Review

Bioapplication and activity of fullerenol $C_{60}(OH)_{24}$

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Here we summarize current investigations about a relatively new group of compounds mainly composed of carbon atoms - fullerenes and their derivatives. One of the fundamental characteristics of fullerene is its ability to quench various free radicals, behaving as a "free radical sponge". Moreover, the dual nature of fullerenes to act as either quenchers or generators of cell - damaging ROS could be exploited for development of cytoprotective agents on one side or cytotoxic anticancer/antimicrobial agents on the other. In addition, several derivatives have shown immunomodulating, neuroprotective and radioprotective effect. Fullerenes are hydrophobic molecules best dissolved in organic solvents, so potential biomedical applications are restricted by their extremely poor solubility in polar solvents. One of the strategies for improving poor solubility is derivatization. Fullerenol $C_{60}(OH)_{24}$ is a water-soluble derivative of C_{60} with improved chemical properties and potential bioapplicability as a free radical scavenger in biological systems, in oxidative stress induced by xenobiotics or radioactive irradiations. However, solubility of $C_{60}(OH)_{24}$ in water (44 mg/l) is not satisfactory and presents a major drawback in its application as an organo-protector. Improvement of physicochemical characteristics of $C_{60}(OH)_{24}$ and chronic investigations on different animal models as well as in human trials are recommended for establishing its antioxidant effect.

Key words: Carbon atoms, cytoprotective, derivatization, antioxidant effect.

INTRODUCTION

Fullerenes are a relatively new group of compounds and represent a class of sphere-shaped molecules made entirely of carbon atoms. The fullerenes discovered by Peter Harris and colleagues were completely closed and comparatively large, containing around 6,000 to 10,000 carbon atoms each (Djordjevic et al., 2006). The molecule has the shape of an icosahedron, containing 12pentagons and 20 hexagons, in which every carbon atom is bound to three other adjacent atoms through sp^2 hybridization (Kratschmer et al., 1990).

Fullerenes are hydrophobic molecules best dissolved in organic solvents, so potential biomedical applications are restricted by their extremely poor solubility in polar solvents. Different methods can be used for improving poor water-solubility of fullerene, such as addition of artificial or natural surfactants (Deguchi et al., 2001), long-term stirring in water (Brant et al., 2006), and incorporation into water-soluble supramolecular structures such as γ-cyclodextrin (Makha et al., 2006). In addition, liposomes with incorporated fullerene (C_{60}) and photoactive C_{60} were also prepared in order to facilitate poor solubility of this molecule (Husebo et al., 2004). However, these procedures result in formation of water stable C_{60} aggregates

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a)

$$C_{60} \xrightarrow{h\upsilon} {}^{1}C_{60}^{\cdot} \longrightarrow {}^{3}C_{60}^{\cdot} \xrightarrow{e^{-}} C_{6}^{\cdot -} \xrightarrow{O_{2} \quad O_{2}^{\cdot -}} C_{60}$$

singlet   triplet   radical anion

b)

$$C_{60}(OH)_{24} \xrightarrow{h\upsilon} C_{60}(OH)_{24}^{\cdot} \xrightarrow{O_{2} \quad {}^{1}O_{2}} C_{60}(OH)_{24} \xrightarrow{e^{-}} C_{60}(OH)_{24}^{\cdot -} \xrightarrow{O_{2} \quad O_{2}^{\cdot -}} C_{60}(OH)_{24}$$

$$O_{2}^{\cdot -} \xrightarrow{H^{+}} HOO^{\cdot}$$

$$2O_{2}^{\cdot -} + 2H^{+} \xrightarrow{SOD} H_{2}O_{2} + O_{2}$$

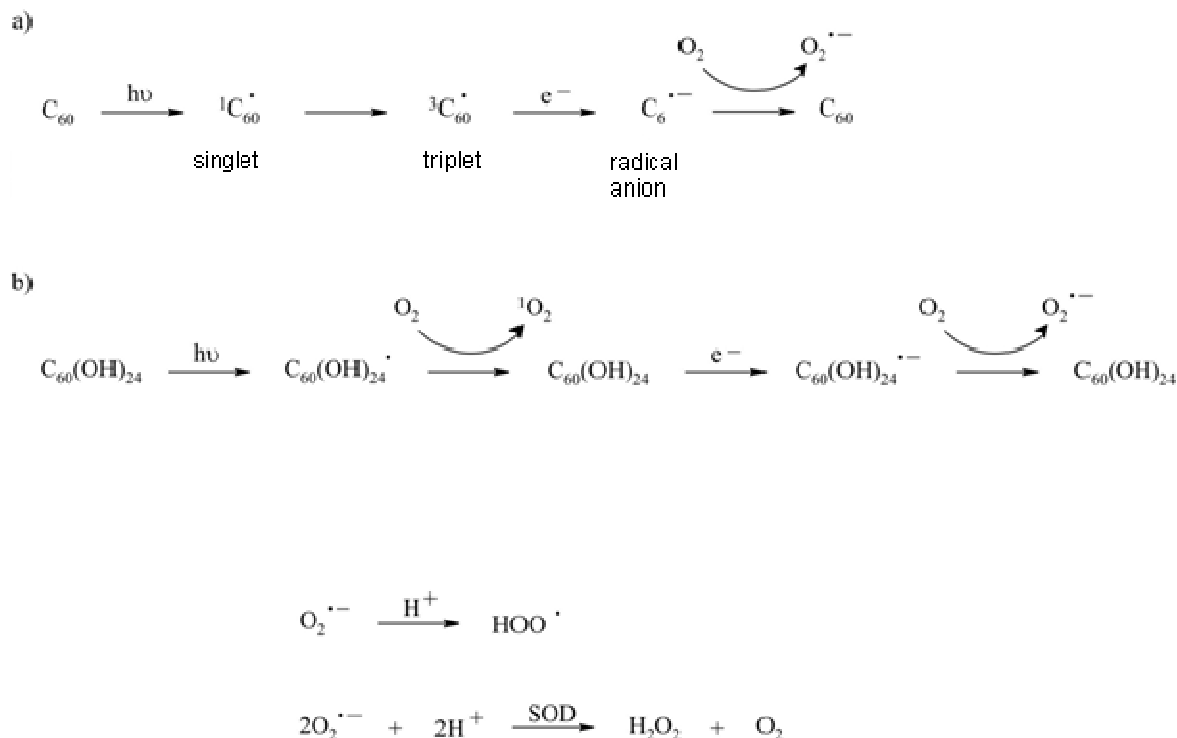**Figure 1.** Fotoexcitation of C_{60} (a) and $C_{60}(OH)_{24}$ (b) molecules and formation of reactive oxygen species; SOD – superoxide dismutase.

