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PRODUCTION OF NITRIC OXIDE BY HYPOXIC RADIOSENSITIZER SANAZOLE

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Aim: **In this work we investigated the ability of hypoxia-selective radiosensitizer — sanazole to produce nitric oxide (NO).** ***Methods:*** **NO formation was determined by spectophotometric method in the reaction with sanazole and oxyhemoglobin. In suspensions of lymphoma EL-4 and mastocytoma P 8815 cell NO production was estimated indirectly as nitrite concentration in the supernatant fraction.** ***Results:*** **Transformation of oxyhemoglobin by sanazole to methemoglobin suggested the dissociation of nitro group in aqueous solution and denitration of molecules. Addition of sanazole to hypoxic tumor cell suspension resulted in the increase of nitrite content in tissue culture medium.** ***Conclusion:*** **Presented data suggest the ability of sanazole to produce NO that may be important in a probable mechanism for antitumor and immunomodulating properties of this radiosensitizer.**
Key Words: **tumor cells, hypoxic radiosensitizer, sanazole, nitric oxide.**

The major class of hypoxia–selective radiosensitizers — nitroazoles is widely used in clinical oncology. These compounds acting indirectly can kill radioresistant hypoxic cells and increase the efficiency of both radiotherapy and cell cycle active chemotherapeutic agents [6, 23]. The clinical use of the majority of radiosensitizers such as metronidazole and misonidazole is limited by their neurotoxicity. However, in last decade a number of new nitrotriazole derivatives with radiosensitizing activity were developed [7, 10, 20]. Sanazole, 3–nitrotriazole derivatives (substance AK–2123), was presented as a new perspective hypoxic cell radiosensitizer due to its lower toxicity and higher radiosensitizing effect [18].

Sanazole, as other electron affinic sensitizers, substantially increased the efficiency of radiation therapy, cytostatic drugs and hyperthermia [1, 16]. Nitroazole–like radiosensitising drugs appear to exhibit pronounced toxicity towards hypoxic cells resulted from multiple biochemical effects including DNA damage and binding to cellular macromolecules [8, 22]. The antitumor effect of sanazole has been shown on B–16 melanoma cells [11]. Many of the above effects are associated with electron transfer to nitro group of the sensitizer and generation of reduction products [22]. However, subsequent metabolic transformation of AK–2123 is not clear yet. It has been previously suggested that reduction of nitrocompounds in cells may be accompanied by production of nitric oxide (NO) [2]. NO has a variety of biological functions including regulation of signal transmission pathways and influence on different cellular responses such as vascular relaxation, platelet aggregation, inflammation [24], proliferation and apoptosis [9]. It is important that NO donors induced cytostatic effect in human cancer cells [17] and their apoptosis [14]. NO has been shown to be generated in the reaction of denitration registered during formation of hemoglobin nitrosocomplexes. The present study was undertaken to determine whether sanazole might be a donor of NO in different biological systems.

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Abbreviations used: NO — nitric oxide.

Spectrophotometric assays. Changes in the visible spectrum during the interaction of NO with oxyhemoglobin were measured using spectophotometer Specord M40 (Germany). NO formation was determined in the reaction with sanazole and oxyhemoglobin according to [15]. The absorbance decrease is directly proportional to the amount of NO formed [3]. Spectrophotometric measurement of oxyhemoglobin conversion to methemoglobin was carried out with sodium nitrite (10^{-4} M) for 15 min (every 5 min) and with sanazole (0.02%) for 2 h (every 30 min). The oxyhemoglobin–methemoglobin conversion (indicating NO formation) was evaluated by change in the absorbance at 578 nm *vs* 592 nm using a molar absorption coefficient of 11.2 $mM^{-1}cm^{-1}$.

