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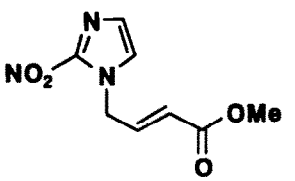
CELLULAR NON-PROTEIN THIOL DEPLETION AND RADIOSENSITIZATION OF HYPOXIC CELLS BY A NOVEL 2-NITROIMIDAZOLE DERIVATIVE POSSESSING AN NPSH-REACTIVE SIDE CHAIN

Sei-ichi Nishimoto* and Ling Zhou

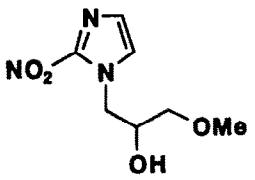
Division of Energy and Hydrocarbon Chemistry
Graduate School of Engineering, Kyoto University
Sakyo-ku, Kyoto 606-01, Japan

Abstract: *The reaction of methyl 4-(2'-nitroimidazolyl)crotonate (1) with glutathione (GSH) in phosphate buffer solution was examined to form the corresponding 1,4-addition products which have been isolated and fully characterized by spectroscopy. The same reaction took place within EMT6/KU cells with intracellular GSH to give the drug-GSH conjugates. The enhanced radiosensitizing activity of 1 on hypoxic EMT6/KU cells by alteration in both D_0 and n parameters of dose-survival curve was suggested as the result of the intracellular NPSH-depletion by the α,β-unsaturated carbonyl side chain of 1.*

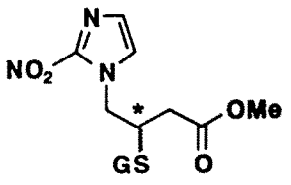
Endogenous non-protein thiols (NPSH), mainly glutathione (GSH, γ-*L*-glutamyl-*L*-cysteinylglycine), play an important role in determining the response of cells to ionizing radiation, especially under hypoxic conditions.[1,2] The degree of ionizing radiation damage to cells is governed by the competitive processes between the actions of damage repair by thiols and the damage fixation by oxygen or oxygen-mimic chemical modifiers.[3,4] The enhancing effect on radiosensitivity of hypoxic cells under reduced thiol level has been observed when thiol-depleting agents are used alone,[5] or in combination with electron-affinic radiosensitizers such as misonidazole (2).[6,7] A number of strategies have been sought to achieve NPSH depletion, including oxidation of GSH to GSSG,[8] formation of GSH conjugate through covalent bond,[9] and inhibition of intracellular GSH synthesis.[5] However, little direct evidence has been hitherto obtained for the intracellular GSH reaction. In connection with our interests in developing electron-affinic compounds with thiol-



1



2: misonidazole



3a,b

depleting action for radiosensitization of hypoxic cells, we have synthesized a series of nitroazole derivatives bearing an α,β-unsaturated carbonyl group in the side chains. Among them, compound **1** demonstrated extraordinarily large enhancement ratio *in vitro*, compared with the corresponding saturated analog. In this communication, we report on the reaction of **1** with GSH both in phosphate buffer solution and in biological cells, and the effect of intracellular NPSH depletion by **1** on radiosensitization of hypoxic EMT6/KU cells.

Compound **1** was synthesized by reaction of 2-nitroimidazole with methyl γ-bromocrotonate in absolute ethanol under basic conditions. Reaction of **1** with GSH in phosphate buffer solution (pH 7.2, 37°C) followed a second-order kinetics with the rate constant (k_2) of 0.74 $mM^{-1} \cdot h^{-1}$. The reaction mixture was separated by HPLC[10] and the structures of the two products were determined by ^{13}C NMR and MS spectra to be the diastereomers of the conjugate addition products **3a,b** in ca. 1:1 ratio.[11] The absolute stereochemistry at the β-carbon of **3a,b** has not been established. Since equal amounts of diastereomers were formed, the chiral centers in GSH have no inductive effect on the newly forming chiral center at the β-carbon atom of **3a,b**. This Michael addition reaction of intracellular GSH to **1** was also examined using EMT6/KU tumor cells. Figure 1 shows the dependence of total intracellular NPSH level in EMT6/KU cells on the concentration of compounds **1** and **2**, when drug exposure was performed for 1 h at 37 °C in air. Thus, treatment of **1** resulted in gradual decrease of the intracellular NPSH level. Compound **1** at 1.0 mM depleted NPSH by more than 80% (from 68.8 nmol/10^7 cells to 12.3 nmol/10^7 cells, see Fig. 1). Similar depletion of the total NPSH in cellular system was also obtained

