Antioxidant and genoprotective properties of the antidiabetic drug metformin under X-ray irradiation

E E Karmanova, S A Abdullaev, V E Ivanov, G M Minkabirova and V I Bruskov
Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, 142290, Russian Federation
Email: bruskov_vi@rambler.ru

Abstract. The antioxidant and genoprotective properties of the antidiabetic drug metformin have been investigated. The research was performed both in vitro and in vivo under X-ray irradiation. It was shown that metformin reduced the formation of hydroxyl radicals and hydrogen peroxide in aqueous solutions irradiated with X-rays. The particular features of its influence on the formation of H2O2 were revealed. It has been established that metformin reduced the formation of 8-oxoguanine, a key biomarker of oxidative damage to DNA, in vitro. The effect of metformin on the frequency of micronucleus formation in polychromatic erythrocytes of bone marrow of irradiated mice was investigated. Its genoprotective and radiomimimatory properties at different concentrations and times of application are shown.

1. Introduction
Sources of radioactive radiation are widely used in various branches of human activity. Therefore, it is urgent to study the physico-chemical mechanisms of their damage to biological systems and the possibilities of neutralizing their harmful effects on living organisms. A number of effective radioprotectors are now known and well-studied. They exhibit protective properties when administered shortly before exposure to radiation [1, 2]. However, the results of their radioprotective actions strongly depend on time of administration prior to irradiation, which largely limits the possibilities of their practical use. Many of these drugs have side actions, and the long-term effects of their use are still unclear [3]. Another class of radioprotective compounds is radiomimitors. These drugs are used after irradiation and have a therapeutic effect. Radiomimitors are applied to compensate and repair damage caused by ionizing radiation [4]. In medicine, a lot of drugs are actively used to treat various diseases. A new and actual area of prevention and treatment of different forms of radiation damage is the search for effective radiomimitory agents among them. This approach does not require the study of their toxicity when used in the therapeutic concentrations for treatment. In this paper, some mechanisms of the radioprotective action of the drug metformin have been investigated.

Metformin (1,1-dimethylbiguanidine hydrochloride) is widely used for treatment of hyperglycemia in patients with type two diabetes. According to numerous published data, metformin has a multifactorial effect on the living organism [5]. It is able to modulate the cellular metabolism and production of mitochondrial reactive oxygen species (ROS) by activation of AMP protein kinase (AMPK) [6, 7]. It has also geroprotective properties [8, 9] and anticarcinogenic activity [10, 11]. There are some data on the radioprotective properties of metformin obtained in animals [12] and cell
cultures [13]. Although metformin has been used for many years in medicine and in fundamental research, the mechanisms of its multiple actions are yet little understood and contradictory [14]. In this paper, the antioxidant and genoprotective properties of metformin have been studied in vivo and in vitro at X-ray irradiation.

2. Methods

2.1. X-ray irradiation
The animals and solutions were irradiated at the Common Use Centre – Group of Radiation Sources of the Institute of Cell Biophysics, Russian Academy of Sciences, using an RUT-15 therapeutical X-ray device (MosRentgen, Russia) at a dose rate of 1 Gy/min (200 kV, 20 mA, focal distance 37.5 cm). Mice (5 animals per group) were irradiated at a dose of 1.5 or 2 Gy.

2.2. Measurement of Hydrogen Peroxide
The concentration of hydrogen peroxide in irradiated solutions of metformin (Sigma-Aldrich, USA) was measured by the method of enhanced chemiluminescence using a luminol–4-iodophenol–peroxidase system [15]. A counter for liquid scintillation Beta-1 (MedApparatura, Ukraine) operating in the mode of single photon counting was used as a highly sensitive chemiluminometer [16]. The high sensitivity of this method allows registration of hydrogen peroxide at concentrations as low as 1 nM. The content of H$_2$O$_2$ was determined by using the calibration curves of chemiluminescence dependence on the known concentration of H$_2$O$_2$ in the solution. The concentration of hydrogen peroxide used for calibration was quantitatively estimated by spectrophotometry at 240 nm using a molar absorption coefficient of 43.6 M$^{-1}$×cm$^{-1}$. All used reagents were Sigma-Aldrich, USA. Freshly prepared bidistilled water used for dilution had pH of 5.6 and conductivity of 120 μS/m.