Preparation of tumor cells. Mouse mastocytoma P815 and lymphoma EL–4 tumor cells were maintained by weekly transplantation ($7 \cdot 10^6$ cells/mouse) in the abdominal cavity of DBA/2 and C57Bl/6 mice, respectively. The ascitic fluid was harvested from the abdominal cavity of tumor–bearing mice on the 7th day after transplantation and tumor cells were washed three times with RPMI –1640 medium (Vector, Russia). Cell viability determined by trypan blue exclusion was over 97%. Finally, the cells were suspended in RPMI–1640 medium supplemented with 10% heat–inactivated fetal bovine serum (ICN, USA), 2 mmol/L l–glutamine and 100 μg/ml gentamycin.

For experiments cells were kept in air: CO_2 (95 : 5) incubator at 37 °C. Hypoxia was induced by nitrogen gas passing through the air over tumor cell suspension. The gas flow was continued during the whole incubation period.

Determination of nitrite. In tumor cell suspension NO production was determined indirectly as nitrite concentration in the supernatant fraction. The nitrite concentration was determined using Greiss reagent [5] containing equal volumes of 0.1% N–(1–naphtil) ethilenediamine (Sigma, USA) and 1% sulfanilamide (Sigma, USA) in 5% phosphoric acid (Sigma, USA). 100 μl of supernatant fraction was mixed with an equal volume of Greiss reagent and incubated for 10 min at room temperature. Absorbance was determined at 543 nm on Multiscan EX plate reader (Thermo Labsystems). A calibration curve for the nitrite concentration was established using sodium nitrite solution (0.1–1.0 μM).

Statistics. All the data shown are average of three separate experiments. The statistical evaluation was achieved by Student's *t*–test to identify significant differences.

Spectrophotometric analyses of oxyhemoglobin–methemoglobin conversion were performed to investigate a possibility of NO to be formed from sanazole. Fig. 1 shows the absorption spectrum of oxygemoglobin, and spectral changes after sanazole addition to hemoglobin solution. The spectrum of oxyhemoglobin had two maximums, at 578 nm and 540 nm. After 2 h incubation of oxyhemoglobin with sanazole (1 mM) we observed decrease in the absorbance. The rate of NO production by sanazole was 176 μM/min. Similar changes in spectrum were observed after addition of 0,1 mM of sodium nitrite to solution of oxyhemoglobin. Although the rate of NO production by sodium nitrite was higher and was about 7.28 mM/min, similar transformation of oxyhemoglobin by sanazole proved the dissociation of nitro group in aqueous solution and denitration of molecules.

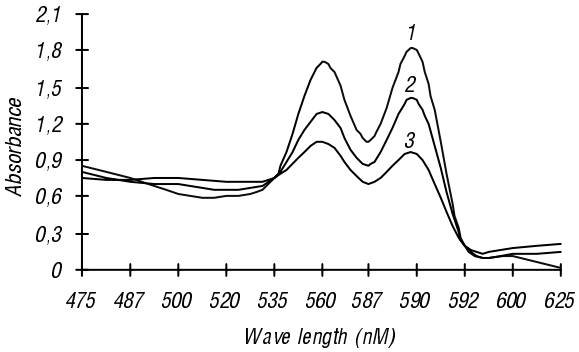

Fig. 1. Light absorbance spectrum of oxyhemoglobin (*1*) and its conversion by 1 mM of sanazole (*2*) and 0,1 mM of sodium nitrite (*3*)

To investigate whether NO was also formed in tumor cell suspension following their incubation with sanazole, we evaluated the nitrite accumulation as the final product of NO formation. Addition of sanazole at concentration 1 mM to P 815 mastocytoma and lymphoma EL–4 tumor cells resulted in accumulation of nitrite in both tissue culture mediums (Fig. 2). The concentration of nitrite raised from 3.00 ± 0.25 μM/10^6 cells in lymphoma EL–4 cell culture supernatant to 5.44 ± 0.38 μM/10^6 cells in mastocytoma P 8815 cell culture supernatant after 3 h incubation with 0.02% sanazole. An increase of nitrite content was observed only in hypoxic tumor cells, whereas its content in cells with normal oxygenation was equal to control and ranged 2.2 μM and 4.7 μM in lymphoma EL–4 and mastocytoma P 8815 cells, respectively.

