radioprotection of living organisms by pharmacological substances particularly depends on their ability to reduce intracellular concentrations of free radicals and peroxides produced over first milliseconds after irradiation. Substances, which could be used in the protection of healthy tissues from ionizing radiation effects in radiation therapy, should adhere to the following two basic conditions: 1/ they must selectively protect normal tissues (without affecting tumour cells) and 2/ they must exert minimum toxicity.

2.1. Radioprotection due to hypoxia

The degree of radiation damage to tissues directly correlates with their oxygenation. Substances able to reduce the oxygenation can therefore have protective effects. A reduction of oxygen levels to 3 – 10 % in the inhaled air during the course of irradiation can exert protective effects in mice and rats, comparable to those achieved with traditional radioprotectants (Vacek et al. 1971). Radioprotectants taking advantage of hypoxia as the main mechanism of the effect include indolylalkylamines (serotonin and mexamine). The mechanism of their protective effect is explained by the post-vasoconstriction hypoxia

(Zherebchenko and Suvorov 1963). However, given their side effects, such as decrease in arterial blood pressure, decrease in body temperature, teratogenic effect or degenerative changes in testicles (Kuna 1985) these substances did not find any use in clinical practice.

2.2. Inactivation of oxidative radicals in water

Strongly reactive oxygen radicals produced during irradiation by the water radiolysis display harmful effects on the cell. The radical scavenging is a basic mechanism of many chemical substances and enzymes protecting biological targets against ionising radiation. It is essentially a competition for a radical between the protective substance and the biological molecule. In aqueous solutions, protective substances and enzymes react with free radicals and peroxides, produce stable non-toxic products and thus reduce amounts of these species. Many of these radioprotectants are very good extinguishers of oxygen radicals. Compounds containing sulphur are well known radioprotectants exerting the highest protective effects but also a considerable toxicity. In contrast, antioxidants of natural origin can be characterized by a relatively low toxicity, but also lower radioprotective properties.

3. Radioprotective agents

3. 1. Sulphur containing compounds

Sulphur containing radioprotectants are chemical analogues of cysteine (a thiol group containing amino acid). Similar to cysteine, these substances have their SH group separated by two to three carbon atoms from the basic amino group. To provide effective radioprotection, these substances must be present in the organism prior to irradiation. The optimum radioprotection is achieved in the case of an intravenous administration 15 – 30 min before irradiation. Sulphur containing compounds act through the mediation of three main mechanisms: as extinguishers of free radicals, as carriers of oxygen and last but not least also as substances inducing hypoxic effects. The most known radioprotectants of this group are cysteamine, cystamine AET and WR2721 (Bacq 1954, Dostál 1967, Kuna 1985).

In the Walter Reed Military Institute in the USA, 4000 compounds have been produced and tested. In 1969, a substance marked WR-2721 was synthesized (Piper et al. 1969). WR-2721 (amifostine, ethyol) is an organic thiophosphate prodrug (2-(3-aminopropylamino)ethylsulphanyl phosphonic acid) which is hydrolysed in vivo by alkaline phosphatase to the active cytoprotective thiol metabolite, WR-1065 (2-((aminopropyl)amino)ethanethiol). The selective protection of non-malignant tissues is believed to be related to higher alkaline phosphatase activity, higher pH, and vascular

permeation in normal tissues. The combination of hypovascularity, low pH, and reduced enzyme levels results in low accumulation of active drug in tumour tissues (Kouvaris et al. 2007). Mean lethal doses were established for accurate determination of the radioprotective effects. These doses are typically related to the 30th day after irradiation. LD50/30 is a lethal dose after which 50 % of animals survive up to the 30th day after irradiation. The DRF (Dose Reducing Factor) is a ratio of the mean lethal dose in protected animals to that in non-protected ones. Table 1 presents DRF values of different radioprotectants used in mice. In the case of whole-body gamma-ray irradiation, WR-2721 administered at a dose of 300 mg/kg is obviously the most effective radioprotectant (Kuna et al. 1983, Kuna 1985). No radioprotective effect of WR-2721 was found in the case of its administration at a dose of 160 mg/kg (intravenously or intramuscularly) to rats 15 – 20 min before their whole-body exposure to fission neutrons (Kuna et al. 2004). This is probably due to the fact that neutrons primarily damage biological molecules directly. WR-2721 also considerably reduces the toxicity of chemotherapeutic agents, particularly of cisplatin (Yuhas 1980).