2.3. Measurement of Hydroxyl Radicals
The concentrations of hydroxyl radicals were determined using coumarin-3-carboxylic acid (CCA), a highly specific to OH-radicals fluorescent probe [17]. The CCA (Sigma-Aldrich, USA) concentration used in the test solutions was 0.5 mM. The intensity of fluorescence was measured on a Cary Eclipse spectrofluorimeter (Varian, Australia) with $\lambda_{ex}$ = 400 nm and $\lambda_{em}$ = 450 nm. Calibration for formation of hydroxyl radicals was done using a commercial preparation of 7-OH-CCA (Sigma-Aldrich, USA) [18]. 240 nM/Gy was used as standard for the radiation-chemical yield of OH radicals [19]. The measurements were carried out in the Common Use Centre of the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences.

2.4. Immunoenzyme assay for determination of 8-oxoguanine (8-oxoG) in DNA
The method used for immunoenzymone assay and properties of 8-oxoG-specific antibodies have been described in detail previously [16, 20]. DNA from salmon sperm (Sigma-Aldrich, USA) was used at a concentration of 400 μg/ml. Metformin was added to DNA solution immediately before the irradiation. The DNA samples were denatured in a water bath for 9 min and cooled on ice. Aliquots of 50 μl were transferred to the wells of immunoassay plates (Costar, USA). DNA was immobilized by a simple dry adsorption procedure with incubation for 3 h at 80°C until the solution was fully evaporated [21]. The blocking of nonspecific sites was performed using 300 μl of solution containing 1% skimmed milk powder (Sampo, Russia) in 0.15 M Tris-HCl buffer, pH 8.5, and 0.15 M NaCl. Then the plates were incubated on a plate shaker for 2 h at 37°C. Formation of antigen complexes anti-mouse IgG-peroxidase conjugate with 8-oxoG-specific antibodies (50 μl/well, 1:1000 (Bio-Rad, USA)) was carried out in the blocking solution by incubation with stirring for 2 h at 37°C. The samples were washed three times (300 μl/well) with a solution of 50 mM Tris-HCl buffer, pH 8.5, and 0.15 M NaCl on a shaker for 3 minutes. After that, a chromogenic substrate (18.2 mM ABTS (Sigma-Aldrich, USA) and 10 mM hydrogen peroxide in 75 mM citrate buffer, pH 4.2, were added. The optical density of the samples was measured with a microwell plate reader Multiscan FC (Thermo
Fisher Scientific, Finland) at 405 nm. The details of the calibration procedure and determination of the concentration of DNA solutions have been published elsewhere [20, 22].

2.5. Micronucleus (MN) test
The method for determination of micronuclei was described in detail previously [15]. Histological samples for MN-test were prepared as in [23], with minor modifications. Males of random-bred white Kv:SHK mice aged 5-6 weeks weighing 25–29 g (nursery Kryukovo, Russian Academy of Medical Sciences) were used. They were maintained under controlled conditions of temperature (23±2 °C) and were given standard commercial mouse feed (Arno, Russia) and drinking water ad libitum. More than 2000 polychromatophilic erythrocytes (PCE) per mouse were counted. Isotonic metformin solutions (Sigma-Aldrich, USA) were injected intraperitoneally (i.p.) to mice 15 min after the irradiation with a dose of 1.5 Gy. In another series of experiments, mice were irradiated at a dose of 2 Gy and suspension of tableted metformin (Merck Serono, France) was administered orally at 300 mg/kg before or after irradiation. The mice were killed by cervical dislocation 28 h after irradiation. Animals handling were done according to institutional guidelines for animal care. Experiments with mice were approved by the bioethics committee of ITEB RAS.

3. The Results and Discussion

3.1. The antioxidant properties of metformin

![Figure 1](image)

Figure 1. (A) Effect of metformin on the generation of hydroxyl radicals in phosphate buffer (1 mM, pH 7.4) irradiated with 5 Gy of X-rays. (B) Effect of metformin on the formation of hydrogen peroxide in phosphate buffer (1 mM, pH 7.4) (1) and in physiological saline (150 mM NaCl) containing phosphate buffer (1 mM, pH 7.4) (2) under the influence of X-ray radiation at a dose of 10 Gy. The background values of hydroxyl radicals and hydrogen peroxide are subtracted from the results. The data are the mean ± SEM (n = 10).

