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Evidences for Adduct Formation between Intracellular Non-Protein Thiols and Nitroazoles Possessing an α,β-Unsaturated Carbonyl Side Chain and the Effects on Radiosensitization of Hypoxic Cells

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Abstract—Reactivity of a number of nitroazole derivatives bearing an α,β-unsaturated carbonyl group on the side chain toward non-protein thiols (NPSH) was examined both in the phosphate buffer solution and in the biological system. These α,β-unsaturated compounds reacted with NPSH, such as glutathione (GSH) and L-cysteine (Cys), in the buffer solution to afford the 1,4-addition products. The reaction gave a second-order rate constant. The adducts of methyl 4-(2′-nitroimidazol-1′-yl)crotonate (**1**) with GSH and Cys were isolated and characterized as two diastereomers (**7a,b** and **8a,b**) in ca. 1:1 ratio, respectively. Similarly, exposure of EMT6/KU cells to **1** at 1.0 mM for 1 h resulted in depletion of the intracellular NPSH by more than 80%. Over 50% of the depleted NPSH was attributed to the formation of the conjugated diastereomeric adducts. On the other hand, incubation of EMT6/KU cells with **1** at 1.0 mM under hypoxic conditions before X-ray irradiation caused concurrently a sharp reduction of the shoulder of the dose-survival curves (reduced the extrapolation number (n) from 8.0 to ca. 1.0) and an increase in the slope (decreased the mean lethal dose (D_o) to ca. 50% of the control level). The observed effects of **1** on the dose–survival curves were due to the NPSH depletion through the Michael addition occurred in the cellular system. A fairly linear relationship was obtained between the n value and the reduced intracellular NPSH level. It indicated that the shoulder effect of the dose-survival curves of hypoxic cells should be the result of the NPSH depletion by the α,β-unsaturated carbonyl group attached to the nitroazoles.

Introduction

It has been known that the endogenous non-protein thiols (NPSH) play an important role in determining the radiation response of DNA.[1] In mammalian cells, about 90% of NPSH is glutathione (GSH). Thus, the cellular GSH level is a crucial factor that significantly influences the degree of radiation damage. According to the fixation–repair competition hypothesis proposed by Alper,[2] molecular oxygen can enhance the radiation damage on DNA while the endogenous NPSH can protect the irradiated cells. The extent of radiation-induced damage on DNA depends on the relative concentrations of molecular oxygen and NPSH presented in the biological system, particularly at low oxygen tensions.[3,4] The radiation-generated radicals can undergo the competitive reactions with the cellular oxidizing and reducing species. The radicals can be oxidized by the highly reactive oxidizing species such as molecular oxygen, leading to a fixation of damage and cell death. On the other hand, the intracellular reducing species such as GSH reduce the radicals, resulting in a restitution of damage.[5,6] The possible roles of GSH in the radiation response of cells include hydrogen-transferring to DNA radicals, radical scavenging,[7–9] and involving in the enzymatic repair processes.[10]

Since the protective effect of intracellular NPSH to ionizing radiation becomes much more significant under hypoxic conditions,[3,4,10] it raised great interest in studying the influences of cellular thiol contents on radiation response of hypoxic tumor cells. The latter are known to be radiation resistant. Modification of the

Key words: Nitroazoles; NPSH depletion; hypoxic cell; radiosensitization.

Abbreviations: NPSH = non-protein thiols; GSH = glutathione; Cys = L-cysteine; MEA = β-mercaptoethylamine; NEM = *N*-ethylmaleimide; DEM = diethyl maleate; DMF = dimethyl fumarate; BSO = L-buthionine *S*,*R*-sulfoximine; DTNB (Ellmen's reagent) = 5,5′-dithiol-bis(2-nitrobenzoic acid); .

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intracellular GSH level may provide a novel strategy for cancer radiotherapy. A number of studies showed that a reduction in the intracellular thiol contents by GSH-reacting agents, such as *N*-ethylmaleimide (NEM), diethyl maleate (DEM) and L-buthionine *S*,*R*-sulfoximine (BSO), could increase radiosensitivity of tumor cells.[11–14] These studies demonstrated that intracellular NPSH depletion by DEM, or BSO increased the radiosensitization activity of the electron-affinic radiosensitizers such as misonidazole and SR-2508 both in vitro and in vivo when administrated in combination.[15,16] At present, the mechanisms involved in radiosensitizing hypoxic cell by GSH-depleting agents have not been fully understood. They are known to be different from that of the electron-affinic nitroimidazole derivatives. The survival curves of both types of agents were found to have different patterns. The GSH-depleting agents, such as the diamide and BSO, are known to act as "shoulder modifier" and therefore to be much more effective at low radiation doses.[17–20] In contrast, the electron-affinic nitroimidazole derivatives were proposed to sensitize hypoxic cells via a redox-mediated free radical process, giving an oxygen-mimic effect.[21] Their radiosensitizing effects were found to be the same as molecular oxygen and dose-dependent and became less effective at low radiation-dose region.[22–25] Considering the importance of cell responses to radiation at the therapeutically relevant low doses, it is of interest to fully understand the mechanism of radiosensitization of hypoxic cells by NPSH depletion.

In connection with our efforts in developing new compounds with thiol-depleting action for radiosensitization of hypoxic cells, we synthesized a series of nitroazole derivatives bearing an α,β-unsaturated carbonyl group on the side chain.[26,27] These compounds were found to reduce the intracellular NPSH level and to exhibit an enormously high enhancement ratio in vitro (SER_{vitro}), compared with the ordinary electron-affinic nitroazole radiosensitizers. These compounds sensitized hypoxic EMT6/KU cells by not only making the slope steep but also reducing the shoulder of cellular survival, thereby providing a high radiosensitizing capacity at both low and high radiation doses.[27] It is desirable to understand the impacts of the combined effects of NPSH depletion and electron affinity originating from the same molecules on the radiation response of hypoxic cells, particularly in the low radiation dose region. In this study, we examined the NPSH depleting activity of the nitroazole compounds bearing an α,β-unsaturated carbonyl side chain by reacting with a number of non-protein thiols such as glutathione (GSH), L-cysteine (Cys), and β-mercaptoethylamine (MEA) both in the phosphate buffer solution and in the cellular system. The effects of NPSH depletion by these nitroazole compounds on radiosensitization of hypoxic cells and on the profiles of dose–survival curves were discussed.

