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Abstract

Protection of Escherichia coli NIHJ and C57BL mice from the effects of 60Co gamma-rays provided by S-alk(en)yl-L-cysteines and their hydantoin derivatives was examined. E. coli (10(6) cells/ml) suspended in a 20 mM aqueous solution of one of the drugs was irradiated with 60 Gy of gamma-rays. Five week-old male mice were exposed to 5.0-9.5 Gy of gamma-rays after a single intraperitoneal injection of 0.75 mmol/kg body weight of each compound. In both E. coli and mice, S-allyl compounds afforded more effective radioprotection than S-propyl compounds. The replacement of the alpha-hydrogen of S-substituted cysteines by methyl groups decreased the radioprotective effect. Hydantoin derivatives were much more radioprotective than the original sulfur-containing amino acids. Especially, DL-5-allylthiomethyl-5-methylhydantoin had a remarkable radioprotective effect in mice. The gamma-radiolysis mechanism of thiomethylhydantoin derivatives was discussed in connection with the radioprotective effect of the drugs.

KEYWORDS: radioprotector, thiomethylhydantoin, sulfur amino acids, dose reduction factor, ?-radiolysis

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Radioprotective Effects of Thiomethylhydantoin Derivatives on *Escherichia coli* and Mice.

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Protection of *Escherichia coli* NIHJ and C57BL mice from the effects of ^{60}Co γ-rays provided by S-alk(en)yl-L-cysteines and their hydantoin derivatives was examined. *E. coli* (10^6 cells/ml) suspended in a 20 mM aqueous solution of one of the drugs was irradiated with 60 Gy of γ-rays. Five week-old male mice were exposed to 5.0-9.5 Gy of γ-rays after a single intraperitoneal injection of 0.75 mmol/kg body weight of each compound. In both *E. coli* and mice, S-allyl compounds afforded more effective radioprotection than S-propyl compounds. The replacement of the α-hydrogen of S-substituted cysteines by methyl groups decreased the radioprotective effect. Hydantoin derivatives were much more radioprotective than the original sulfur-containing amino acids. Especially, DL-5-allylthiomethyl-5-methylhydantoin had a remarkable radioprotective effect in mice. The γ-radiolysis mechanism of thiomethylhydantoin derivatives was discussed in connection with the radioprotective effect of the drugs.

Key words : radioprotector, thiomethylhydantoin, sulfur amino acids, dose reduction factor, γ-radiolysis

The radiation dose used in the radiotherapy of cancer patients is limited to a dose insufficient to kill radiation-resistant hypoxic tumor cells due to the intolerance of normal tissues. An agent which protects normal tissues more than tumor cells from radiation damage could increase the therapeutic effectiveness of radiation. Since the discovery of radioprotective effects of cysteine and glutathione (1,2), many sulfur compounds have been tested for their radioprotective action on various biological systems (3-10). Some, such as cysteamine (MEA) and glutathione are very strong radioprotectors, although they are toxic to mammals at their maximum protective doses (11-15).

In a previous paper (16), we reported two new types of sulfur-containing radioprotectors and radiosensitizers in mice: S-alk(en)yl-L-cysteines and their hydantoin derivatives. In the course of the search for radioprotectors less toxic to mammals, we found that synthesized thiomethylhydantoin derivatives were possible candidates. This paper deals with the effects of thiomethylhydantoin derivatives on γ-irradiated *Escherichia coli* and mice.

Materials and Methods

Bacteria. *E. coli* NIHJ was cultured on 0.5% agar medium which contained 1% meat extract, 1% peptone and 0.5% NaCl at 37°C for 24 h. Broth containing *E. coli* (5 mg, wet weight) was

diluted to 100 ml with sterilized water. A 0.1 ml aliquot of the solution was added to 0.9 ml of a 22.2 mM drug solution in sealed vessels and irradiated with γ-rays (dose rate, 6.1×10^2 Gy/h) from ^{60}Co. The viable cell number was determined by counting visible colonies. The concentration of *E. coli* before irradiation was about 10^6/ml. The irradiation dose affording 90% lethality (LD_{90}) was 60 Gy. The survival ratio was defined as the ratio of the number of colonies of the drug-treated group to that of the control group.