The initial target for the biological impact of ionizing radiation is the aqueous matrix of the organisms with the formation of reactive oxygen species (ROS) as a result of radiolysis of water. Among the ROS the most significant are hydrogen peroxide, as the longest-lived ROS, and hydroxyl radical, as
the most reactive. The manifestation of the antioxidant properties of metformin was studied by the formation of hydroxyl radicals and hydrogen peroxide during the radiolysis of 1 mM phosphate buffer, pH 7.4, at X-ray irradiation. It is shown that metformin at a concentration of 10 mM reduces by 95% the formation of hydroxyl radicals under the influence of X-ray radiation at a dose of 5 Gy (figure 1A). Metformin at a concentration of 1 mM and 5 mM reduces the formation of hydrogen peroxide by X-ray irradiation at a dose of 10 Gy by 35% and 55%, respectively. In physiological saline solution, 35% more hydrogen peroxide was formed than in the control sample of 1 mM phosphate buffer, pH 7.4 (figure 1B). The obtained data indicate that metformin is an effective antioxidant serving as scavenger of hydroxyl radicals, whereas formation of hydrogen peroxide can occur not as a result of the recombination of hydroxyl radicals, but largely as a result of the dismutation of hydroperoxide radicals [16].

3.2. The genoprotective properties of metformin
Oxidative damage to DNA is responsible for the genotoxic effects of ionizing radiation. They lead to such biological processes as mutagenesis, carcinogenesis, aging and age-related degenerative diseases. In this work, the genoprotective properties of metformin were investigated in vitro and in vivo. The most sensitive biomarker of oxidative damage to DNA in vitro - the formation of 8-oxoG - was used to determine the genoprotective properties of metformin. It is found that metformin at a concentration of 250 µM in solution of the salmon sperm DNA exposed to X-ray radiation at a dose of 10 Gy reduced the formation of 8-oxoG by 66%, showing genoprotective properties (figure 2).

Figure 2. Influence of metformin on the formation of 8-oxoguanine in the solution of DNA of salmon sperm in phosphate buffer (1 mM pH 7.4) in vitro under X-ray-irradiation at a dose of 10 Gy. The data are the mean ± SEM (n = 10).

The results of the MN-test showed that metformin, when administered orally at 300 mg/kg, exhibited an obvious genoprotective effect during 6 h with a maximum protection at 15 min after irradiation (figure 3, insertion). Recently it was shown that the same treatment of mice by metformin prolongs their survival rate [24]. Further, the concentration dependence of genoprotective effects of metformin was studied by the MN-test, when metformin was administered to mice at 15 min after irradiation (figure 3). It is shown that metformin (3 mg/kg and 30 mg/kg) administered i.p. after X-ray irradiation at a dose of 1.5 Gy reduces the frequency of formation of PCE with MN by 65 and 75%, respectively. It follows from these data that intraperitoneal injection of metformin at a dose of 30 mg/kg is equivalent to oral administration of metformin at 300 mg/kg.
Figure 3. Influence of metformin on the frequency of micronucleated polychromatic erythrocytes of bone marrow of X-ray irradiated mice: 1 – 1.5 Gy, 2 – 1.5 Gy and metformin intraperitoneally 3 mg/kg, 3 – 1.5 Gy and metformin intraperitoneally 30 mg/kg, 4 – intact control 0 Gy. Insert: Dependence of the frequency of micronucleus formation in polychromatic erythrocytes of bone marrow of mice irradiated at a dose of 2 Gy on time of administration of metformin (per os, 300 mg/kg). The boxplot (or box-and-whiskers plot) consists of a box from the lower quartile of the $x_i$ to their upper quartile, with a crossbar at the median of the $x_i$. Outside of the box, the upper fence is given by $Q_2 + 4(Q_3 - Q_2)$ and the lower fence by $Q_2 + 4(Q_1 - Q_2)$, where $Q_j$ is the $j$th quartile hence $Q_2$ is the median [25]. The whiskers are the horizontal lines going from the box to the most extreme values inside the fences. The dots indicate the position of the data.

According to Stone et al., all the agents that are able to correct radiation injuries can be classified into protectors, mitigators, and treatments depending on the time of their administration [2]. The obtained data demonstrate the possible radiotherapeutic effect of metformin given orally and intraperitoneally immediately and shortly after irradiation. Thus, the antidiabetic drug metformin shows antioxidant, genoprotective and radiomitigating properties under X-ray irradiation and is a promising compound for further studies of its radioprotective potential.