Results

Nucleophilic addition in phosphate buffer solution

A number of nitroazoles **1**–**5** possessing an α,β-unsaturated carbonyl group on the side chain listed in Table 1 were used to react with the typical NPSH, GSH, Cys, and MEA, in the phosphate buffer solution (pH 7.2, at 37°C). Among the nitroazoles tested, **5** having an α-fluoro-α,β-unsaturated ketone side chain acted similarly as the well-known misonidazole (**6**) and did not react with the thiols at all. In all other cases, the reactions followed the second-order kinetics and the rate constants are summarized in Table 1. The reactivity of NPSH compounds with nitroazoles **1**–**4** commonly increased in the following order of GSH < Cys < MEA. The parent ring structure of the nitroazoles also influenced the reactivity toward NPSH in the order of **3** (3-nitro-1,2,4-triazolyl) < **2** (2-methyl-4-nitro-1,3-imidazolyl) < **1** (2-nitro-1,3-imidazolyl) bearing the same α,β-unsaturated ester side chain. Compound **4** with a β-(aminocarbonyl)-α,β-unsaturated carboxylic acid side chain showed a much lower reactivity toward the thiols. These results indicated that different side chain structures as in nitroazoles **1**, **4** and **5** had a quite diverse effect on the reactivity. The α,β-unsaturated ester side chain in compounds **1**–**3** showed a higher reactivity with NPSH.

Table 1. Second-order rate constants (k_2) for the reactions of nitroazoles **1**–**6** with NPSH in phosphate buffer solution (pH 7.2, 37°C)

Compd	Structure	k_2/mM^{-1} h^{-1}		
		GSH	Cys	MEA
1	**Ar**-$CH_2CH{=}CHCO_2Me$	0.74	1.88	2.70
2	**Ar′**-$CH_2CH{=}CHCO_2Me$	0.60	1.48	2.17
3	**Ar″**-$CH_2CH{=}CHCO_2Me$	0.47	1.20	1.39
4	**Ar**-$CH_2CH(OH)CH_2NHCOCH{=}CHCO_2H$	0.14	0.12	0.20
5	**Ar**-$CH{=}CFCOCH_2CH_2CONH_2$	0	0	0
6	**Ar**-$CH_2CH(OH)CH_2OCH_3$	0	0	0

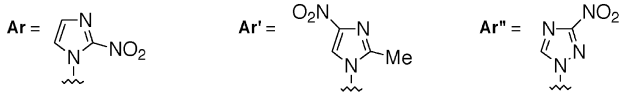


Figure 1 shows variations of the second-order rate constants (k_2) for the nucleophilic 1,4-addition of GSH, Cys, and MEA with **1** as a function of pH. The reaction rates increased rapidly with increasing pH. It is consistent with the nucleophilic addition mechanism. The pH effect on the reactivity of these thiols should be similar and the observed reactivity order of GSH < Cys < MEA with **1** is correlated with the bulkiness of the nucleophiles. All NPSH used in this study contain two functional groups, i.e. the mercapto group and the amino group. Both of them may act as a nucleophile and react with the α,β-unsaturated carbonyl compounds. According to the kinetic study by Friedman and co-workers,[28] at comparable p*K* values and steric environments, sulfur anions were about 280-fold much more reactive than amino groups, the mercaptide group in aminothiols attacked at the electrophilic center of α,β-unsaturated carbonyl compound preferentially. Thus, the possible reaction of the amino group of the NPSH with **1** could be ignored. Scheme 1 illustrates the Michael addition of RSH to the α,β-unsaturated ester moiety on the side chain of nitroimidazole **1** to form a mixture of adducts **7a**,**b**, **8a**,**b**, and (±)-**9**, respectively.

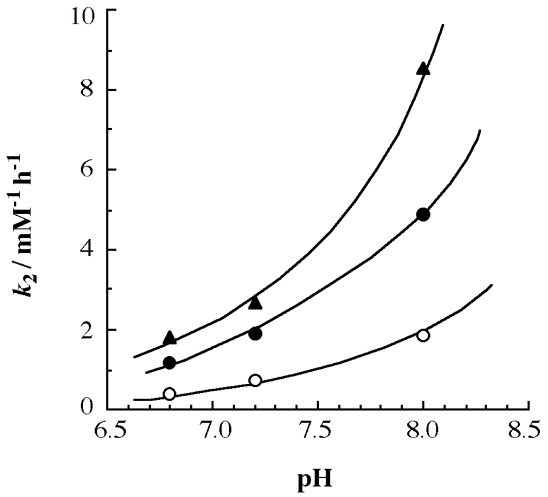


Figure 1. The pH dependence of the second-order rate constants (k_2) for reactions of NPSH compounds and **1** in the phosphate buffer solution at 37°C: GSH (open circles); Cys (solid circles); MEA (solid triangles).

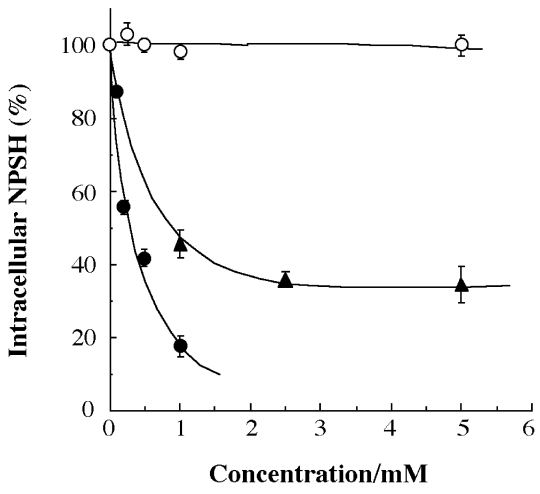
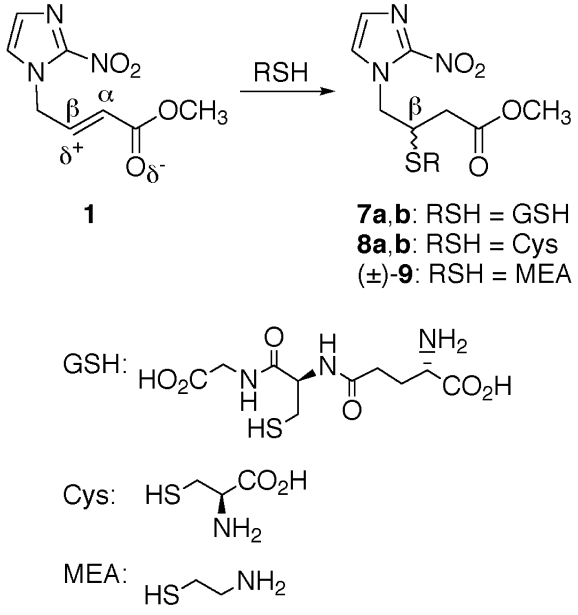