Mice. Five week-old male C57BL mice were exposed to several doses (5.0-9.5 Gy) of γ-rays (dose rate, 0.464 Gy/min) from ^{60}Co in 30 min after a single intraperitoneal injection of 0.75 mmol/kg body weight of each compound. On the 30 th day after irradiation, mice which survived were counted. The dose reduction factor (D. R. F.), which is defined as the ratio of the LD_{50} (median lethal irradiation dose) for the drug-treated group to that for the drug-free control group, was calculated from a graph showing the relationship between the dose of γ-rays and the percent lethality 30 days after irradiation.

Synthesis of S-Alk(en)yl-L-cysteines. The synthetic procedure used was a modification of the method of du Vigneaud *et al.* (17) for preparing methionine from homocystine. S-Alk(en)yl-L-cysteines were prepared from L-cystine by reduction with metallic sodium in liquid ammonia, followed by alk(en)yl bromide treatement.

S-*n*-Propyl-L-cysteine: mp 210-212°C (dec); IR(KBr), 2965-2860, 2580, 2120, 1622 (NH_3^+), 1590 (COO^-) cm^{-1}; MS m/z (%), 163 (M^+, 12.4), 118 (21.7), 90 (40.3), 89 (100), 74 (31.0), 61 (46.5), 47 (65.1) and 43 (89.9); ^{1}H-NMR (D_2O-NaOD), δ0.90 (3H, t, $J=7.0$ Hz, CH_3), 1.55 (2H, m, CH_2), 2.50 (2H, t, $J=7.2$ Hz, SCH_2 at *n*-propyl), 2.75-2.85 (2H, dd, $J=6.2$ Hz, SCH_2 at C-β), and 3.45 (1H, dd, $J=6.2$ Hz, CH at C-α). *Anal.* Calculated for $C_6H_{13}NO_2S$: C, 44.16; H, 8.03; N, 8.58. Found: C, 44.09; H, 7.99; N, 8.60.

S-Allyl-L-cysteine: mp 208-210°C (dec); IR (KBr), 3020-2870, 2590, 2120, 1620 (NH_3^+), 1580 (COO^-) cm^{-1}, 990 and 918 (allyl double bond); MS m/z (%) 161 (M^+, 14.2), 116 (8.4), 88 (55.8), 87 (100), 74 (90.0), 45 (46.8), 41 (87.6), and 39 (32.4); ^{1}H-NMR (D_2O-NaOD), δ2.61-2.71 (2H, dd, $J=6.2$ Hz, SCH_2 at C-β), 3.10 (2H, dd, $J=7.0$ Hz, SCH_2 at allyl), 3.26 (1H, dd, $J=6.2$ Hz, CH at C-α), 5.01-5.22 (2H, dd, $J=17.0$ and 10.0 Hz, vinylic CH_2), and 5.71 (1H, m, vinylic CH). *Anal.* Calc. for $C_6H_{11}NO_2S$: C, 44.71; H, 6.88; N, 8.69. Found: C, 44.52; H, 6.81; N, 8.60.

Synthesis of DL-5-Alk(en)ylthiomethyl-5-methylhydantoins. The synthetic route is shown in Fig. 1. Alk(en)ylthiopropanones were prepared from alk(en)yl mercaptan. Equimolar amounts of alk(en)ylmercaptan and monochloroacetone were reacted in sodium ethoxide-ethanol solution. The mixture was allowed to stand overnight at room temperature. The reaction mixture was diluted with a large amount of water and the product was extracted with ether. The extract was dried, and the ether was evaporated. The product was converted to 2,4-dinitrophenylhydrazone (2,4-DNPH) to confirm the structure.