(Zhao et al., 2008). Early experiments demonstrated that modifications of fullerenes resulting from interactions with solutes present in environmental and biological systems may have a significant influence on the metabolism, transport and reactivity of fullerene (Brant et al., 2006). One practical solution for improving negligible solubility of fullerene in water is derivatization. Derived fullerenes usually form stable molecular dispersed suspensions as a result of hydrophilization or encapsulation of C_{60} in order to prevent contact between the fullerene cores (Gao et al., 2005). However, previous investigations have noted that derivatization does not completely prevent cluster formation (Gao et al., 2005; Brant et al., 2007; Vileno et al., 2004). On the other hand, the unique electronic π-system of fullerenes makes them potential photosensitizers upon absorption of UV or visible light.

The ground state adsorption spectrum of C_{60} is characterized by intense absorption in the UV region with weaker bands extending throughout the visible spectrum up to 700 nm. The short–lived singlet state is formed by irradiation of C_{60} and converts almost quantitatively into a longer-lived triplet state. Furthermore, singlet oxygen is often generated in the process of transferring energy to molecular oxygen. The triplet lifetime is essential for photoactivity in cells. In fact, it was suggested that only triplet states with lifetimes longer than 100 μs have enough cytotoxic potential. Singlet oxygen possesses intensive oxidative potential, and is, therefore, considered

to be responsible for several biological activities of dissolved C_{60}. Consequently, the damaging effects are usually inhibited by the presence of singlet oxygen quenchers (Prat et al., 1999). Zhao et al. (2008) have shown that a variety of water-soluble fullerenes can efficiently generate singlet oxygen ($^{1}O_{2}$) upon irradiation via energy transfer (type II photochemical mechanism) from the excited triplet of fullerene to oxygen (Nagano et al., 1994; Sera et al., 1996; Arbogast et al., 1991). There are also reports that photoirradiation of fullerenes in aqueous systems results in the production of the radical anions ($C_{60}^{\cdot}$) followed by generation of superoxide anion radical ($O_{2}^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$) via electron transfer (type I photochemical mechanism), especially in the presence of electron donors (such as NADH or amines) (Nakanishi et al., 2002a; Nakanishi et al., 2002b, Yamakoshi et al., 2003; Stasko et al., 1996). These two photochemical mechanisms, occurring during photodynamic therapy (PDT), demonstrate the main pathways for the photoinduced toxicity of fullerenes (Tokuyama et al., 1993). Possible pathways are presented in the Figure 1. This dual nature of C_{60} to either quench or generate cell-damaging ROS could therefore be used for its development as a cytoprotector or as a cytotoxic anticancer/antimicrobial agent (Markovic and Trajkovic, 2008). Recent studies describe increased toxicity of pure C_{60} against dermal fibroblasts and liver carcinoma HepG2 cells, in comparison to fullerenol and other water-soluble

fullerenes. It was also demonstrated that the cytotoxic activity of C_{60} colloid was caused by ROS-mediated cell membrane lipid peroxidation (Sayes et al., 2004). In addition, Isakovic et al. (2006) demonstrated that pure C_{60} and fullerenols $C_{60}(OH)_x$ apparently employ different cytotoxic mechanisms resulting in preferential induction of caspase-independent necrosis and caspase-dependent apoptosis, respectively. More precisely, C_{60} induces production of oxygen radicals, which are involved in lipid peroxidation, and consequently stimulates necrotic cell death (vacuolation of the cytoplasm, breakdown of the plasma membrane, release of cellular components and pro-inflammatory molecules). On the other hand, $C_{60}(OH)_x$-triggered apoptosis (chromatin condensation, activation of caspases, fragmentation of DNA without membrane breakdown, absence of inflammation) seems to be ROS independent (Isakovic et al., 2006). Pro-oxidant activity of pure C_{60} is related to its chemical structure. It was found that derivatization of C_{60} decreases ROS generation and cytotoxicity (Sayes et al., 2004).