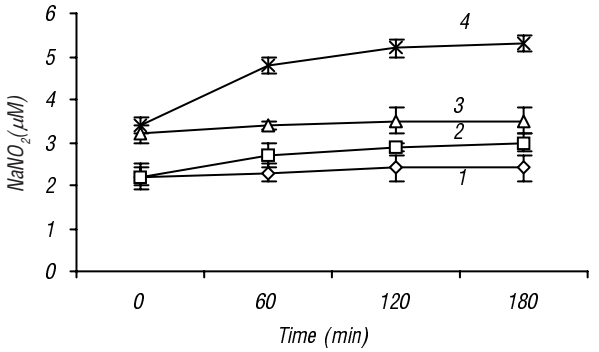

Fig. 2. Nitrite formation in lymphoma EL–4 (*2*) and mastocytoma P815 (*4*) cellular suspension in the presence and in the absence (*1, 3*) of sanazole

The present findings provide the evidence of NO formation from sanazole. The release of NO was proven by experiments with oxyhemoglobin. Similar changes of oxyhemoglobin spectra after addition of sanazole and sodium nitrite give the evidence in the increase of NO–hemoglobin complex. Because the rate of oxyhemoglobin conversion by sodium nitrite was significantly higher than by sanazole, our data suggest that the effects of these compounds differ in time. Reaction of oxyhemoglobin with sodium nitrite occured within some minutes whereas formation of NO from sanazole required hours.

Incubation of ascitic tumor cells with sanazole clearly demonstrated the time–dependent increase of NO formation. The augmentation of NO content was observed in only hypoxic tumor cells. Several enzymes have been found to participate in bioactivation including cytochrome P450 reductase, cytochrom B_5 reductase, adrenodoxin reductase and xanthine oxidase [19]. Previous studies have shown that nitroazoles are more toxic to mammalian cells under hypoxia than under aerobic conditions [22, 23]. Our findings point that at least a part of this toxicity may be associated with NO production. Toxic effect of NO on tumor cells is well known. NO has been demonstrated to induce fragmentation of DNA [4], to alter ribonucleotide reductase activity in adenocarcinoma cells [12], and to reduce thymidine incorporation in DNA of melanoma cells [13].

There are many evidences that production of NO is very important for different cellular functions. NO is a biological messenger which is involved in different regulatory processes in tissues and plays the crucial role in intracellular communications. Antitumor, antimetastatic and immunomodulatory effects of sanazole were demonstrated by some authors [11, 21].

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ПРОДУКЦИЯ ОКСИДА АЗОТА ГИПОКСИЧЕСКИМ РАДИОСЕНСИБИЛИЗАТОРОМ — САНАЗОЛОМ

Цель: **в работе исследована способность гипоксического радиосенсибилизатора — саназола продуцировать оксид азота.** ***Методы:*** **образование оксида азота определялось спектрофотометрическим методом в реакции саназола и оксигемоглобина. В суспензии клеток лимфомы EL-4 и мастоцитомы P 8815 продукция оксида азота оценивалась косвенно как концентрация нитрита во фракции супернатанта.** ***Результаты:*** **превращение оксигемоглобина в метгемоглобин под действием саназола свидетельствует о диссоциации нитрогруппы в водном растворе и денитрировании молекул саназола. Добавление саназола к суспензии гипоксических опухолевых клеток приводило к увеличению содержания нитрита в культуральной среде.** ***Выводы:*** **представленные данные свидетельствуют о способности саназола продуцировать оксид азота, что может быть важным в механизме противоопухолевого и иммуномодулирующего действия этого радиосенсибилизатора.**

Ключевые слова**: опухолевые клетки, гипоксический радиосенсибилизатор, саназол, оксид азота.**