Undesirable side effects of WR-2721 are related to application of high doses. The LD50/48 value for mouse strain H after i.p. (intra peritoneal) administration was 764 – 1054 mg/kg. The best radioprotective effect was achieved by i.p. application of 300 mg/kg, when DRF was 2.11 – 2.39. Decrease in the dose to 100 mg/kg caused a significant decrease in protective effect (DRF = 1.3) (Kuna 1985). WR-2721 side effects include hypotension, hypocalcaemia, diarrhoea and nausea (France et al. 1986). Hwang et al. (2004) applied WR-2721 to patients during myeloablative conditioning therapy for allogenic bone marrow transplantation. WR-2721 was administered at a dose of 1000 mg/day of conditioning and was well tolerated if the attention was paid to serum calcium level, blood pressure and antiemetics.

3.2. Antioxidants of natural origin

There are a few substances of natural origin that are able to protect cells from negative effects of free radicals and reactive oxygen species. These substances can be divided into two groups: 1/ low-molecular substances acting as scavengers of oxygen radicals and 2/ enzymes detoxifying reactive oxygen radicals and peroxides.

The low-molecular compounds acting as oxygen radical scavengers include vitamins A and E, which are lipophilic, and vitamin C, which is hydrophilic. Several studies have established the radioprotective values of vitamins A, C and E and carotenoids in normal cells (Prasad et al. 2002, Malick et al. 1978, Konopacka et al. 1998). In these compounds, the DRF

values range between 1.1 and 1.2. Lipophilic vitamins A and E administrated i.p. and hydrophilic vitamin C administered i.m. to mice for 14 days (3 days before immunoradiotherapy and 11 days after immunoradiotherapy) reduced body weight loss and myelosupression associated with radio-immunotherapy (Blumenthal et al. 2000).

The group of enzymes with antioxidant properties include superoxide-dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase (Citrin et al. 2010) and is presented below.

3.2.1. Superoxide-dismutase (SOD)

Superoxide-dismutase is an enzyme important for the detoxification of reactive oxygen radicals catalyzing the superoxide radical conversion to hydrogen peroxide and hydrogen. H_2O_2 is subsequently removed by a reaction with a participation of two enzymes (catalase and glutathione peroxidase). The administration of superoxide-dismutase 2 h prior to irradiation (200 mg/kg) and 1 h after irradiation (35 mg/kg) provides a relatively high radioprotective effect - DRF = 1.58 (Petkau 1978). In contrast to radioprotectants containing sulphur, these enzymes exert only a minimum toxicity.

In terms of radiation damage, not only the DNA impairment but also the damage to mitochondria mediated through the production of superoxide and other reactive oxygen species (ROS) derived from superoxide is of importance. An increased ROS production can be observed in the irradiated tissues 6 months after the exposure (Epperly et al. 2008). The damage to the mitochondria is manifested by induction of apoptosis. Manganese superoxide dismutase (MnSOD), which is an enzyme present in human cells, is actually the first line of defense against an increased superoxide production in mitochondria (Belikova et al. 2009). Thus, antioxidant gene therapy studies have utilised manganese superoxide dismutase-plasmid liposomes (MnSOD-PL). Overexpression of the mitochondria localized MnSOD gene product have been shown to decrease ionizing radiation-induced apoptosis of cells *in vitro* (Epperly et al. 2002). In the case of intravenous application of MnSOD-PL to mice before their exposure to 9.5 Gy (antioxidant gene therapy - the mice received intravenously 100 µl of liposomes containing 100 µg of human MnSOD-transgene plasmid 24 hours prior to irradiation), increased animal survival was observed 30 days as well as 340 days after irradiation (Epperly et al. 2008).

3.3 Fullerene - derivatives

Compounds developed based on nanotechnology, as e.g. carbon nanospheres named fullerenes (C60, C70, C80 - C200) also represent an important group of antioxidants due to possible absorption of many oxygen radicals in a single fullerene molecule (Bosi et al. 2003). The most abundant form of fullerenes is buckminsterfullerene C60 (Fig 1) with 60 carbon atoms arranged in a spherical structure (Markovic and Trajkovic 2008). C60 is soluble in aromatic solvents and carbon disulfide but essentially insoluble in water and alcohol (Jensen et al. 1996). For their use in biology, it is necessary to obtain fullerene derivatives, which are soluble in polar solvents. Chemical modification of the fullerene carbon cage by attachment of various functional groups (e.g.-OH, NH_2, -COOH) enables fullerene molecule to establish bounds with water via hydrophilic functional adducts (Markovic and Trajkovic 2008). Johnston et al. (2010) reviewed analyses of fullerenes toxicity in detail. Manipulating fullerene water solubility has included the use of surface modifications, solvents, extended stirring, and mechanical processes. However, the ability of these processes to impact fullerene toxicity requires further assessment, especially when considering the use of solvents, which particularly enhance the toxicity of fullerene derivates (Johnston et al. (2010).