Figure 2. Changes in total intracellular NPSH level of EMT6/KU cells upon exposure to **1** (solid circles), **2** (solid triangles), and **6** (open circles) for 1 h at 37°C in air. Total amount of NPSH before treatment with nitroazole compounds was 68.6 nmol/10^7 cells. Vertical bars represent standard errors (SE), calculated from more than three separate determinations.



Scheme 1. The Michael addition of NPSH with **1**.

The product mixtures were purified by preparative HPLC, and then characterized by ^{1}H and ^{13}C NMR and MS. Compounds **7a** ($[\alpha]_D^{20} = -20.11$) and **7b** ($[\alpha]_D^{20} = +0.34$) were confirmed to be the diastereomeric adducts of **1** with GSH. Similarly, compounds **8a** ($[\alpha]_D^{20} = -20.71$) and **8b** ($[\alpha]_D^{20} = +1.55$) were the adducts of **1** with Cys. The ratios of both pairs of diastereomers **7a**,**b** and **8a**,**b** were nearly 1:1. The configuration of the newly generated chiral β carbon cannot be determined. The adduct (±)-**9** is a 1:1 mixture of enantiomers.

Reactions within EMT6/KU cells

The reaction of intracellular NPSH toward nitroazoles possessing an α,β-unsaturated carbonyl group side chain was examined using the EMT6/KU cells. Figure 2 shows the dependence of total cellular NPSH level in EMT6/KU cells on the concentration of **1**, **2** and **6**. The initial amount of total intracellular NPSH in EMT6/KU cells before treatment with nitroazole compounds was 68.6 nmol/10^7 cells. After 1 h exposure to **1** and **2** at 37°C, total intracellular NPSH decreased with increasing drug concentrations. The remaining intracellular NPSH after treatment with **1** (1.0 mM) for 1 h was 12.3 nmol/10^7 cells, accounting for only 17.9% of the initial intracellular NPSH level. Administration of **2** up to 5.0 mM resulted in a decreased NPSH level of 34.5%. Treatment using **3** at 1.0 mM for 1 h also caused a reduction of intracellular NPSH to 47.8% of the control (data not shown). In contrast, there was no depletion of intracellular NPSH in EMT6/KU cells after administration of compounds **4** and **5**. Similarly, treatment of EMT6/KU cells with misonidazole (**6**) up to 5.0 mM for 1 h resulted in little decrease in the intracellular NPSH level (Fig. 2). The extent of intracellular NPSH depletion by these nitroazole compounds having a carbonyl-conjugated double bond in the side chain is apparently well correlated to the NPSH reactivity (k_2 value) determined in the phosphate buffer solution (Table 1). The remarkable effect of **1** on the reduction of intracellular NPSH is attributed to the thiol-reacting capability of the conjugated carbonyl side chain.

In order to identify the products formed within the EMT6/KU cells, the reaction of **1** with intracellular NPSH was monitored by HPLC using authentic samples. Figure 3 presents typical HPLC chromatograms indicating change of total intracellular NPSH level before and after drug incubation. After 1 h treatment with **1** at 37°C, the cellular supernatant solution (pH 3.0) containing low molecular weight (< 10,000) components in EMT6/KU cells was filtered through a Millipore filter and subjected to HPLC analysis. Figure 3a shows the HPLC profile of the cellular supernatant of the control without drug treatment. To confirm that there was no reaction between **1** and cellular thiols occurring during the processes of post treatment under

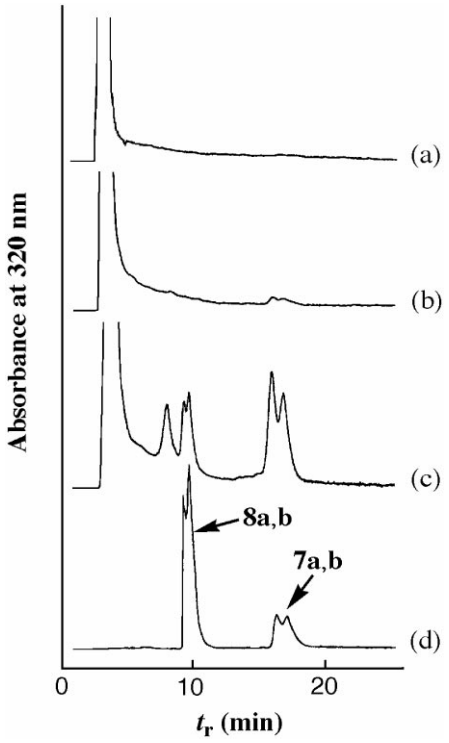


Figure 3. Typical HPLC chromatograms indicating the formation of diastereomeric adducts in the nucleophilic 1,4-addition of cellular GSH and Cys to **1** within EMT6/KU cells. After treatment with **1** (1.0 mM) at 37°C, the cellular supernatant solution (pH 3.0) containing low molecular weight (< 10,000) components was filtered through a Millipore filter and was subjected to HPLC analysis using phosphate buffer solution (pH 4.5) containing 15% MeOH as the eluent with UV detection at 320 nm. (a) control, without drug treatment; (b) at $t = 0$ min, cells were washed by phosphate buffer solution (pH 3.0) immediately after treatment with **1**; (c) at $t = 60$ min, cells were exposed to **1** for 60 min, and then washed by phosphate buffer solution (pH 3.0); (d) authentic samples synthesized from **1** and GSH (**7a**,**b**) or Cys (**8a**,**b**).

