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RADIATION RESPONSE OF E. COLI AFTER COMBINED TREATMENT WITH MISONIDAZOLE AND WR-2721 AT VARIOUS OXYGEN CONCENTRATIONS

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Abstract

It has been reported that the aminothiol compound WR-2721 is a promising radioprotective agent and in combination with misonidazole (MISO) seems to be of therapeutic benefit. Since the radiomodification is oxygen-dependent, the actual oxygen status of cells and the surrounding media is an important factor influencing their effectiveness. *Escherichia coli* B/r radioresponse was studied either alone or in combination with these compounds at various oxygen concentrations ranging from anoxia to high oxygen content. WR-2721 had a protective effect under anoxic conditions and gave overall protection when oxygen was present. The maximum protection was seen at 3.2% O_2 in N_2 (PF 2.08). In combination with MISO the hypoxic sensitization of MISO was completely abolished by WR-2721, resulting in radioprotection under hypoxic conditions as well. Under euoxic conditions MISO was able to reduce the protective effect of WR-2721 by about 21%. According to our results MISO and WR-2721 influence each other in their radiomodifying effect in either fixation or repair of the radiation-induced damage.

Key words: Radiation modification, WR-2721, misonidazole, oxygen, E. coli.

The possibility of chemically modifying radiation sensitivity has been recognized for several years. However, such an effect with clinical potential in cancer therapy can be evoked either by selective sensitization of tumor cells or selective protection of normal tissues. It would be preferable for the sensitizing agents to sensitize hypoxic tumor cells, with the protective agents selectively protecting normal tissues. It can be assumed that the combination of these agents would also confer therapeutic benefits. Misonidazole (MISO), the well-known electron-affinic sensitizer, is able to enhance the radiosensitivity of hypoxic cells in an 'oxygen-mimetic' manner by interacting with short-lived radiation-induced free radicals (1). The sulfhydryl radioprotectors have been shown to decrease radiation-induced damage in normal tissues. It is thought that they can enhance radioresistance by repairing lesions formed directly in vital molecules or by scavenging free radicals that could react with biologically important targets (2).

WR-2721, the thiophosphate derivate of cysteamine, is one of the most effective less toxic agents with a protective effect widely documented (3, 4). Mechanistic studies have been complicated due to the apparent need for dephosphorylation ('activation') of the compound and lack of uptake by certain cells and lack of a quick, convenient and specific assay for the parent and dephosphorylated forms of the drug both in vitro and in vivo (5). Conventionally, WR-2721 and its free thiol form (WR-1065) have been shown to be more effective under euoxic conditions, both in vitro and in vivo (3–5).

It has been demonstrated, too, that the addition of MISO reduces the protective ability of WR-2721 (6). Rojas et al. (2) reported that MISO and WR-2721 are not independent in their radiomodifying action on tumor or normal tissues. Oxygenation is obviously an important factor in thiol radioprotection as well as in MISO sensitization (2). The actual oxygen status of cells and the surrounding media are critical for the effectiveness of these drugs and correlate with the endogenous sulfhydryl concentration as well (7).

It was hoped that the present studies would contribute to our knowledge on the mechanism of the oxygen-dependent radiomodification of MISO and WR-2721 in combination. The combined treatment was carried out on *Escherichia coli* cells. Bacteria are excellent tools for inves-

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tigating the radiomodifying effects of protectors and sensitizers at the cellular level, eliminating the gross concentration gradients of other metabolites inevitably present in organized tissue (8). The present experiments were designed to get detailed information about the behavior of these two compounds at well-defined oxygen concentrations, covering a wide range.

Material and Methods

E. coli B/r (ATCC No. 23227) were grown to stationary phase in liquid minimal medium (9). Cells were washed three times by centrifugation and resuspended in 0.15 mol/l TRIS buffer at pH = 8. The plating medium was minimal medium supplemented with 1.5% oxoid agar No. 1. Colonies were counted after overnight incubation at 37°C. Gamma radiation facility was an RH-γ-30 ^{60}Co apparatus at a dose rate 60.74 Gy min^{-1} as determined by Fricke dosimetry. Cell suspensions containing 3×10^7 cells were equilibrated with various oxygen-nitrogen gas mixtures (0.7%, 1.4%, 1.9%, 3.2%, 5.1%, 21.00%, O_2 in N_2 and pure nitrogen) for 30 min before irradiation. The oxygen concentration was measured by oxygen electrode (Biological microanalisator, Radelkis, Hungary). The gas flow was continued throughout irradiation. The irradiations were carried out at 4°C. Misonidazole, (Angvar Chem. Inc., New York) was dissolved in TRIS buffer and 10 mmol/l was added to the suspension 30 min before irradiation.