References

[1] Weiss J F and Landauer M R 2009 History and development of radiation-protective agents Int. J. Radiat. Biol. 85 539–73

[2] Stone H B et al. 2004 Models for evaluating agents intended for the prophylaxis, mitigation and treatment of radiation injuries: Report of an NCI Workshop, 3–4 December 2003 Radiat. Res. 162 711–28

[3] Hosseinimehr S J 2007 Foundation review: Trends in the development of radioprotective agents Drug Disc. Today 12 794–805

[4] Greenberger S, Clump D, Kagan V, Bayir H, Lazo J S, Wipf P, Li S, Gao X and Epperly M W 2012 Strategies for discovery of small molecule radiation protectors and radiation mitigators Frontiers in oncology 1 59

[5] Xu G et al 2015 Metformin ameliorates ionizing irradiation-induced long-term hematopoietic
stem cell injury in mice *Free Radical Biology and Medicine* **87** 15–25

[6] Zhou G et al 2001 Role of AMP-activated protein kinase in mechanism of metformin action *The Journal of clinical investigation* **108** 1167–74

[7] Toyama E Q, Herzig S, Courchet J, Lewis T L, Losón O C, Hellberg K and Shaw R J 2016 AMP-activated protein kinase mediates mitochondrial fission in response to energy stress *Science* **351** 275–81

[8] Anisimov V N 2013 Metformin. Do we finally have an anti-aging drug? *Cell cycle* **12** 3483–9

[9] Cabreiro F et al 2013 Metformin retards aging in C. elegans by altering microbial folate and methionine metabolism *Cell* **153** 228–39

[10] Lee H, Park H J, Park C S, Oh E T, Choi B H, Williams B, Lee C K and Song C W 2014 Response of breast cancer cells and cancer stem cells to metformin and hyperthermia alone or combined *PloS One* **9** e87979

[11] Koritzinsky M 2015 Metformin: a novel biological modifier of tumor response to radiation therapy *Int. J. of Radiat. Oncology• Biology• Physics* **93** 454–64

[12] Miller R C, Murley J S and Grdina D J 2014 Metformin exhibits radiation countermeasures efficacy when used alone or in combination with sulphhydryl containing drugs *Radiat. Res* **181** 464–70

[13] Muaddi H, Chowdhury S, Vellanki R, Zamiara P and Koritzinsky M 2013 Contributions of AMPK and p53 dependent signaling to radiation response in the presence of metformin *Radiotherapy and Oncology* **108** 446–50

[14] Pietrocola F and Kroemer G 2017 Metformin: a metabolic modulator *Oncotarget* **8** 9017

[15] Asadullina N R, Usacheva A M, Smirnova V S and Gudkov S V 2010 Antioxidative and radiation modulating properties of guanosine-5’-monophosphate *Nucleosides Nucleotides Nucleic Acids* **29** 786–99

[16] Bruskov V I, Malakhova L V, Masalimov Zh K and Chernikov A V 2002 Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA *Nucleic Acids Res* **30** 1354–63

[17] Manevich Y, Held K D and Biaglow J E 1997 Coumarin-3-carboxylic acid as a detector for hydroxyl radicals generated chemically and by gamma radiation *Radiat. Res* **148** 580–91

[18] Kachur A V, Manevich Y and Biaglow J E 1997 Effect of purine nucleoside phosphates on OH-radical generation by reaction of Fe^{2+} with oxygen *Free Radic. Res* **26** 399–408

[19] Ward J F 1988 DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation and reparability *Progr. Nucl. Acid Res. Mol. Biol.* **35** 95–125

[20] Smirnova V S, Gudkov S V, Shhtarkman I N, Chernikov A V and Bruskov V I 2005 The genotoxic action of uranyl ions on DNA in vitro caused by the generation of reactive oxygen species *Biofizika* **50** 456–63

[21] Gudkov S V, Shhtarkman I N, Chernikov A V, Usacheva A M and Bruskov V I 2007 Guanosine and inosine (riboxin) eliminate the long-lived protein radicals induced X-ray radiation *Dokl. Biochem. Biophys.* **413** 50–3

[22] Shhtarkman I N, Gudkov S V, Chernikov A V and Bruskov V I 2008 Effect of amino acids on X-ray-induced hydrogen peroxide and hydroxyl radical formation in water and 8-oxoguanine in DNA *Biochemistry-Moscow* **73** 470–8

[23] Karp O E, Gudkov S V, Garmash S A, Shhtarkman I N, Chernikov A V and Bruskov V I 2010 Genotoxic effect of long-lived protein radicals in vivo generated by X-ray irradiation *Dokl. Biochem. Biophys.* **434** 250–3

[24] Abdullaev S, Minkabirova G, Karmanova E, Bruskov V and Gaziev A 2018 Metformin prolongs survival rate in mice and causes increased excretion of cell-free DNA in the urine of X-irradiated rats *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **831** 13–18

[25] Rousseeuw P J, Ruts I and Tukey J W 1999 The bagplot: a bivariate boxplot. *The American Statistician* **53** 382–7