WATER-SOLUBLE C_{60} DERIVATIVES

The potential environmental and health effects of fullerenes have attracted increasing attention in recent years, especially with development of water-soluble forms that alleviate their use in biological systems (Zhao et al., 2008). These compounds have potential for suppression of metastases, treatment of cerebral conditions such as Alzheimer's and Parkinson's diseases, type-C hepatitis therapy, and HIV treatment. Water-soluble C_{60} derivatives, synthesized by attaching various functional groups (-OH, -COOH, -NH$_2$) to the C_{60} cage, are novel candidates for many biomedical applications, including cytoprotection, DNA photocleavage, enzyme inhibition, diagnostic imaging, and antimicrobial and anticancer therapy (Bosi et al., 2003).

The chemical modification of fullerenes by adding the OH groups to their carbon surface yields a variety of polyhydroxylated structures $C_{60}(OH)_x$ exhibiting different degrees of solubility and antioxidant activity in the aqueous environment (Deguchi et al., 2006; Brettreich and Hirsch, 1998).

Their electronic properties and reactivity strongly depend on the number of hydroxyl groups, as well as their precise position on the carbon cage. The specific behavior of fullerenols is a consequence of their structural flexibility, the rotation of the OH groups around the axes going through the C-O bonds and the distribution of these groups across different carbon sites of the fullerene surface (Fileti et al., 2008).

Fullerols simultaneously have both attractive (C–OH) and repulsive (C–O–) sites. The acidic protons may be involved in attractive hydrogen bonding interactions with other fullerenol molecules, driving cluster formation. Indeed, fullerenol has been found to form clusters in water

despite increasing the number of hydroxyl groups on the molecular cage, which would decrease the hydrophobic portion of the molecular surface area (Vileno et al., 2006). Studies related to physicochemical properties of fullerenols confirmed that hydroxylated fullerene C_{60} forms colloids, and is not likely to form a molecular solution in water, which is in conflict with the description of this material as water-soluble (Brant et al., 2007). Moreover, Husebo et al. (2004) demonstrated that fullerenol clusters seem to constitute large, loosely associated, amorphous aggregates in water. However, the formation of aggregates was exhibited in micromolar concentrations, suggesting that fullerenol, is most likely to form supramolecular clusters (Husebo et al., 2004). These clusters have a mean size of 100 nm or more and are often of different shapes, which depend on the characteristics of the hydroxyl groups (length). The effect of the length is connected with molecular packing and balance between attractive and repulsive interactions (Brant et al., 2007).

In the relation to biological activity of polyhydroxylated fullerenes $C_{60}(OH)_x$ it is of value to mention its protective effect against oxidative stress in RAW 264.7 macrophage cell line, ichemia-reperfused rat lungs (Chen et al., 2004), and its ability to significantly reduce doxorubicin toxicity against human breast cancer cell lines (Bogdanovic et al., 2004). On the contrary, fullerenol alone has also suppressed proliferation of human breast cancer cells in a cell line-dependent manner (Bogdanovic et al., 2004). Possible mechanism of its cytotoxic and cytostatic action is inhibition of protein tyrosine kinase activity (Lu et al., 1998).

BIOLOGICAL APPLICATIONS OF DIFFERENT WATER-SOLUBLE C_{60} DERIVATIVES

Antioxidant activity

Oxidative stress and oxidative damage are mediators of cellular injury in many pathological conditions. Harmful effects of oxidative stress are associated with mitochondrial depolarization and subsequent release of small pro-apoptotic molecules such as cytochrome c, leading to activation of caspase cascades and apoptosis (Orrenius, 2007). Polyhydroxylated and malonic acid derivatives of C_{60} are able to associate with mitochondria and induce cytoprotective antioxidant effect. The antioxidant effect of water-soluble fullerenes is used in protection of different organs such as the brain from age-associated oxidative stress (Quick et al., 2008), lungs from ischemia-reperfusion injury (Chen et al., 2004), heart and liver from different pathological changes caused by doxorubicin (Injac et al., 2008a; Injac et al., 2008b). Certain number of papers also deals with direct NO-scavenging activity of C_{60} derivatives. Mono and di-malonic acid derivatives of C_{60} showed inhibitory effect on NO-induced relaxation of rabbit aorta, which is connected with production of supero-
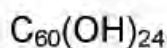
Figure 2. Structure of $C_{60}(OH)_{24}$.

xide anion radical and neutralization of NO by superoxide anion (Sato et al., 1997). However, nitric oxide (NO) reacts with superoxide anion in diffusion controlled rates, competing with endogenous SOD for substrate. In that case, fullerenol may prevent the formation of peroxynitrite anion, a reactive and short-lived species, by decreasing the bioavailability of NO, one of the peroxynitrite precursor radical species (Radi et al., 2001). Recently it has been shown that fullerenol $C_{60}(OH)_{24}$ (FRL) exhibited significant NO-antiradical activity (Mirkov et al., 2004; Bogdanovic et al., 2004).