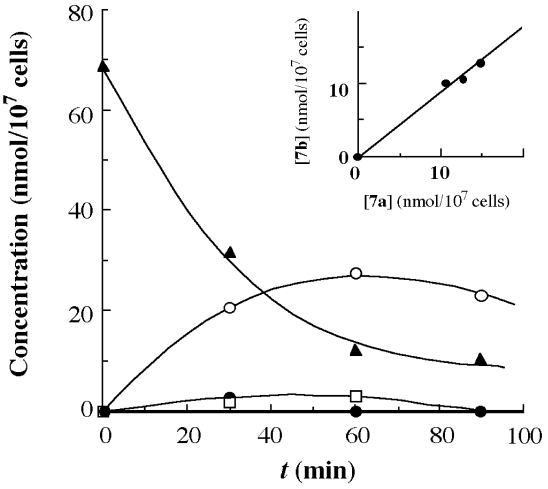


Figure 4. Time-courses of the decrease in total intracellular NPSH concentration (solid triangle), the formation of **7a**,**b** (open circles) and **8a**,**b** (open squares), and concentration of the penetrated **1** (solid circles) within EMT6/KU cells after treatment with 1.0 mM of **1** at 37°C. The insert illustrates almost equal yields of diastereomers **7a** and **7b** derived from nucleophilic addition of cellular GSH to the α,β-unsaturated carbonyl side chain of **1**.

acidic condition (pH 3.0), cells after short time exposure to **1** ($t = 0$ min) were immediately washed with PBS (−) solution and the supernatant was then extracted with phosphate buffer solution (pH 3.0). The HPLC chart of this sample is given in Figure 3b, showing almost absence of the adducts. Figure 3c illustrates formation of the adducts after incubation of **1** with the cells for 1 h at 37°C. The new peaks at $t_r = 9.0$ and 9.4 min are confirmed, compared to the HPLC profiles of the authentic samples given in Figure 3d, to be the diastereomeric adducts **8a**,**b** formed from **1** and Cys while the pair of peaks at $t_r = 16.7$ and 17.5 min are the adducts **7a**,**b** between **1** and GSH. The biological reaction products between **1** and MEA were not detected in this cell line. In addition, the unreacted compound **1** within the cells could be detected at a retention time of $t_r = 20.6$ min, when phosphate buffer solution containing 20% MeOH was used as the eluent (data not shown).

Figure 4 shows the decrease of the total intracellular NPSH level and the formation of nucleophilic addition products as a function of incubation time during treatment of EMT6/KU cells with **1** (1.0 mM) at 37°C, as monitored by HPLC analysis. The concentrations of GSH- and Cys-derived products **7a**,**b** and **8a**,**b** formed within the EMT6/KU cells (open circles and squares, respectively) increased with decrease of the total intracellular NPSH concentration (solid triangle) during the course of incubation with **1**. Up to a maximum of 26 nmol/10^7 cells for **7a**,**b** and 2.2 nmol/10^7 cells for **8a**,**b**, respectively, were produced at 60 min incubation. The yield of **7a**,**b** is ca. 12.5-fold higher than that of **8a**,**b**. In view of the higher reactivity of Cys compared to GSH (k_2(Cys) > k_2(GSH)) as given in Table 1, the results indicate that GSH is the major component of the intracellular NPSH compounds presented in the EMT6/KU cell line. On the other hand, the penetrated intracellular concentration of **1** increased gradually at the beginning of incubation, up to 2.6 nmol/10^7 cells at 30 min incubation, and then disappeared after 60 min incubation. It indicates that the penetration of **1** into the interior of EMT6/KU cells should facilitate its reaction with the cellular NPSH. After prolonged incubation, the concentrations of both adducts decreased, suggesting that decomposition of the adducts occurred possibly through the retro-Michael addition to return to GSH and Cys followed by unknown reaction pathways. This could be true on the fact that the total concentration of products **7a**,**b** and **8a**,**b** is 28.2 nmol/10^7 cells at maximal, which only accounts for about 50% of the depleted NPSH (56.3 nmol/10^7 cells). It is possible that the NPSH depletion by the nitroazole compounds bearing an α,β-unsaturated carbonyl side chain might involve some different mechanisms other than the nucleophilic 1,4-addition. The insert of Figure 4 shows the HPLC analysis results of the GSH adducts, indicating that the two diastereomers **7a** and **7b** were produced in almost 1:1 ratio (about 13 nmol/10^7 cells for each) through the reaction course. It has been reported that the nucleophilic addition of α,β-unsaturated carbonyl compounds with GSH occurring within cells is usually catalyzed by enzyme(s), especially GSH-S-transferase, to afford one of the stereoisomers in excess yield.[29] However, it seems that the influence of enzyme(s) or the asymmetric environment of the nucleophile on the formation of the

diastereomeric adducts **7a**,**b** would be at least minor in this study.

Radiosensitizing effects in vitro

EMT6/KU cells were used to investigate the influence of the intracellular NPSH depletion by nitroazoles having an α,β-unsaturated carbonyl side chain on the radiosensitization of hypoxic cells. After administration with graded concentrations of compounds **1**, **2**, and misonidazole (**6**), the cells were exposed to varying doses of X-ray under hypoxic conditions, and survival was determined by colony assay. The survival data were fitted to the single-hit multi-target model: $S = 1 - (1 - e^{-D/D_o})^n$, as described in our previous study,[26] where D is the radiation dose, n is the extrapolation number, and D_o is the dose required to reduce cell survival to e^{-1} (or 37%), or the mean lethal dose.

Figure 5 shows values of D_o, D_q and n as a function of the drug concentration. The D_o values for compounds **1**, **2**, and misonidazole (**6**) showed similar concentration dependence (Fig. 5a). Treatment of EMT6/KU cells with 1.0 mM of **1** decreased the mean lethal dose (D_o) by 50%, from 3.2 Gy for control to 1.6 Gy. Figure 5b demonstrates that the decrease of D_q is dependent both on the drug concentration and on the structure of nitroazole compounds, the extent of which is in the order of compound **1** < **2** < misonidazole (**6**). Treatment with **1** resulted in decrease of the extrapolation number (n) from 8.0 to 2.0 with increasing drug concentration, as seen in Figure 5c. A similar shoulder reduction of survival curves was also observed in the cases of **2**. In contrast, misonidazole (**6**) up to 5.0 mM did not show the shoulder reduction effect of the dose–survival curves. The different efficacy of nitroazoles with and without an α,β-unsaturated carbonyl group on the profiles of dose–survival curves, with respect to the changes in n and D_q values, reveals their different radiosensitizing actions. Compound **1** not only altered the slope but also reduced the initial shoulder of the survival curve.