3.3.1. Inhibition HIV replication

These substances were also shown to possess considerable antiviral effects. In 1993, the water-soluble derivative of C60 [bis(monosuccinimide) derivative of bis(2-aminoethyl)diphenyl-C60] was found to be a substance inhibiting HIV-1 protease (Schinazi et al. 1993). A derivative of tris-hydroxymethyl methano-fullerene was later discovered to exert an even higher antiviral activity (Jensen et al. 1996). The antiviral activity seems to be characteristic for many non-toxic derivatives of the C60 fullerene (Friedmann et al. 1998, Cheng et al. 2010).

3.3.2. Photodynamic therapy of tumors

Mroz et al. (2007) showed that C60 molecule mono-substituted with a single pyrollidinium group is a remarkably efficient photosensibilizer and can mediate killing of a panel of mouse cancer after exposure to white light. Following intravenous injection of C60-PEG (polyethylene glycol (PEG) conjugated with C60) to tumour-bearing mice, coupled with exposure of the tumor site to visible light, the volume increase of tumour mass was suppressed and C60-PEG conjugate exhibited a stronger suppressive effect than Photofrin (Tabata et al.1997, Liu et al. 2007). These data demonstrate the potential use of these compounds as photosensibilizers for photodynamic therapy of tumours.

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3.3.3. Antioxidant activity

Oxidative stress and associated oxidative damage are mediators of cellular injury in many pathological conditions, including autoimmunity, atherosclerosis, diabetes, and neurodegenerative disorders (Markovic and Trajkovic 2008). In many studies, the capability of water-soluble fullerene derivates to act as antioxidant substances scavenging oxygen radicals (including ROS generated by ionising radiation) and protecting cells and/or tissues against ROS damage have been shown. Known antioxidant activity of water soluble C60 derivatives is summarized in Table 2.

For instatnce, polyhydroxylated fullerenes - $C_{60}(OH)_x$, also referred to as fullerenols, were studied by Trajkovič et al. (2007) and Cai et al. (2010). Trajkovič et al. (2007) demonstrated a radioprotective effect of fullerenol ($C_{60}(OH)_{24}$) administered to rats intraperitoneally in a dose of 100 mg/kg 30 min prior to 8 Gy irradiation. Fullerenol protected rats´ haemopoietic and lymphoid tissues. Cai et al. (2010) studied radioprotective effects of repeated (for a period of 14 days) fullerenol administrations in a dose of 40 mg/kg on mouse immune system. It was found that 2-week $C_{60}(OH)_{24}$ pretreatment effectively reduced whole body irradiation-induced mortality without apparent toxicity. $C_{60}(OH)_{24}$ pretreatment also showed significant protective effects against ionizing-radiation-induced decreases in immune and mitochondrial function and antioxidant defense in the liver and spleen. This suggests that the polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$ protects against ionizing-radiation-induced mortality, possibly by enhancing immune function, decreasing oxidative damage and improving mitochondrial function.

Antioxidant and protective properties of carboxy-fullerenes were descirbed by Ali et al. (2008) and Dugan et al. (1996). Both studies showed that carboxy-fullerenes are able to protect neurons against oxidative stress associated with Parkinson disease and ischaemic brain injury. Moreover, Ali et al. (2008) compared the structure of 6 carboxy-fullerenes and found the best antioxidative effect in tris–adduct malonic acid derivate of fullerene - $C_{60}(C(COOH)_2)_3$. Ali et al. (2008) described carboxy-fullerenes as three-dimensional carbon carriers with the antioxidative properties depending not only on the number of bound carboxylic groups but also on their distribution on the fullerene ball.

The ability of fullerenes to modulate cytokine production and cellular damage was shown in bis-adduct malonic acid derivate and water-soluble C60 fullerene (polyvinylpyrrolidone wrapped C60). Bis-adduct malonic acid derivate inhibited the TNF-alpha initiated apoptosis in HeLa cells (Li et al. 2011). On the other hand, findings by Yudoh et al. (2009) indicate that polyvinylpyrrolidone wrapped C60 reduces pro-inflammatory

cytokine production from synovial inflammation-related cells and mitigates resultant synovitis *in vitro*. Intra-articular treatment with this compound significantly attenuates synovitis and joint destruction in the rat model of arthritis.