Anticancer and antimicrobial activity

The potent ability of fullerenes to induce production of ROS makes them promising candidates for the photodynamic killing of cancer cells. There have been many studies confirming the efficient photodynamic action of various water-soluble C_{60} derivatives against different types of cultured cancer cell lines (cervical, larynx, lung and colon carcinoma) and malignant tumors *in vivo* (Mroz et al., 2007). The anticancer activity is a result of generation of both singlet oxygen and superoxide anion. This effect is inversely correlated with the extent of derivatization of the fullerene cage (Mroz et al., 2007; Yang et al., 2002). Furthermore, generated ROS are responsible for the "programmed" cell death (Type I), known as apoptosis. Beside fullerenols, other water-soluble derivatives exhibited potential anticancer activity, such as malonic acid derivatives (Yang et al., 2002; Rancan et al., 2002) pyrrolidinium derivatives (Mroz et al., 2007), C_{60}-porphyrin and sugar derivatives (Alvarez et al., 2006; Mikata et al., 2003).

Immunomodulatory activity

One of the possible ways for improving cancer therapy is enhancement of immune function. Resent studies evaluated immunomodulatory activity of $C_{60}(OH)_x$ by measuring activity of arginase (Arg) and acid phosphatase (Acp) of peritoneal macrophages. The increased activity of these two lysosomal enzymatic markers confirmed that $C_{60}(OH)_x$ treatment significantly stimulates

the activation of macrophages and consequently contributed to the inhibition of tumor growth (Zhu et al., 2006). Furthermore, $C_{60}(OH)_x$ could increase levels of TNF-α, which is an important host defense cytokine with antitumor and immunomodulatory properties. Production of TNF-α induced by $C_{60}(OH)_x$ subsequently causes the activation of peritoneal macrophages and enhanced anticancer activity (Terlikowski, 2002).

However, further investigations of different fullerene derivatives would concentrate more on their interactions at the cellular level. The reason for this is its ability to cross the cell membrane and preferentially bind to mitochondria. Basic evidence, which demonstrates intracellular localization of these derivatives, is structural analogy between the C_{60} cage and that of the clathrin-coated vesicles. These last structures are known to be essential in the endocytosis pathway (Foley et al., 2002).

Fullerenol $C_{60}(OH)_{24}$

FRL is slightly water-soluble derivative of C_{60} with improved chemical properties and potential bioapplicability as a free radical scavenger in biological systems, where oxidative stress is induced by xenobiotics as well as radioactive irradiations (Figure 2). Because of its poor solubility in water (44 mg/ml), it is necessary to add surfactant or co-solvent to the solution for parenteral application (Figure 3).

FRL was synthesized in alkaline media (NaOH, pH = 10) by complete substitution of bromine atoms form $C_{60}Br_{24}$ (Djordjevic et al., 2004).

Investigation of biodistribution of $C_{60}(OH)_{24}$ strongly confirms that FRL rapidly distributes through blood circulation and other pathways to all organs except for the brain. Clearance from heart, lung, muscle, skin and the intestine shows the same trend as that of blood. To the contrary, accumulation in the liver, bone, kidney, spleen and stomach is significantly higher than that in other organs. In these organs clearance rate of FRL is much slower (Qiang Ji et al., 2006).