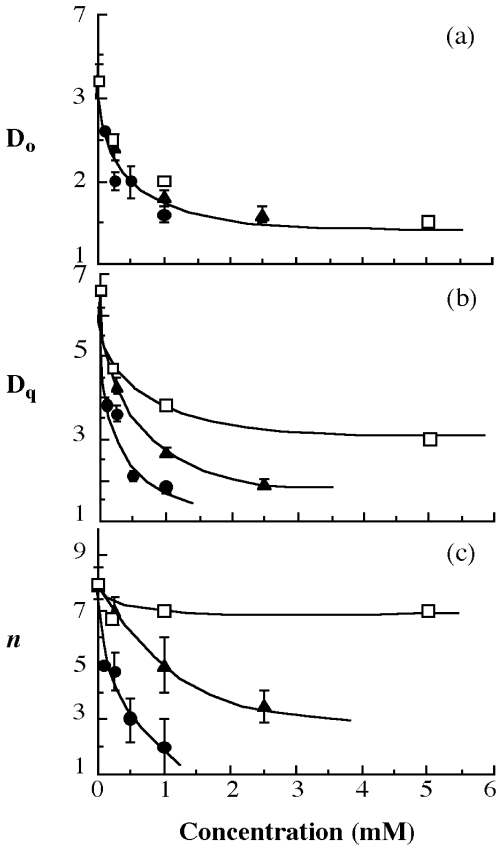


Figure 5. Deferences in (a) the mean lethal dose (D_o), (b) quasithreshold dose (D_q), and (c) extrapolation number (n) of cell survival as a function of drug concentrations: **1** (solid circles); **2** (solid triangles); **6** (open squares). Vertical bars represent standard errors (SE), calculated from more than five separate determinations.

Relationship between *n* value and intracellular NPSH depletion

With an attempt to reveal the influence of intracellular NPSH depletion on the shape of dose–survival curves of hypoxic cells, especially initial shoulder, and to understand the mechanisms by which nitroazole compounds with α,β-unsaturated carbonyl side chains sensitize hypoxic cells, we plotted the extrapolation number (n value) of survival curves as a function of the NPSH depletion of EMT6/KU cells as showed in Figure 6. The data for n value were obtained from the experiments described in Figure 5, and the percentages of decreased intracellular NPSH were quoted from Figure 2. A fine linear relationship was found between the n value and the decreased NPSH level. Independent of concentration and the structure of the nitroazole compound used, a depleted NPSH level in EMT6/KU cells preceeded by the NPSH-reactive nitroazoles resulted in a decrease in the n value. This relationship suggests that the decrease of the shoulder of dose–survival curves is closely associated with reduction of the cellular NPSH level.

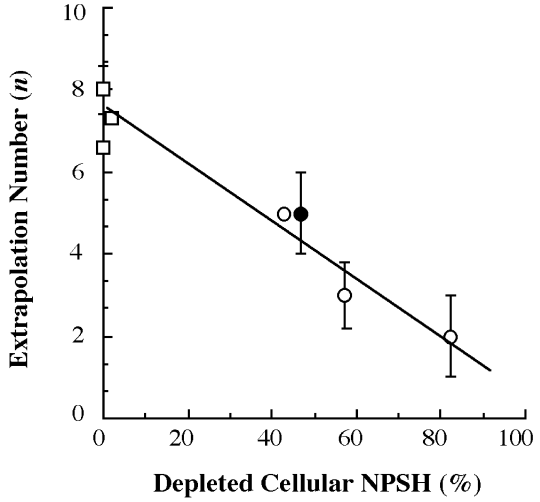


Figure 6. Relationship between extrapolation number (n) of dose–survival curves and intracellular NPSH depletion in EMT6/KU cells upon treatment with varying concentrations of 1 (open circles), 2 (solid circles), and 6 (open squares) for 1 h, respectively. Vertical bars represent standard errors (SE), calculated from more than five separate determinations.

Discussion

As the results of these studies, we have identified and characterized the properties of nitroazoles bearing a NPSH-reactive side chain: (a) the evidence to support the Michael addition of the intracellular non-protein thiols (NPSH) toward the α,β-unsaturated carbonyl moiety attached to the nitroazole compounds; and (b) the unique effects of intracellular NPSH-depletion on radiosensitization of hypoxic cells.

Studies on effects of endogenous thiols on radiosensitization of hypoxic cells have led to the development of the thiol-depletion agents, and their reaction with intracellular thiols, especially GSH, has been reported in varied ways, for example, oxidizing GSH to GSSG by diamide,[30] removing NPSH by covalent bond formation by *N*-ethylmaleimide (NEM), diethyl maleate (DEM), and dimethyl fumarate (DMF),[17,31] and blocking GSH synthesis by L-buthionine *S*,*R*-sulfoximine (BSO).[20,32] Enhancement in radiosensitivity of hypoxic cells has been obtained with the use of these thiol-reactive agents. However, only indirect evidence, mainly the decrease in the NPSH level measured by Ellman's reagent after drug treatment[33,34] has been employed to investigate the mechanism of hypoxic cell radiosensitization in terms of the NPSH depletion. In this study, with the use of HPLC analysis, the reaction products **7a**,**b** and **8a**,**b** of **1** with intracellular GSH and Cys have been successfully detected by UV absorbence at a maximum wavelength of 320 nm, at which most of intracellular protein components did not show UV absorption. These adducts **7a**,**b** and **8a**,**b** were isolated and their chemical strucutres have been characterized by the conventional ^{1}H and ^{13}C NMR and MS spectroscopical methods. The reaction pathway of the intracellular nucleophilic addition has been found to be similar to that occurred in the phosphate buffer solution. Thus, the endogenous thiols such as GSH and Cys react with α,β-unsaturated ester functionality of the nitroazole compound **1** to produce the diastereomers of the 1,4-addition products in ca. 1:1 ratio (Scheme 1 and Fig. 3).