Another promising fullerene derivate is dendro (60) fullerene-1 (DF-1). The derivate is characterised by a single branched dendromer architecture containing 18 terminal carboxylic groups attached to fullerene ball. DF-1 is readily soluble in water, non-toxic and has radioprotective effects (Brown et al. 2010, Theriot et al. 2010). Theriot et al. (2010) showed that DF-1 protects lymphocytes as well as cells in intestinal crypts from the radiation-induced cell death. In this study, human lymphocytes and rat intestinal crypt cells (IEC-6) were incubated with 100 μM DF-1 one hour prior to irradiation, rinsed and immediately exposed to a single dose of 4 Gy in the fresh medium. The study shows that 1-hour incubation with DF-1 reduces number of micronuclei (indicator of DNA damage) in both types of cells compared to the irradiated non-protected groups. Brown et al. (2010) evaluated DF-1 DNA protective effects via expression of phosphorylated histone H2AX (gamma H2AX). Gamma H2AX serves as an indicator of DNA double strand brakes. In the DU145 cell culture, a 30-min pre-treatment with 100 μM DF-1 resulted in a significant decrease in the number of gamma H2AX foci 1 and 6 h after 4 Gy irradiation. Both studies demonstrate that there is a reduction in DNA damage after DF-1 incubation and that DF-1 acts not only against the oxidative stress but also against the DNA damage generated by ionizing radiation.

4. Conclusion

Polyamino- and polyhydroxy-fullerenes show that the water-solubility increases with the number of groups introduced into the molecule. It is possible to conclusively state that water-soluble fullerene derivatives exert considerable protective effects against the oxidative stress as scavengers of free radicals *in vitro* as well as *in vivo* (Bakry et al. 2007, Ali et al. 2004, Dugan et al. 2001, Injac et al. 2008). The radioprotective effects were demonstrated in fullerenols, carboxy-fullerenes, polyvinylpyrrolidone wrapped fullerene, and DF-1. Table 1 summarizes a comparison of the DRFs after a single water-soluble dendrofullerene DF-1 application 30 min before irradiation (DRF = 1.22) with effects of other radioprotectants. Given the fact that these substances (fullerenol, DF-1) have no or only slight side effects, they offer a great potential to become radioprotectants with a possibility of repeated administration, which is necessary in standard fractionated radiotherapy.

Acknowledgements

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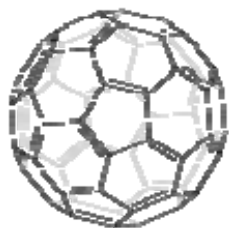Table 1: Comparisson of radioprotective effect of DF-1 dendrofullerene with other known radioprotectants

Drug	dose	irradiation	animals	DRF	author
WR-2721	300 mg/kg, i.m. 15-20 m before irrad.	gamma	mice	2.39	Kuna 1985
WR-2721	100 mg/kg i.m. 15-20 m before irrad.	gamma	mice	1.3	Kuna 1985
cystamine	175 mg/kg i.m. 5-15 m before irrad.	gamma	mice	1.83	Kuna 1985
Superoxide-dismutase	i.v. 2 h before irrad. (200 mg/kg) and 1 h after irrad. (35 mg/kg)	X-rays	mice	1.56	Petkau 1987
Hypoxia – 8 % O_2	In the course of irrad.	gamma	mice	1.5	Vacek 1971
DF-1 dendrofullerene nanoparticle	300 mg/kg 15 m before irrad.	X-rays	mice	1.22	Brown 2010

The DRF (the Dose Reducing Factor) is a ratio of the mean lethal dose (LD50/30) in protected animals to that in non-protected ones.

Table 2: Water soluble C60 derivates and their antioxidative effects

Fullerene type	Biological effects	author
C60 (OH)$_{24}$	radioprotection protection from doxorubicin toxicity	Cai 2004, Trajkovic 2007, Injac 2008
C60 (OH)$_{22}$	protection from H_2O_2 induced oxidative injury	Yin 2008
Malonic acid C60 derivates carboxyfullerenes	neuroprotection	Dugan 1996, 2001, Ali 2008
C60 dendrofullerene	radioprotection	Brown 2010, Theriot 2010
Polyvinylpyrrolidone wrapped C60	reduces synovitis	Yudoh 2009

Figure 1: Structure of fullerene C60