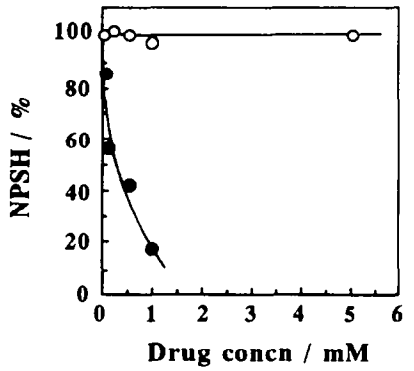


Figure 1. Changes in total intracellular NPSH level of EMT6/KU cells upon exposure to several concentrations of (●) **1** and (○) **2** for 1 h at 37 °C in air. Total amount of NPSH in EMT6/KU cells without drug exposure was 68.6 nmol / 10^7 cells.

with **1** under hypoxic conditions (data not shown). On the other hand, treatment with compound **2** for 1 h at concentrations up to 5.0 mM did not change the intracellular NPSH level (Fig. 1). The remarkable effect of **1** on the reduction of intracellular NPSH level, in contrast to **2**, is attributable to a thiol-reactive function of the α,β-unsaturated carbonyl side chain of **1**. In a separate experiment, formation of the conjugates **3a,b** between **1** and intracellular GSH within EMT6/KU cells was confirmed by HPLC,[10] by reference to the authentic samples prepared from the reaction in phosphate buffer solution as above.

The radiosensitizing effect of **1** possessing the NPSH-reactive side chain on the

hypoxic EMT6/KU tumor cells was then investigated,[12] comparing to misonidazole **2** as a well-documented radiosensitizer. The profiles of dose-survival curves of EMT6/KU cells observed upon X-irradiation with compounds **1** and **2** are shown in Fig. 2. Treatment of hypoxic EMT6/KU cells with 1.0 mM of **1** for 1 h followed by X-irradiation resulted in cell sensitization with 50% decrease of the mean lethal dose (D_0: reciprocal of the slope of linear portion in the dose-survival curve) from 3.2 Gy of control to 1.6 Gy, and reduction of the extrapolation number (n) from 9.0 to 1.5 as well (Fig. 2(a)). Disappearance of the shoulder (non-linear portion at lower doses) of survival curve indicates that compound **1** is capable of sensitizing hypoxic cells efficiently even in the lower radiation dose region. While treatment of 1.0 mM of **2** decreased the D_0 from 3.2 Gy to 2.0 Gy, little alteration of n value (n = 8.0) was achieved in the dose-survival curve, as shown in Fig. 2(b). The sensitizer enhancement ratios (SER: see the legend in Fig. 2) at 1.0 mM were 2.6 for **1** and 1.5 for **2**, respectively. Obviously, the high sensitizing activity of **1**, which is comparable to oxygen enhancement ratio (OER = 2.8), originates from the combined effect on the shoulder and the slope of the dose-survival curve.

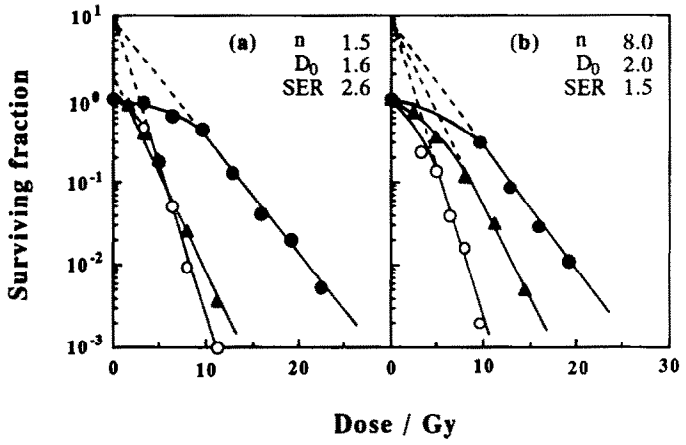


Figure 2. Survival of hypoxic EMT6/KU cells upon X-irradiation after 1h incubation with (▲) 1.0 mM of (a) **1** and (b) **2** under hypoxic conditions (95% N_2+5% CO_2): (●) hypoxic and (○) aerobic (95% air+5% CO_2) controls. The SER was calculated from the ratio of the doses reducing the survival to 1.0 % without and with drug. Oxygen enhancement ratio (OER) was 2.8±0.2. The n values for both hypoxic and aerobic controls were 9.0. The D_0 values were 3.2 Gy for hypoxic control and 1.4 Gy for aerobic control, respectively.