Propylthiopropanone: bp 80°C (22 mmHg); its 2,4-DNPH, mp 118-119°C. Anal. Calcd. for $C_{12}H_{16}N_4O_4S$: C, 47.51; H, 6.98; S, 15.85. Found: C, 47.76; H, 6.95; S, 15.71. Allylthiopropane: bp 87°C (16 mmHg); its 2, 4-DNPH, m.p. 84-85°C. *Anal.* Calcd. for $C_{12}H_{14}N_4O_4S$: C, 46.44; H, 4.55; S, 10.33. Found: C, 46.60; H, 4.58; S, 10.39.

DL-5-Alk(en)ylthiomethyl-5-methylhydantoins were prepared as follows. DL-5-Propylthiomethyl-5-methylhydantoin: propylthiopropanone (0.4 mole, 53 g) dissolved in 500 ml of 70-80% ethanol was added to a mixture of 0.5 mole of potassium cyanide and 1.5 moles of ammonium carbonate in a round-bottomed flask. With occasional shaking, the mixture was kept at 60-65°C for 4-5 h. The temperature was raised to 85°C, and maintained for one hour, and then the ethanol was evaporated under a reduced pressure. On cooling, 78 g of crude crystals of the product were obtained and recrystal-

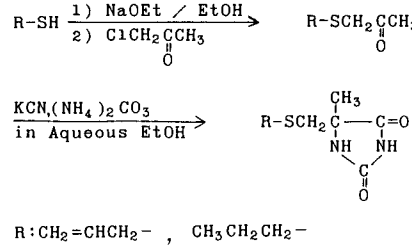


Fig. 1 Scheme of the syntheses of DL-5-alk(en)ylthiomethyl-5-methylhydantoins.

lized as needles from ethanol/water; yield 87%.

DL-5-Methylthiomethyl-5-methylhydantoin: mp 138-140°C. *Anal.* Calcd. for $C_6H_{10}N_2O_2S$: C, 41.37; H, 5.79; S, 18.40. Found: C, 41.25; H, 5.86; H, 18.65.

DL-5-Propylthiomethyl-5-methylhydantoin: mp 119-121°C. *Anal.* Calcd. for $C_8H_{14}N_2O_2S$: C, 47.51; H, 6.98; S, 15.85. Found: C, 47.76; H, 6.95; S, 15.71.

DL-5-Allylthiomethyl-5-methylhydantoin: mp 119-120°C. *Anal.* Calcd. for $C_8H_{12}N_2O_2S$: C, 47.98; H, 6.04; S, 16.01. Found: C, 48.14; H, 6.16; S, 16.16.

Synthesis of Glycyl-S-n-propyl-DL-cysteine.

(a) *S-n-Propyl-DL-cysteine ethyl ester* (I). S-n-Propyl-DL-cysteine ethyl ester was prepared from DL-cystine by the method of du Vigneaud *et al.* (17) followed by the method of Fischer (24).

(b) *N-Carbobenzoxy-glycine* (II). N-Carbobenzoxy-glycine was prepared from carbobenzoxy chloride and glycine by the method of Bergmann and Zervas (25).

(c) *N-Carbobenzoxy-glycyl-S-n-propyl-DL-cysteine ethyl ester* (III). A mixture of 10.55 g (0.041 mol) of II, 8.46 g (0.041 mol) of N, N'-dicyclohexylcarbodimide and 8.0 g (0.041 mol) of I dissolved in 100 ml of tetrahydrofuran was allowed to react for 4 h at room temperature. The mixture was shaken occasionally, and after 4 h, 3 ml of glacial acetic acid was added to decompose excess N, N'-dicyclohexylcarbodimide. The mixture was stored in a refrigerator overnight. N, N'-Dicyclohexylurea was removed by filtration, the solvent was evaporated under a reduced pressure, the residue was dissolved in 80 ml of ethyl acetate, and insoluble material was filtered off. The ethyl acetate extract was washed with 0.5 M sodium bicarbonate and a 0.5 M citric acid solutions and dried over anhydrous sodium sulfate (Na_2SO_4). The solvent was evaporated under a reduced pressure, and an oily residue was obtained; yield 12.3 g (79%).