WR-2721/S-2-(3 aminopropylamino) ethyl phosphorothioic acid/ was kindly provided by Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, Natl. Cancer Inst. Bethesda, USA. It was stored at −20°C and freshly prepared for each irradiation. The desphosphorylation was carried out by acidic hydrolysis described by Fatome et al. (10) and was added to the cells at a concentration of 6 mmol/l, 30 min before the start of irradiation. Dose–effect curves were constructed from experimental points each being determined by at least four replicate plate counts. Each point was calculated from 5–7 experiments. The survival curves were described by the following equation, which relates to the fractional survival (S/S_0) and dose (D):

$$S/S_0 = n \exp(-kD) \quad (1)$$

The inactivation constant (k) and the extrapolation number (n) are characteristic of the organism and the irradiation conditions. To compare the degree of protection of *E. coli* B/r by WR-2721 alone or in combination with MISO, the protection factor (PF) and the sensitizer enhancement ratio (SER) of MISO were calculated from the survival curves. The glutathione (GSH) content of *E. coli* cells was measured by the method by Cohn & Lyle (11).

10^8–10^9 cells ml of *E. coli* B/r in stationary phase were centrifuged and resuspended in 0.15 mmol/l TRIS buffer containing 6 mmol/l dephosphorylated WR-2721 or 10 mmol/l MISO respectively. The cells were incubated with the agents in ice for one or two hours and washed with 0.02 mol/l EDTA Na_2. After removal of the supernatant the pellets were stored in liquid N_2. The pellet was thawed in 2 ml 0.02 mol/l EDTA (Na_2) after 24 h and sonicated for 10 min. The protein content of cells was determined conventionally.

Results and Discussion

The present study was undertaken to investigate the oxygen-dependent radiomodifying action of MISO sensitization and WR-2721 protection either alone or in combination, at a wide range of oxygen concentration. It has been previously postulated by Denekamp et al. (12) that the effect of these compounds is oxygen-dependent and that they in combination are in a sense mirror images of each other. Thiols protect by competing with oxygen or similar sensitizers for repair or fixation of free radical-induced lesions. By formation of peroxyl radicals, oxygen would lead to fixation of damage whereas hydrogen atom donation from thiol group would result in chemical repair of damage (13).

A series of experiments were carried out to determine the relationship between radiation response of *E. coli* B/r and oxygen. The effect of MISO and WR-2721 alone and in combination were also studied on the radiosensitivity of *E. coli* B/r at different oxygen concentrations.

A plot of the inactivation constants as a function of the oxygen concentration is depicted in Fig. 1. The survival curve without any agent shows that the oxygen effect increased rapidly up to 1.9% O_2 in N_2 (OER = 2.01). Further increase in oxygen concentration caused a gradual rise in the response in k value of 22.05 at 21% O_2 in N_2 (OER = 2.61).

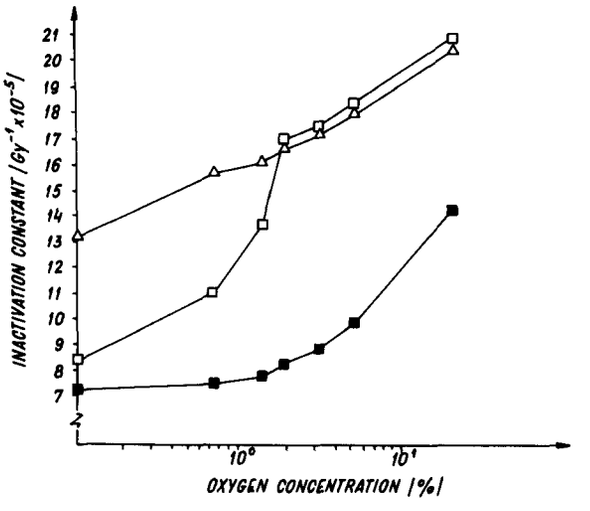

Fig. 1. The radiation response of *E. coli* in the presence of misonidazole and WR-2721. Without agent (□), 6 mmol/l activated WR-2721 (■), 10 mmol/l misonidazole (△).

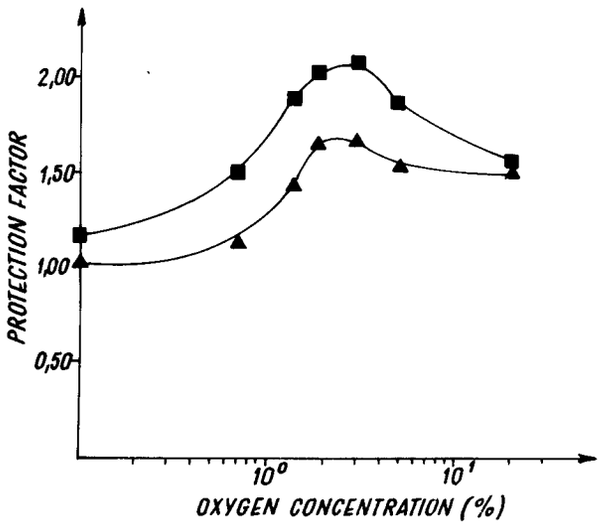

Fig. 2. The combined effect of misonidazole and WR-2721. 6 mmol/l activated WR-2721 alone (■), 6 mmol/l activated WR-2721 and 10 mmol/l misonidazole in combination (▲).