A linear relationship between the extrapolation number *n* of the dose–survival curve and reduction of cellular NPSH level, as shown in Figure 6, indicates that the shoulder reduction effect of nitroazoles bearing an α,β-unsaturated carbonyl side chain originates from the NPSH-depletion activity. Electron-affinic compounds have been known to enhance radiosensitivity of hypoxic cells by increasing the slope but not affecting the shoulder of the dose–survival curve and they are less effective in the low radiation dose region.[23,35,37] Such a reduction also becomes a serious limitation for their clinical application in tumor radiotherapy. There have been reported that a prolonged exposure of mammalian cells to nitroimidazole such as misonidazole (**6**) under hypoxic conditions reduced the intracellular level of GSH, and resulted in reduction of the shoulder as well as the slope of survival curves.[23,36] It is likely that intracellular GSH binds to hydroxylamine, an important reductive intermediate of 2-nitroimidazole in biological system, to form hydoxylamine-GSH conjugate, since biological reduction is an important pathway for 2-nitroimidazole and the reduction process at least leads to the hydoxylamine and sometimes to the amine stage.[36] Such a bioreduction of nitro group would result in the loss of its oxygen-mimic effect due to its diminished electron affinity, in compensation for the gain of GSH depletion activity.

Among the thiol-depleting agents developed, diamide and NEM alter the shoulder of hypoxic cellular survival curve,[17,31] and BSO reduces shoulder even under aerobic conditions.[20] Skov et al. reported that GSH level affects the shoulder portion of the survival curves in both V79 and CHO cell lines. They found that the enhancement ratios were much greater at low doses (0–4 Gy) than at high doses in hypoxic cells after treatment with BSO.[20] Analysis of DNA damage by an alkaline elution assay indicated that DEM enhanced radiation-induced single-strand breaks but did not significantly affect on the repair, while diamide and NEM inhibited repair in addition to enhancing radiation-induced single-strand breaks.[37] Inhibition of repair might explain the effect on the shoulder of the dose–survival curves, as observed with the nitroazole compounds bearing an α,β-unsaturated carbonyl side chain investigated in this study.

Compounds with a NPSH-reactive side chain sensitizes hypoxic EMT6/KU cells by not only changing the slope but also reducing the initial shoulder of cellular survival, thereby leading to higher SER_{vitro} at both low and high radiation doses. This shoulder modification effect should be contributed from the NPSH reactivity of these nitroazole compounds possessing an α,β-unsaturated ester moiety through a chemical reaction with GSH occurring within the cells, since electron-affinic radiosensitizers do not affect the shoulder of the radiation-survival curves.[25,38] On the other hand, the nitro group attached on the parent nuclei remains its electron afinity and thereby plays a fixation effect of radiation–induced damage on DNA at higher doses. In view of the clinical importance of fractionation regimens in the radiotherapy for tumor control, it is important to develop a sensitizer which can sensitize hypoxic cells in low radiation dose regions without inducing serious toxicity. A radiosensitizer capable of enhancing radiosensitivity of hypoxic cell at low and therapeutic relevent doses is promising in clinical application. The effectiveness of the nitroazole compounds bearing α,β-unsaturated carbonyl side chains at both low and high radiation dose regions would provide a new approach to the development of hypoxic cell radiosensitizers with potential application to the fractionation radiotherapy.

Experimental

Materials

Methyl 4-(2′-nitroimidazol-1′-yl)crotonate (**1**), methyl 4-(2′-methyl-4′-nitroimidazol-1′-yl)crotonate (**2**), and methyl 4-(3′-nitro-1′,2′,4′-triazol-1′-yl)crotonate (**3**) were prepared from reaction of the corresponding nitroazole

with methyl 4-bromocrotonate in absolute ethanol containing sodium hydride.[27] Compounds **4** and **5** were supplied by Daikin Industries Ltd. (Japan). Misonidazole (**6**) was obtained from Nippon Roach (Japan). Reduced glutathione (GSH), L-cysteine (Cys), β-mercaptoethylamine (MEA), and Ellman's reagent (DTNB; 5,5′-dithiobis(2-nitrobezoic acid)) were purchased from commercial sources. All other reagents are of the best available grades from commercial sources and were used as received.

Kinetic measurements

The reactivity of NPSH compounds toward various nitroazole compounds were measured by the Ellman's method.[33] Nitroazole compound was dissolved in the phosphate buffer (pH 7.2) to give a 0.01 M solution, to which 0.01 M NPSH dissolved in 0.02 M aq EDTA·2Na solution was added. The mixture was purged with N_2 and then was kept at 37°C for the desired periods of reaction time. Aliquot (0.1 mL) was withdrawn from the reaction mixture at appropriate time intervals and was poured immediately into the phosphate buffer solution (0.9 mL) containing 0.2 mM of the Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), DTNB). The concentration of unreacted GSH in each aliquot was determined by UV absorption spectroscopy at the maximum absorption wavelength of 412 nm, resulting from 3-carboxy-4-nitrophenylmercaptide anion, the reaction product of DTNB with GSH. Shimadzu MPS-2000 multi-purpose spectrophotometer equipped with a Shimadzu graphic printer PR-3 was used for UV measurement. The reaction of compound **1** with GSH in the presence of rat liver glutathione-*S*-transferase (EC 2.5.1.18, 4.4×10^{-2} mg protein) was also determined in a similar manner as described above.

Product analysis and characterization

The reaction of nitroazoles with thiol compounds such as GSH, Cys, and MEA was performed in phosphate buffer solution, and was monitored by high performance liquid chromatography (HPLC) at the wavelength of 210 nm or 320 nm using a Shimadzu SPD-6A solvent delivery system equipped with a reverse-phase column (M and S pack C18, 4.6 mm ϕ×150 mm). The products were separated and purified by HPLC using automatic sampler (AS-8000) equipped with Wakosil 10 C18 column (10 mm ϕ×300 mm) and collected by fraction collector (FC-8000). 5% MeOH in H_2O was delivered as the mobile phase at a flow rate of 6.0 mL/min. Proton nuclear magnetic resonance spectrum (^{1}H NMR) and carbon nuclear magnetic resonance spectrum (^{13}C NMR) were obtained in the indicated solvents with TMS as the internal reference. Mass spectrum (MS) and high resolution mass spectrum (HRMS) were obtained under +FAB condition. Specific rotations were determined on a HORIBA SETA-200 high sensitive polarimeter.