The finding of the distinct effects between compounds **1** and **2** on the profiles of dose-survival curves suggests the importance of NPSH depletion in the radiosensitization of hypoxic cells, particularly in lower radiation dose region. It is reasonable to predict that compound **1** sensitizes hypoxic cells by at least two mechanisms. The effect on the slope is apparently related to the electron affinity of the compound originating from the 2-nitroimidazole ring structure. Since **1** possesses the similar electron affinity with **2**,[13] it

is likely that compound **1** is able to exhibit oxygen-mimic activity in the process of radiation-induced damage of DNA, thereby promoting the radiation inactivation of hypoxic cells.[14] The effect of **1** on the reduction of shoulder in the dose-survival curve might be related to the depletion of cellular NPSH compounds to inhibit their radical scavenging or hydrogen donating action as the protectors.[15] Among several known NPSH-depleting agents, diamide and NEM (*N*-ethylmaleimide) have been found to alter the shoulder of the hypoxic-cell survival curves.[9,16] In view of the clinical importance of dose fractionation regimens in the radiotherapy for tumor control, it is necessary to find less toxic sensitizers which can sensitize hypoxic cells in low radiation dose region. Such sensitizers should be able to remove thiols that compete with intracellular oxidizing species, thus decreasing cell survival upon irradiation.[17] In the clinical study, the radiosensitizer **2** has been revealed to be less effective in the low radiation dose region.[18] The high SER value of the novel compound **1** possessing a thiol-reactive side chain in both low and high radiation dose regions would provide potential application in the fractionation radiotherapy. Further studies in this direction are in progress.

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10. HPLC conditions: column, M&S pack C_{18}, Φ 4.6 x 150 mm; solvent, 15% MeOH in phosphate buffer solution (pH 4.5); delivery rate, 6.0 mL/min; detection, 320 nm. Retention times for **3a,b** are 16.7' and 17.5'.
11. **3a**: $[\alpha]^{20}_D$ -20.11° (c = 0.22, H_2O); ^{13}C NMR (25 MHz, DMSO-d_6) δ 176.2, 175.0, 174.4, 173.0, 172.5, 141.0, 127.8, 122.0, 54.6, 54.0, 53.9, 53.1, 42.5, 41.4, 37.3, 33.3, 31.8, 25.2; MS *m/z* 519 (M^++H). **3b**: $[\alpha]^{20}_D$ +0.34° (c = 0.15, H_2O); ^{13}C NMR (25 MHz, DMSO-d_6) δ 176.0, 175.0, 174.2, 173.0, 172.5, 141.0, 128.0, 122.0, 58.0, 54.5, 53.8, 53.0, 42.3, 41.4, 36.9, 33.4, 31.9, 25.2; MS *m/z* 519 (M^++H).
12. Experimental details of the radiation survival studies on hypoxic EMT6/KU cells can be found in our previous report: Shibamoto, Y.; Nishimoto, S.; Shimokawa, K.; Hisanaga, Y.; Zhou, L.; Wang, J.; Sasai, K.; Takahashi, M.; Abe, M.; Kagiya, T. *Int. J. Radiat. Oncol. Biol. Phys.* **1989**, *16*, 1045.
13. One-electron reduction potentials [$E_{1/2}$ ($S/S^{-\bullet}$) / V vs. Ag/Ag^+] measured in DMF are -1.01 V and -1.04 V for **1** and **2**, respectively. For measurement, see: Shibamoto, Y.; Nishimoto, S.; Mi, F.; Sasai, K.; Kagiya, T.; Abe, M. *Int. J. Radiat. Biol.* **1987**, *52*, 347.
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