Cardioprotective effect of FRL in therapy with Dox

Doxorubicin (Dox), an anthracyclin antineoplastic agent, is principally used in treatment of heaematological malignacies as well as solid tumours, exerting its effect by interrcalating into DNA. However, cardiotoxicity induced by Dox is one of the most severe side effects that importantly limits its use (Minotti et al., 2004). The generally accepted mechanism of Dox-induced cardiotoxicity includes generation of free oxigen radicals, expression of nitric oxid synthase, changes in calcium homeostasis, damage of myocardial mitochondrion and alteration of molecullar signalling (Kalivendi et al., 2005; Takemura and Fujiwara, 2007). Free radical mediated damage is thought to be the most severe side effect (Minotti et al.,
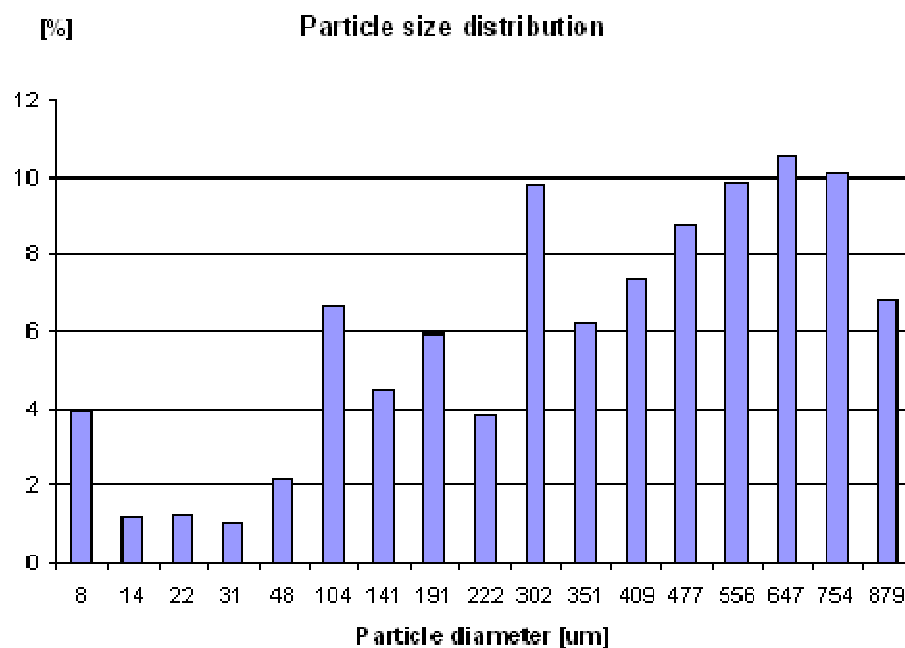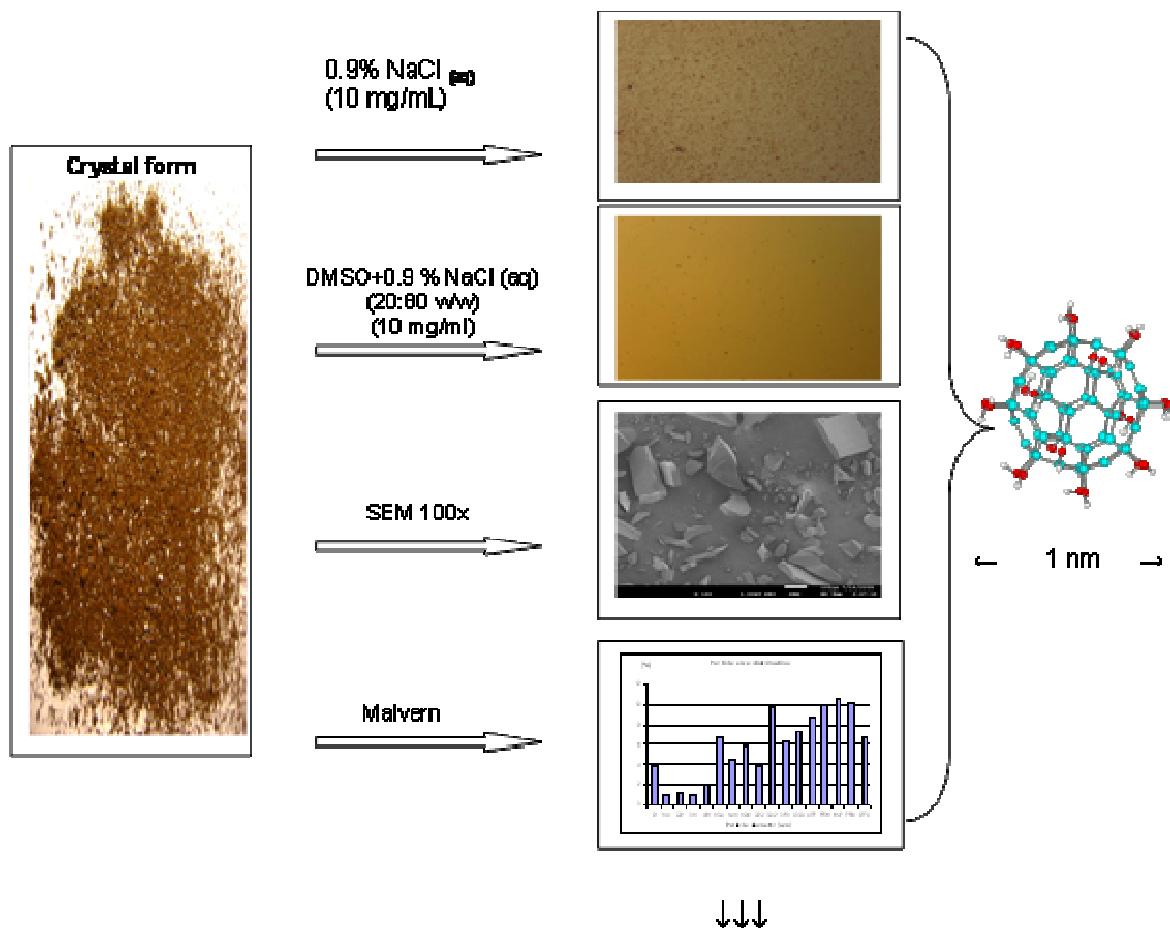
Figure 3. Fullerenol $C_{60}(OH)_{24}$: Macroscopic view (crystal form); Dissolution in physiological fluid (optical microscope; a lot of aggregates of fullerenol with a size of 50 -100 μm); Dissolution in DMSO/physiological fluid (optical microscope; some aggregates of fullerenol; < 20 μm); SEM view; Particle size distribution (Malvern method); structure scheme.
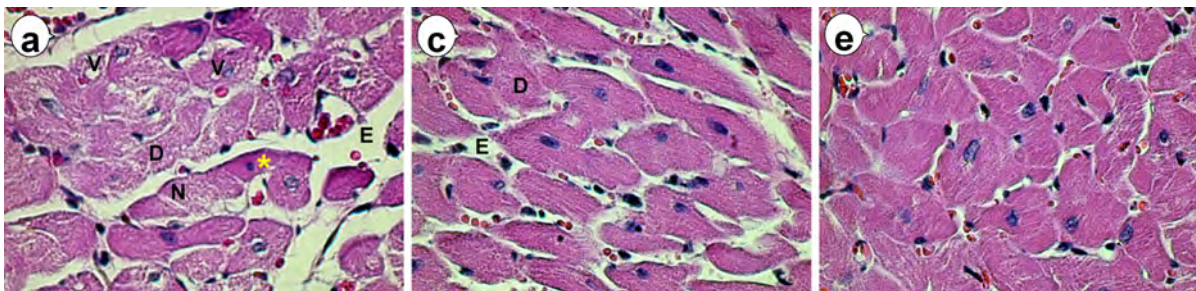
Figure 4. The histologic sections of the heart of the rat treated with Dox (a), Dox pretreated with FRL (25 mg/kg i.p.) (c) and saline only - healthy control (e); H and E, magnification 400x (a). The moderate parenchymal degeneration (D) with small vacuoles (V) in myocytes, necrotic (N) myocytes, apoptotic (*) myocytes, interstitial myocardial edema (E) were representative of the Dox-treated group (c). The milder degree of parenchymal degeneration (D) with no vacuoles in myocytes was present in FRL pre-treated group (e). No histologic lesions were found in the cardiac control group.