(d) *N-Carbobenzoxy-glycyl-S-n-propyl-DL-cysteine* (IV). The crude ester III (12.3 g) was treated with a mixture of 140 ml of dioxane and 100 ml of 1N sodium hydroxide for 6 h at room temperature. After the solution was evaporated under a reduced pressure, the residue was dissolved in water (500 ml). After filtration, the solution was acidified with 1N hydrochloric acid. The oily substance which separated was extracted with ethyl acetate, washed with water and dried over Na_2SO_4. The solvent was evaporated under a reduced pressure. The residue was dissolved in a small amount of ethyl acetate and recrystallized by the addition of diethyl ether and light petroleum; yield 3.64 g (31.9%), mp 118-119°C.

(e) *Glycyl-S-n-propyl-DL-cysteine* (IIa). A solution of 3.6 g of IV in 90% acetic acid (50 ml) was hydrogenated over Palladium black (0.4 g) as a catalyst. The filtrate was evaporated under a reduced pressure, and a crystalline product was obtained. It was recrystallized from water and ethanol; yield 1.23 g (54.9%); mp 191-200°C (dec); IR(KBr) 1674, 1564-1546, 1273 cm^{-1} (amide). *Anal.* Calcd. for $C_8H_{16}N_2O_3S$: C, 43.62, H, 7.32; N, 12.72; S, 14.55. Found: C, 43.43; H, 7.28; N, 12.92; S, 14.47.

Synthesis of Glycyl-S-allyl-DL-cysteine

S-Allyl-DL-cysteine ethyl ester (V). S-Allyl-DL-cysteine ethyl ester was prepared from DL-cystine and allyl chloride by the same method used for the preparation of I.

N-Carbodenzoxy-glycyl-S-allyl-DL-cysteine ethyl ester (VI). A mixture of 10.6 g (0.04 mol) of II, 8.5 g (0.04 mol) of DCC and 7.8 g (0.04 mol) of V dissolved in 100 ml tetrahydrofuran was allowed to react for 4 h at room temperature. The mixture was shaken occasionally, and after 4 h, 3 ml of glacial acetic acid was added to decompose excess DCC. The mixture was stored in a refrigerator overnight. The insoluble N, N'-dicyclohexylurea was removed, the solvent was evaporated under a reduced pressure, the residue was dissolved in 60 ml ethyl acetate, and insoluble material was filtered off. The ethyl acetate solution was washed with 0.5 M sodium bicarbonate and 0.5 M citric acid aqueous solutions, and dried over Na_2SO_4. The solvent was evaporated under a reduced pressure, and an oily residue was obtained; yield 13.6 g (87%).

N-Carbobenzoxy-glycyl-S-allyl-DL-cysteine (VII). The crude VI (13.6 g) was treated with a mixture of 140 ml dioxane and 100 ml 1N sodium hydroxide for 6 h at room temperature. After the solution was evaporated under a reduced pressure, the residue was dissolved in water (550 ml). After filtration, the solution was acidified with 1N hydrochloric acid. The oily substance which separated was extracted with ethyl acetate. The ethyl acetate solution was washed with water, dried over

Na_2SO_4, and the solvent was evaporated under a reduced pressure. The residue was dissolved in a small amount of ethyl acetate and recrystallized by the addition of diethyl ether and light petroleum; yield 10.5 g (74.4%), mp 116-121°C.

Glycyl-S-allyl-DL-cysteine (IIb). To 200 ml of liquid ammonia in a 500 ml round-bottomed flask equipped with a mechanical stirrer and a soda lime tube, and cooled with dry ice and ethanol, was added 10.5 g of VII. Sodium was then added in small portions until the blue color persisted for 5 min. After the addition of ammonium bromide, 17 ml of allyl bromide was added and the mixture was further stirred for 2 h. The ammonia was removed and the residual solid was dried under a reduced pressure. This material was dissolved in 200 ml water. To this solution, 20 ml acetic acid was added, and the peptide was absorbed by passing the solution through a column of Dowex 50W X-2 (H^+ form). The resin was washed with water. The peptide was then eluted with 2 M ammonia. The solution was then concentrated under a reduced pressure to 20 ml, yielding a crystalline mush. After standing in a refrigerator overnight, the crystals were separated from the solution by filtration. The product was recrystallized from water and ethanol; yield 1.35 g (20.7%); mp 191-196 °C (dec); IR(KBr) 1682, 1565-1540, 1270 cm^{-1} (amide), 995 and 923 cm^{-1} (allyl double bond). *Anal.* Calcd. for $C_8H_{14}N_2O_3S$: C, 44.02; H, 6.46; N, 12.83; S, 14.69. Found: C, 43.74; H, 6.49; N, 12.84; S, 14.46.