10 mmol/l MISO enhanced the sensitivity of *E. coli* irradiated in nitrogen by about 35% of maximum oxic sensitization (SER = 1.58). With increasing oxygen concentration the sensitizing effect of MISO gradually decreased. The compound gave no sensitization above 1.9% O_2 in N_2.

6 mmol/l WR-2721 had an overall protective effect which was maximal for the cells irradiated at the oxygen concentration of 3.2% O_2 in N_2 (PF 2.08). The protection was reduced in anoxia and in air with protection factors of 1.16 and 1.54 respectively. Radioprotection of *E. coli* cells by 6 mmol/l WR-2721 and 10 mmol/l MISO in combination is demonstrated in Fig. 2. In anoxia the activated aminothiol completely eliminated the hypoxic sensitizing effect of MISO. Overall protection was obtained with this combination of the electron affinic sensitizer and radioprotective drug, when oxygen was present in the medium during irradiation. The dephosphorylated aminothiol protected cells under hypoxic and normoxic condition as well as at high oxygen concentration, even in the presence of more than equimolar concentration of MISO. The most effective protection could be achieved with this combination under normoxic condition with a PF of 1.66. As indicated in Fig. 2, the protective effect of WR-2721, either alone or in combination with MISO, was highly dependent on the actual oxygen concentration. When trace levels of oxygen are present, the radical scavenging and/or hydrogen donating reactions may be more efficient (13). WR-2721 is effective in competing with such a level of oxygen as a H′ donating compound. However, the radical fixation increases with increasing concentration of oxygen and MISO was not able to express its maximum radiosensitization but reduced the protective effect of WR-2721 by about 21% in the presence of 1.9% O_2. Under euoxic condition there is a competition between oxygen and WR-2721, which acts by oxygen-depleting mechanism, as a result of fast thiol oxidation in the extracellular medium as well (14). In our system, WR-2721 preserved its protective effect even in the presence of MISO, throughout the range investigated from anoxia to high oxygen content. This supports the theory favored by Denekamp et al. (7) that additional sulfhydryls will reduce the diffusion range of radiation-induced radicals which can interact with the critical biological molecules. At high oxygen content WR-2721 seems not to be able to express its maximum effect. The addition of 'excess' oxygen overwhelms the sulfhydryls in competition for radiation damage. In air, marked protection was obtained by WR-2721, which was preserved in combination with MISO as well. According to the generally accepted view, MISO does not modify the radiation response at this oxygen level. It was reported by Ward (15) that WR-1065 acts in the same manner as endogenous glutathione, but is at least ten times more efficient in the chemical rapair. Furthermore, the radiosensitization by MISO, as a result of its preincubation effect, is also related to the degree of glutathione reduction (16).

Thiol status seems to have a strong association with the cell's initial response to radiation injury. The change in the GSH content of *E. coli* cells as a result of incubation with 6 mmol/l dephosphorylated WR-2721 is demonstrated in the Table. These data show that there is an increase in cellular GSH content after treatment with radioprotective compounds. On the other hand, there was no significant change after two hours' incubation with MISO. However, when MISO was present during cultivation (18 h), the GSH content was reduced by 20-40%. MISO presumably reacted with the endogenous sulfhydryls and decreased them, but this reaction needed more time, as a consequence of its hypoxic metabolism (17). As observed by Malaise (18), MISO and oxygen also compete with glutathione for radiation-induced lesions. In combination with aminothiols, the increased sulfhydryl level caused by WR-2721 could cope with radical fixation of oxygen and/or MISO (7). Mechanistic study of WR-2721 radioprotec-

Table 1

Percentage of GSH increased by WR-2721 and its free thiol form in E. coli system

Agent	Incubation time	Increasing (%)
WR-2721	1 h	158.1
	2 h	171.1
	4 h	180.5
Activated WR-2721*	1 h	239
	2 h	228
	4 h	208

* The effectiveness of acidic hydrolysis was also studied. It was found that 70.2% of WR-2721 were hydrolysed to its free thiol form, WR-1065.

tion reported by Durand (14) showed two mechanism of action. One was oxygen-independent, presumably due to hydrogen donation, and the other a quantitatively larger oxygen-depleting mechanism most evident in cells on the verge of radiobiologic hypoxia. An alternative mechanism proposed for WR-2721 (1065) is intracellular scavenging of hydroxyl radicals. As presumed by Ward (15), the active local concentration of the sulfhydryl compound required for $OH^{\cdot}$ scavenging and for $H^{\cdot}$ atom donation is approximately equal. Consequently, the mechanism of radioprotection is probably a composite of both actions.

As our data also indicate, there should be a 'mirror' effect of MISO and WR-2721 as postulated by Denekamp (7). The actual oxygenation is one of the most important factors influencing the radiation response of *E. coli* B/r in the presence of radiosensitizing or radioprotecting agents. We presume that there are critical oxygen concentrations, at which the critical target for WR-2721 radioprotection is available and also the condition for MISO sensitization in hypoxia can exist.

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