2004;Takemura and Fujiwara, 2007). Dox generates oxygen radicals by itself or by forming complexes with ions. Cardiac tissue is highly susceptible to such oxidative damage due to the insuficient capability of eliminating these radicals. In addition, mitochondria as a major site of ROS production, could also be the target suseptible to ROS attack. Defects in mitochondrial architecture lead to the alteration of the mitochondial methabolism, resulting in decreased activities of mitochondrial enzymes, in the Dox intoxicated hearts (Peng et al., 2005).

Injac et al. (2008a) investigated the potential protective activity of FRL towards Dox toxicity using a MNU-induced breast cancer rat model. Typical markers for oxidative heart damage such as MDA, SOD, CAT, GSH, TAS, GSH-PX, GR and TAS have been chosen to confirm or refute a potential antioxidative effect of FRL. The study confirmed that FRL in a dose of 100 mg/kg after i.p. administration, demonstrated a statistically significant cardioprotective effect in acute Dox-induced toxicity. In groups pretreated with FRL, the activities of the investigated enzymes were comparable with the control group. Furthermore, MDA as one of the well-known secondary products of lipid peroxidation, was significantly decreased in groups pretreated with FRL. These results revealed that FRL clearly decreases lipid peroxidation induced in the heart muscle by Dox. The generally accepted explanation for this is direct scavenging of the free oxygen radicals, especially OH• and peroxynitrite (ONOO•) (Injac et al., 2008a). Thus, FRL might be a potential cardioprotector in Dox-treated individuals, only after an improvement of the physical properties of FRL is achieved (especially concerning solubility of the solid-state form in water). There has been a number of papers dealing with acute evaluation of protective effects of FRL against Dox-induced toxicity (Injac et al., 2008a; Injac et al., 2008b). FRL, including chronic investigation in animal and human trials in order to evaluate its protective effects.

The first chronic study with FRL was performed on rats Accordingly, there is need to carry out further studies with with colorectal cancer after a multi dose administration of

Dox (unpublished paper, Injac et al., 2008). According to the results, FRL has shown protective effects against myocardial lesions in doses of 25 and 50 mg/kg (Figure 4). Furthermore, levels of serum specific enzymes such as AST, ALT and the ALT/AST ratio were not significantly different in all groups pre-treated with FRL during three weeks in comparison to the control group. Levels of antioxidative enzymes such as SOD, GR and CAT were not significantly different in comparison to the control group. This kind of chronic *in vivo* evaluation of protective effect of FRL is a great step forward in improving non-specific anticancer therapy. *Ex vivo* investigation in a human or a swine model is strongly recommended to reach the final goal of clinical trials.

Hepatoprotective effects of FRL in therapy with Dox

The imbalance in oxidant-antioxidant systems results in tissue injury, which is demonstrated with lipid peroxidetion and protein oxidation in the tissue. It is known that the combination of inflammatory processes, free radical oxidative stress and lipid peroxidation is related with liver damage, induced by toxic agents such as Dox (Yagmurca et al., 2007). Persistent and irreversible liver injury as a side effect of Dox therapy has been proven and an elevation of the apoptotic processes in liver tissue after a single dose of Dox has been described (Pedrycz et al., 2004a; Pedrycz et al., 2005). It was confirmed that the therapeutic doses of Dox enhance lipid peroxidation in microsomes and mitochondria in the liver, especially in the presence of Fe^{3+} ions (Griffin Green et al., 1988). Dox-mediated hepatotoxicity includes focal damage in hepatocytes, vascular damage and steatosis (Pedrycz et al., 2004b). Screening antioxidants for protective effects against the Dox-induced hepatotoxicity has become a trend recently (Injac et al., 2008c). Current study about antioxidative effect of FRL in Dox-induced hepatotoxicity demonstrated that Dox and Dox/FRL have intensive influence on the balance in intra and extra-cellular compartments.
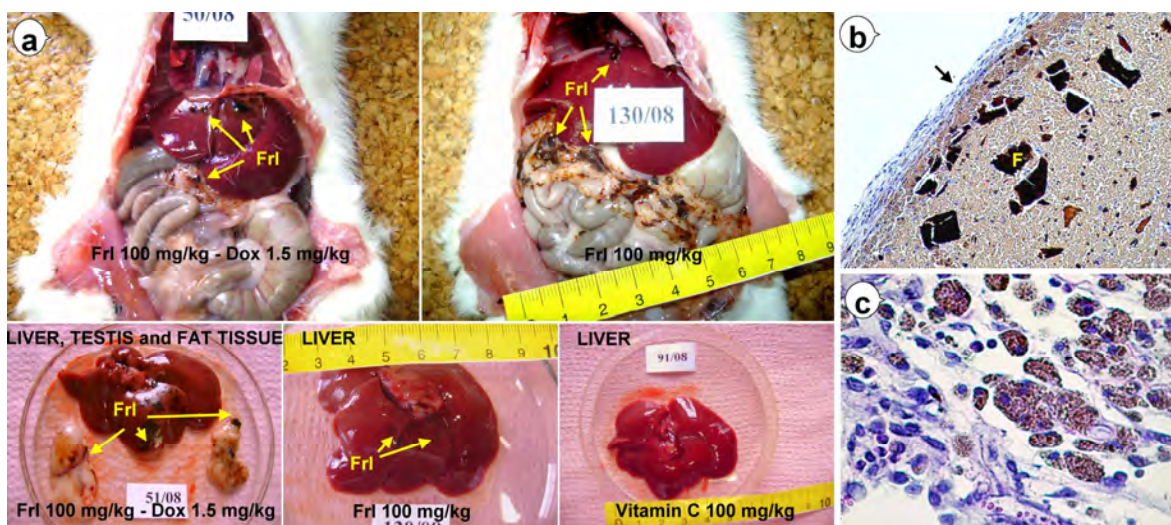
Figure 5. Macroscopic and microscopic changes in the abdominal cavity of rats treated with FRL. (a) Black and brown particles of FRL are seen in the abdomen, particularly on the surface of the liver and the mesenteric fat surrounding the liver. (b) Foreign body granuloma covered with thick peritoneum contains inflammation cells and fibroblasts (black arrow). Granulomatous tissue is predominantly composed of FRL composites in crystal form (F) and macrophages filled with FRL; H and E, magnification 100x. (c) Large number of macrophages with numerous brown granule of FRL in cytoplasm; H and E, magnification 400x.