Isolation and Identification of γ-Radiolysis Products

γ-Radiolysis of 20 mM alk(en)ylthiomethylhydantoin solutions was examined by exposure to γ-rays (dose rate, 6.1×10^2 Gy/h; total doses 60-600 Gy). Two-dimensional thin layer chromatography (Avicel SF) of radiolysis products using *n*-butanol-acetic acid-water (4:1:1, by vol) and phenol-water (4:1, by vol) gave two ninhydrin-positive spots besides the original material. These products were isolated by column chromatography and identified by comparing with the Rf value and mass spectral fragmentation. Volatile products were identified by the combined GC-MS method.

Results and Discussion

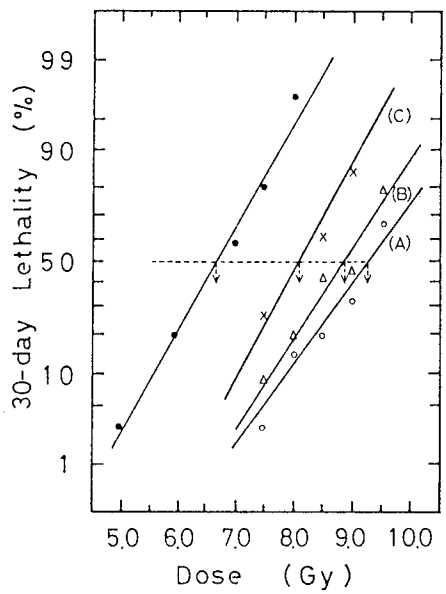
The effects of sulfur-containing amino acids (L-cysteine, S-allyl-L-cysteine, S-propyl-L-cysteine, S-allyl-2-methyl-DL-cysteine, S-propyl-2-methyl-DL-cysteine, S-allyl-L-cysteine sulfoxide and S-propyl-L-cysteine sulfoxide) and their hydantoin derivatives (DL-5-allylthiomethyl-5-methylhydantoin and DL-5-propylthiomethyl-5-methylhydantoin) on the survival of irradiated *E. coli* were examined. S-Allyl-L-cysteine (survival ratio, 1.88) was comparatively more radioprotective among the sulfur-containing amino acids, but was less protective than L-cysteine (1.91). S-alk(en)yl-L-cysteines were more effective than S-Alk(en)yl-2-methyl-DL-cysteines, while sulfoxide amino acids were less effective than sulfide amino acids. However, hydantoin derivatives such as DL-5-allylthiomethyl-5-methylhydantoin (2.21) and DL-5-propylthiomethyl-5-methylhydantoin (2.02) were more radioprotective than L-cysteine and its derivatives. S-Allyl compounds were generally more radioprotective than S-propyl compounds.

The dose reduction factor (D.R.F.) of sulfur-containing amino acids (L-cysteine, S-allyl-L-cysteine, S-propyl-L-cysteine, glycyl-S-allyl-L-cysteine, glycyl-S-propyl-L-cysteine) and their derivatives (5-mercaptomethyl-hydantoin and 5-allylthiomethyl-hydantoin) were derived as described in Materials and Methods (Ref. 27). 5-Mercaptomethyl-hydantoin and 5-allylthiomethyl-hydantoin provided more radioprotection to mice than the original amino acids as was the case with *E. coli*. A large difference in the radioprotection afforded by S-allyl and S-propyl compounds was not found in mice.