Adduct 7a. 36%; $[\alpha]_D^{20}$ −20.11 (*c* 0.22, H_2O); ^{1}H NMR (270 MHz, D_2O) δ 7.54 (s, 1 H), 7.22 (s, 1 H), 4.78 (dd, *J* = 13.5, 5.4 Hz, 1 H), 4.65 (dd, *J* = 13.5, 8.1 Hz, 1 H), 4.55 (dd, *J* = 8.1, 5.4 Hz, 1 H), 3.78–3.71 (m, 3 H), 3.68 (s, 3 H), 3.62 (t, *J* = 8.1 Hz, 1 H), 3.04 (dd, *J* = 13.5, 5.4 Hz, 1 H), 2.89–2.71 (m, 3 H), 2.52 (t, *J* = 5.4 Hz, 2 H), 2.14 (q, *J* = 8.1 Hz, 2 H); ^{13}C NMR (67.8 MHz, D_2O) δ 179.0, 177.8, 176.1, 174.2, 147.4, 131.6, 130.7, 57.1, 56.0, 55.9, 55.4, 46.2, 44.5, 40.3, 35.3, 34.4, 29.4; HRMS (+FAB) calcd for $C_{18}H_{27}N_6O_{10}S$: 519.1509 ($M+H^+$). Found *m*/*z* 519.1482.

Adduct 7b. 49%; $[\alpha]_D^{20}$ +0.34 (*c* 0.15, H_2O); ^{1}H NMR (270 MHz, D_2O) δ 7.53 (s, 1 H), 7.21 (s, 1 H), 4.70 (d, *J* = 8.1 Hz, 2 H), 4.37 (dd, *J* = 10.8, 5.4 Hz, 1 H), 3.80–3.73 (m, 3 H), 3.70 (s, 3 H), 3.60 (t, *J* = 8.1 Hz, 1 H), 3.08 (dd, *J* = 13.5, 5.4 Hz, 1 H), 2.86 (dd, *J* = 8.1, 2.7 Hz, 2 H), 2.74 (dd, *J* = 13.5, 8.1 Hz, 1 H), 2.47 (t, *J* = 5.4 Hz, 2 H), 2.11 (q, *J* = 8.1 Hz, 2 H); ^{13}C NMR (67.8 MHz, D_2O) δ 179.0, 177.6, 176.2, 174.4, 147.3, 131.5, 130.7, 57.1, 56.0, 55.9, 55.6, 46.2, 44.6, 40.7, 35.3, 34.2, 29.5; HRMS (+FAB) calcd for $C_{18}H_{27}N_6O_{10}S$: 519.1509 ($M+H^+$). Found *m*/*z* 519.1547.

Adduct 8a. 41%; $[\alpha]_D^{20}$ −20.71 (*c* 0.21, H_2O); ^{1}H NMR (270 MHz, D_2O) δ 7.59 (br s, 1 H), 7.27 (br s, 1 H), 4.79 (dd, *J* = 14.0, 6.5 Hz, 1 H), 4.68 (dd, *J* = 14.0, 7.6 Hz, 1 H), 4.21 (t, *J* = 7.5 Hz, 1 H), 3.69 (s, 3 H), 3.69–3.61 (m, 1 H), 3.06 (d, *J* = 5.4 Hz, 2 H), 2.87 (dd, *J* = 17.3, 5.4 Hz, 1 H), 2.77 (dd, *J* = 17.3, 7.7 Hz, 1 H); ^{13}C NMR (67.8 MHz, D_2O): δ 176.2, 176.0, (N–*C*(NO_2)=N, not observed), 131.6, 130.9, 60.4, 56.1, 55.5, 44.5, 39.9, 34.3; HRMS (+FAB) calcd for $C_{11}H_{17}N_4O_6S$: 333.0869 ($M+H^+$). Found *m*/*z* 333.0891.

Adduct 8b. 45%; $[\alpha]_D^{20}$ +1.55 (*c* 0.22, H_2O); ^{1}H NMR (270 MHz, D_2O) δ 7.56 (s, 1 H), 7.23 (s, 1 H), 4.79–4.70 (m, 2 H), 4.12 (t, *J* = 5.5 Hz, 1 H), 3.68 (s, 3 H), 3.72–3.60 (m, 1 H), 3.17 (dd, *J* = 14.6, 4.9 Hz, 1 H), 2.99 (dd, *J* = 14.6, 6.2 Hz, 1 H), 2.85 (dd, *J* = 16.5, 6.3 Hz, 1 H), 2.77 (dd, *J* = 16.5, 7.5 Hz, 1 H); ^{13}C NMR (67.8 MHz, D_2O) δ 176.1, 173.6, (N–*C*(NO_2)=N, not observed), 131.6, 130.9, 60.6, 56.3, 55.5, 45.2, 40.3, 34.3; HRMS (+FAB) calcd for $C_{11}H_{17}N_4O_6S$: 333.0869 ($M+H^+$). Found *m*/*z* 333.0898.

In vitro assay

EMT6/KU single cells were grown as monolayer in Eagle's minimum essential medium (MEM) supplemented with 12.5% fetal bovine serum (FBS) and L-glutamine. MEM was adjusted to pH 7.5 by 10% aqueous $NaHCO_3$ solution. Ellman's reagent was adjusted to the appreciated concentrations immediately before use. All nitroazole compounds for evaluation were dissolved in FBS free MEM at appropriate concentrations and filtered through a Millipore filter immediately before use. Cells were seeded into glass cultural dishes (80 mm ϕ) in about $3–5\times10^5$ cells per dish, 24 h prior to experiment, to establish an exponential growth condition which is necessary for cellular NPSH determination and irradiation survival assay.

Intracellular NPSH depletion

Total intracellular NPSH was determined by the reported method[39] with minor modification. About 1.0×10^7 cells trypsinized from monolayer cultures were suspended in a

spinner flask and allowed to expose to the compound at 37°C for a period of time. After removal of the drug by centrifugation (1500 rpm for 5 min), cells were washed with PBS (−) solution twice and then extracted with 0.1 M phosphate buffer solution (pH 3.0) by sonication (160 W) at room temperature for 5 min. The cellular supernatant was filtered by an LGC Millipore filter to cut the proteins with molecular weight higher than 10,000, and then determined for cellular NPSH level with Ellman's reagent by measuring the change in UV absorbency at wavelength of 412 nm before and after treatment. The biological reaction products which were formed within cells during the incubation were identified and quantified by HPLC analysis monitored by UV detection at wavelength of 320 nm as illustrated above.