Furthermore, FRL may have side effects on some of the abdominal organs after i.p. administration because of its unsuitable physical characteristics. Consequently about 20% of FRL accumulates in the form of black and brown particles in the abdomen, especially on the ventral surface of the liver, pancreas and spleen, particularly in the ventral mesenteric fat area surrounding the liver and scrotum area where it caused the formation of granulomas. FRL as a xenobiotic could have a harmful effect on the peritoneal compartments (peritonitis), which can lead to secondary changes of the liver tissue (Injac et al., 2008b).

Obtained results confirmed that FRL could, under certain conditions, play a hepatoprotective role in doxorubicin-induces hepatotoxicity due to its antioxidant properties. Intracellular FRL protects hepatocytes against doxorubicin toxicity, but the levels of MDA and different glutathione forms point to significant cellular injury. Therefore, the pro-oxidant influence of FRL on healthy hepatocytes *in vivo* or *in vitro*, as well as the *in vivo* irritability of the peritoneum and abdominal tissue caused by poor FRL solubility, could hamper the potential cardio- (Injac et al., 2008a; Puhaca, 1999) and hepatoprotector (Injac et al., 2008b). On the other hand, when oxidative stress is too high and induced with very aggressive pro-oxidant such as Dox, the cytotoxic effects of FRL are overcome by its protective role as an effective antioxidant compound. In chronic evaluations of protective effect of FRL in Dox-induced hepatotoxicity, the changes on the liver were especially noticeable in the group treated with Dox and in all FRL treated groups (25, 50, 100 mg/kg). Autopsy also confirmed the inappropriate solubility of applied FRL in dose of 100 mg/kg. Consequently, undissolved FRL

remained in the ventral side of abdomen, as already descrybed in the acute investigation. In addition, the accumulation of undissolved FRL in the abdominal cavity was dose-dependent, because the amount of non-distributed FRL decreased with the decrease of the dose of FRL (from 100 to 25 mg/kg) (Figure 5). In addition, various parameters of oxidative stress (LDH, SOD, GSH-Px, GR and CAT) were in good correlation with levels of the same parameters in control group. According to these results, poor solubility of FRL in water is major drawback for its application as an organo-protector. Improvement of physico-chemical characteristics of FRL is strongly recommended to establish its protective effect as antioxidant (Injac et al., 2008; unpublished paper).

Protective effect of FRL in acute Dox pulmotoxicity

Studies of lung injury produced by airborne agents and environmental toxicants have demonstrated inflammatory process along side elevated cellular concentration of free radicals (Armutchu et al., 2007; Garcon et al., 2001; Meng et al., 2003; Park et al., 2004).

ROS have been implicated in many lung diseases including those associated with exposure to asbestos, nitrogen dioxide, ozone, paraquat, hyperoxia, carbon tetrachloride, and the anticancer drugs bleomycin and Dox. Phagocytic cells have also been implicated in the generation of ROS during inflammation (Matés and Sánchez-Jiménez, 1999; Shull et al., 1991). When studying Dox pulmotoxicity, it is of value to remember that the alveolar epithelial surface is under constant exposure to high oxygen pressure, which makes the lung highly sus-

ceptible to free radical generation. Tissue of the lung is, among other reasons, very sensitive to oxidative stress because of its enlarged oxidative metabolism and reduced antioxidant defense in comparison to other organs, such as the liver (Meadors et al., 2006; Tsai et al., 2006).