In a previous paper (16), it was reported that a methyl group on the 5-position of the hydantoin ring enhanced radioprotection in mice in comparison with alk(en)yl-thiomethyl-hydantoin. Therefore, some DL-5-alk(en)-ylthiomethyl-5-methylhydantoins were examined in detail for radioprotection of mice. The relationship between the dose (Gy) of γ-rays and the percent lethality of mice

which received 5-methyl substituted hydantoin derivatives is shown in Fig. 2. DL-5-Allylthiomethyl-5-methylhydantoin (A) was the most radioprotective of all. S-allyl (A), S-methyl (B) and S-propyl (C) compounds had D.R.F.s of 1.41, 1.32 and 1.20, respectively.

These alk(en)ylthiomethyl-5-methylhydantoins (Fig. 2) were more radioprotective



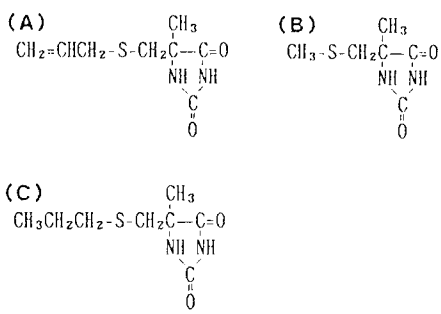


Fig. 2 Protective effect of DL-5-alk(en)ylthiomethyl-5-methylhydantoins on the lethality of C57BL mice after ^{60}Co irradiation. A hydantion derivatives (A), (B) or (C) (0.75 mmole/kg of body weight) was intraperitoneally injected 30 min before the irradiation, and the mice were irradiated at the indicated dose (dose rate: 0.49 Gy/min). Percent lethality (probit scale) in 30 days was plotted against the γ-ray dose. Control, ●. Injected with (A), ○; (B), △ or (C), ×.

than SH compounds such as L-cysteine and its hydantoin derivative. It seems that sulfide compounds are much less toxic to mice than SH compounds such as cysteamine and L-cysteine. In the case of DL-5-allylthiomethyl-5-methylhydantoin (structure A in Fig. 2), even a large amount of the compound (1.5 mmol or 300 mg/kg body weight) resulted in no death of mice within 30 days.

To interpret the radioprotection of thiomethylhydantoin derivatives, γ-radiolysis of the drugs in aqueous solutions was studied. Fig. 3 shows the γ-radiolysis mechanism of alkylthiomethylhydantoins (27). The identification of radiolysis products was carried out by direct comparisons with authentic compounds. Irradiations in the presence of N_2O (specific scavenger for e^-_{aq}), KBr (specific scavenger for ·OH) or NaCN (H radical scavenger) were also done to elucidate the radiolysis mechanism. The yield of 5-methylhydantoins decreased with increasing concentrations of N_2O and increased with increasing concentrations of KBr. Therefore, it is considered that alk(en)ylthiomethylhydantoins react with e^-_{aq} to produce 5-methylhydantoins. On the other hand, the yield of alk(en)yl-thiomethylhydantoin sulfoxides increased with increasing concentration of N_2O and dramatically decreased with increasing concentrations of KBr. This evidence indicates that sulfide hydantoins react with ·OH

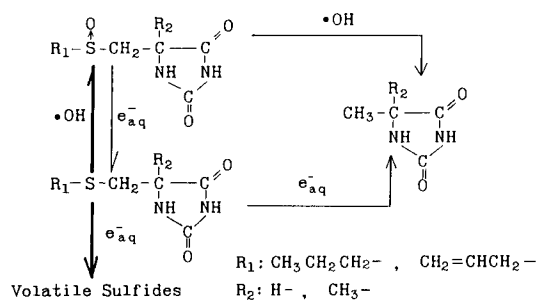


Fig. 3 Possible degradation mechanism of hydantoin derivatives by ^{60}Co irradiation in oxygen-free aqueous solutions. Major routes are indicated by thick lines.

to produce sulfoxides. Sulfide amino acids and their hydantoins react with ·OH so fast that they cause indirectly damage to living cells (26). From this information, it appeares that alkylthiomethylhydantoins play a role as scavengers of the OH radical.

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