Radiosensitizing activity in vitro

Sensitizing effect of nitroazole compounds on EMT6/KU cells was evaluated as described previously.[40] Briefly, each nitroazole compound was added to a dish containing exponentially growing cells, followed by incubation at 37°C for 45 min. The dish was then purged with N_2 containing 5% CO_2 for 15 min before and during irradiation to establish acute hypoxic conditions. Irradiation was performed at a dose rate of 1.42 Gy/min by an X-ray source (250 kV_p, 10 mA) with a 1.0 mm Al filter. After irradiation, nitroazole compound was aspirated off immediately and cells were seeded in plastic cultural dishes (6 mm ϕ) at appreciated amounts, and then were incubated at 37°C in air containing 5% of CO_2. Following a 7-day incubation, cells were fixed with alcohol and stained with diluted Gimusa dye. Cell survival was assayed by a colony-formation method. Any colony consisting of greater than 50 cells was counted. The planting efficiency after treatment with compounds were higher than 70%, similar to that of untreated cells (73%). Each survival curve was obtained from 4 to 7 different doses of irradiation.

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References

1. Liphard, M.; Bothe, E.; Schulte-frohlinde, D. *Int. J. Radiat. Biol.* **1990**, *58*, 603.
2. Alper, T. *Cellular Radiobiology*. Cambridge University Press: Cambridge, 1979.
3. Astor, M. B.; Anderson, V.; Mester, A. *Pharmaco. Ther.* **1988**, *39*, 115.
4. Held, K. D. *Pharmaco. Ther.* **1988**, *39*, 123.
5. Howard-Flanders, P. *Nature* **1960**, *186*, 485.
6. Chapman, J. D.; Greenstock, C. L.; Reuvers, A. P.; Dugle, D. L. *Radiat. Res.* **1975**, *64*, 365.
7. Biaglow, J. E.; Varnes, M. E.; Clarck, E. P.; Epp, E. R. *Radiat. Res.* **1983**, *95*, 437.
8. Jordan, J.; d'Arcy Doherty, M.; Cohen, G. M. *Br. J. Cancer* **1987**, *55*, 627.
9. Britten, R. A.; John, A. G.; Warenius, H. M. *Int. J. Radiat. Oncol. Biol. Phys.* **1992**, *24*, 527.
10. Bump, E. A.; Brown, J. M. *Pharmacol. Ther.* **1990**, *47*, 117.
11. Clark, E. P.; Epp, E. R.; Morse-Gaudio, M.; Biaglow, J. E. *Radiat. Res.* **1986**, *108*, 238.
12. Held, K. D.; Epp, E. R.; Clark, E. P. *Radiat. Res.* **1988**, *115*, 495.
13. Held, K. D.; Epp, E. R.; Awad, S.; E., B. J. *Radiat. Res.* **1991**, *127*, 75.
14. Saunders, E. L.; Meredith, M. J.; Eisert, D. R.; Freeman, M. L. *Radiat. Res.* **1991**, *125*, 267.
15. Edgren, M.; Nishidai, T.; Scott, O. C. A.; Revesz, L. *Int. J. Radiat. Biol.* **1985**, *47*, 463.
16. Ling, C. C.; Wong, R. S. L.; Basas, R. D. *Int. J. Radiat. Oncol. Biol. Phys.* **1990**, *18*, 325.
17. Harris, J. W.; Koch, C. J.; Power, J. A.; Biaglow, J. E. *Radiat. Res.* **1977**, *70*, 585.
18. Harris, J. W.; Power, J. A.; Koch, C. J. *Radiat. Res.* **1975**, *64*, 270.
19. Ho, K.; Skarsgard, L. D.; Harrison, I. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 1415.
20. Skov, K. A.; MacPhail, H. S. *Int. J. Radiat. Oncol. Biol. Phys.* **1992**, *22*, 533.
21. Adams, G. E. *Radiat. Res.* **1992**, *132*, 129.
22. Revesz, L.; Palcic, B. *Acta Radiol.* **1985**, *24*, 207.
23. Watts, M. E.; Hodgkiss, R. J.; Jones, N. R.; Fowler, J. *Int. J. Radiat. Biol.* **1986**, *50*, 1009.
24. Palcic, B.; Korbelik, M.; Trotter, M.; Revesz, L. *Radiat. Res.* **1989**, *117*, 409.
25. Wouters, B. C.; Sy, A. M.; Skasgard, L. D. *Int. J. Radiat. Biol.* **1996**, *70*, 719.
26. Nishimoto, S.; Zhou, L. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 439.
27. Zhou, L.; Nishimoto, S. *Int. J. Radiat. Biol.* **1995**, *67*, 335.
28. Friedman, M.; Cavins, J. F.; Wall, J. S. *J. Am. Chem. Soc.* **1965**, *87*, 3672.
29. Chasseaud, L. F. *Adv. Cancer Res.* **1979**, *29*, 175.
30. Midander, J.; Deschavanne, P. J.; Debieu, D.; Malaise, E. P.; Revesz, L. *Int. J. Radiat. Biol.* **1986**, *49*, 403.
31. Han, A.; Sinclair, W. K.; Kimler, B. F. *Radiat. Res.* **1976**, *65*, 337.
32. Britten, R. A.; Warenius, H. M.; White, R.; Peacock, J. P. *Int. J. Radiat. Oncol. Biol. Phys.* **1992**, *22*, 769.
33. Tietze, F. *Anal. Biochem.* **1969**, *27*, 502.
34. Varnes, M. E.; Biaglow, J. E. *Cancer Res.* **1979**, *27*, 2960.
35. Brown, J. M.; Yu, N. Y. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 1207.
36. Whitmore, G. F.; Varghese, A. J. *Bioch. Phar.* **1986**, *35*, 97.
37. Todd, P.; Nishidai, T.; Revesz, L.; Edgren, M. *Int. J. Radiat. Biol.* **1986**, *50*, 1023.
38. Harris, J. W. *Exp. Cell Res.* **1968**, *50*, 293.
39. Revesz, L.; Palcic, B. *Acta Radiol.* **1985**, *24*, 207.
40. Shibamoto, Y.; Nishimoto, S.; Shimokawa, H.; Hisanaga, Y.; Zhou, L.; Wang, J.; Sasai, K.; Takahashi, M. M. A. *Int. J. Radiat. Oncol. Biol. Phys.* **1989**, *16*, 1045.