Injac et al. (2008a) have confirmed that a single dose of Dox induced pulmotoxicity in rats in acute phase. In the lung of the Dox administered rats, MDA and GSSG levels were significantly elevated at acute stages and supported the hypothesis that a major role is played by radicals in Dox pulmotoxicity (Bogdanovic et al., 2004; Djordjevic-Milic et al., 2006; Djordjevic-Milic et al., 2008; Injac et al., 2008c; Injac et al., 2008a; Injac et al., 2008b; Meadors et al., 2006; Minotti et al., 1990). In all cases biochemical, haematological and histopatological parameters showed a significant decrease in cell injury when FRL was used for pre-treatment during Dox therapy. Very low antioxidant activity in lung tissue is also established by TAS level. TAS test measures the total antioxidant effect of all defense systems in circulation (endogenous and exogenous). A deficiency in any antioxidant will result in a reduction of the TAS. Therefore, high cell injury and low antioxidant activity is found in the Dox treated group and it is significant in comparison to the FRL pre-treated groups. Results for TAS confirm previously presented conclusions for protective influence of FRL on lung, kidney, liver and heart tissue in Dox-induced toxicity (Injac et al., 2008a; Injac et al., 2008b; Injac et al., 2008c). The cardinal advantage of FRL in comparison to other known and investigated antioxidants or protectors is its applicability both as a radioprotector and as an organo-protector (heart, liver, kidney, and lung) during anticancer therapy (radio- and chemo-). However, before a conclusive statement may be made on the potential usefulness of FRL as an adjunct to Dox therapy, there is need for further studies, including human trials.

Protective effect of $C_{60}(OH)_{24}$ in acute Dox nephrotoxicity

In animal trials, Dox demonstrated nephrotoxic activity and produces chronic progressive glomerular disease. In rats with Dox-induced nephropathy, heavy proteinuria associated with swelling and vacuolation of epithelial cells were reported in short-term experiments. Dox-induced nephrosis provides a well-characterized model of progressive renal damage, induced by a uniform challenge at a single point in time. This results in proteinuria and subsequent structural renal damage with a relatively large variability among individual animals. Severe renal damage, extensive glomerular lesions, tubular dilatation, vacuolization of renal glomeruli, protein deposits in tubular lumen, and stromal fibrosis has been observed in long-term studies. These experiments indicated that Dox-induced nephropathy has chronic and self-perpetuating effects leading to terminal renal failure. The dose and the

duration of Dox application required to induce renal diseases vary among investigations. It was demonstrated that a dose of 3 mg/kg of Dox induced renal damage after 6 weeks. On the other hand, nephrotoxicity can be induced by 25 mg/kg of Dox after only 2 days (Dziegiel et al., 2002; Hahn et al., 2004; Liu et al., 2007; Manabe et al., 2001; Oteki et al., 2005; Rook et al., 2005; Yagmurca et al., 2004, Yilmaz et al., 2006). The generally accepted mechanisms of Dox-induced nephrotoxicity are formation of free radicals, iron-dependent oxidative damage of biological molecules, and membrane lipid peroxidation (Liu et al., 2007). For that reason, application of FRL, which is able to react with highly reactive oxygen radical species, could prevent nephrological changes induced by Dox.

Tissue damage after Dox-induced nephrotoxicity was established by changes in the activity of MDA, GSH, GSSG, SOD, CAT, GR, GSH-Px, and α-HBDH, as well as serum and tissue LDH levels. First of all, groups pretreated with FRL have almost normal α-HBDH/LDH ratios compared to the healthy control group. Furthermore, very high levels of LDH activity in the serum without significant changes in α-HBDH activity show strong tissue damage (liver, heart, kidney), after Dox administration, which could be controlled and prevented by application of FRL thirty minutes before Dox-treatment (Injac et al., 2008c).

Radioprotective effect of FRL

Ionizing radiation produces harmful effects on living organisms by inducing enhanced production of free radical species. Exposure of cells to ionizing radiation leads to the formation of ROS that are related to radiation-induced cytotoxicity. The antioxidative enzyme SOD catalyzes the dismutation of superoxide anions into hydrogen peroxide. SOD is a very important parameter, which contributes to increased protection from lethal irradiation to haematopoietic cells. Trajkovic et al. (2005a) investigated potential radioprotective effects of FRL in dose of 100 mg/kg i.p. which was given 30 min before X-irradiation (Trajkovic et al., 2005). Previous reports about radioprotective effect of FRL demonstrated that i.p. administration of 100 mg/kg of FRL 30 minutes before irradiation is satisfactory in comparison to a smaller dose of 10 mg/kg (Bogdanovic et al., 2004). In the relation to FRL radioprotectivity, emphasis must be put on its haemato- and tissue protective effects. The tissue protective effect was especially demonstrated on the spleen, small intestine and lung (Trajkovic et al., 2007).

FUTURE DEVELOPMENTS

It is well known that anticancer the rapy includes either

radio- or chemo- therapy, which are harmful to healthy tissue. In that manner, FRL may act as a cardioprotector (Djordjevic-Milic, 2006; Injac et al., 2008a), hepatoprotector (Injac et al., 2008b; Jacevic et al., 2007), nephroprotector and radioprotector (Trajkovic et al., 2005; Trajkovic et al., 2007), and thus has benefits beyond the other antioxidants described in literature. The findings presented here confirm the protective role of fullerenol $C_{60}(OH)_{24}$ in different *in vivo* Dox-induced cytotoxicity. Despite intensive antioxidative activity, poor solubility is a major disadvantage for its bioapplication. In the future it is necessary to thoroughly investigate all of the functions of the OH groups on the fullerene cage. It is also required to study chronic effects of $C_{60}(OH)_{24}$ on different animal models, in order to establish its protective effects and possible application as a supplement agent in anticancer therapy.

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