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A Radioprotective Stereostructure–Activity Study of *cis*- and *trans*-2-Mercaptocyclobutylamine Analogs and Homologs of 2-Mercaptoethylamine

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For purposes of studying stereostructure–activity relationships at the molecular, cellular, and animal levels and probing the mechanism of 2-mercaptoethylamine (MEA) radioprotection we synthesized several conformationally constrained cyclobutyl analogs. The comparative radioprotective properties for MEA, *cis*- and *trans*-2-mercaptocyclobutylamine (**2**), *cis*- and *trans*-2-mercaptocyclobutylmethylamine (**3**), and *trans*-2-mercaptomethylcyclobutylamine (**4**) are discussed in terms of their ability to chemically reduce transient free radicals, the formation of single strand breaks in DNA, and protect Chinese hamster cells (*in vitro*) and mice against the lethal effects of ionizing radiation. The results are interpreted in light of current proposed mechanisms of action for MEA. No correlation exists between ability of these analogs to enhance mice survival times and their ability to protect against the induction of DNA single strand breaks and the inactivation of proliferative capacity of hamster cells growing *in vitro*. Analysis of two isomers (*cis*- and *trans*-**3**) on the repair of single strand breaks showed both isomers only marginally influenced the rate and did not influence the extent of single strand break rejoining. The results are consistent with a mode of action involving chemical repair of transient radicals and protection against DNA and critical enzymatic sites.

Previous reports from these laboratories describe the activity of several mercaptocyclobutylamine analogs and homologs of 2-mercaptoethylamine (MEA, **1**) on bradykinin-induced contraction of the guinea pig ileum[1] and drug-stimulated glycerol release from rat adipose tissue.[2] While MEA exerts a multitude of actions in a variety of biochemical and pharmacological systems, this agent is largely noted for its relatively potent radioprotective properties.[3-6] In this article we discuss the results of a comparative analysis of the radioprotective effects of *cis*- and *trans*-2-mercaptocyclobutylamine (**2**), the aminomethyl homologs (*cis*- and *trans*-**3**), and *trans*-2-mercaptomethylcyclobutylamine (**4**) *in vitro* and *in vivo*.

Specifically, MEA and mercaptocyclobutylamines **2–4** were evaluated for their ability to reduce (by H donation) free radicals generated in DNA by the indirect action of ·OH;[7,8] for their ability to prevent DNA damage in Chinese hamster fibroblast cells by measuring the incorporation of tritiated thymidine during unscheduled DNA synthesis[9,10] and for their ability to prevent single strand break induction as measured by sedimentation in alkaline sucrose gradients;[11] for their toxic and radioprotective effects in Chinese hamster cells; and for their ability to protect mice against a lethal dose of γ radiation.[4] The results of these studies are discussed in light of current proposed mechanisms of action for MEA.

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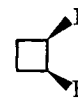
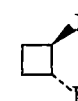


cis-**2**, R = NH_2; R^1 = SH
cis-**3**, R = CH_2NH_2; R^1 = SH



trans-**2**, R = NH_2; R^1 = SH
trans-**3**, R = CH_2NH_2; R^1 = SH
trans-**4**, R = NH_2; R^1 = CH_2SH

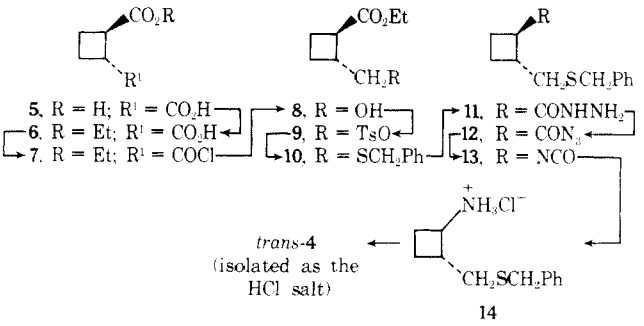
Experimental Section

Melting points were determined by using a calibrated Thomas-Hoover melting point apparatus. All compounds were analyzed by means of uv (Perkin-Elmer 257 spectrophotometer), nmr (Varian A-60A nuclear magnetic resonance spectrometer), and glpc (F and M 402 biomedical gas chromatograph equipped with glass columns). Elemental analyses were performed by Clark Microanalytical Labs, Urbana, Ill.

A. Synthetic Aspects. The synthesis for *cis*- and *trans*-2-mercaptocyclobutylamine (**2**)[2] and *cis*- and *trans*-2-mercaptocyclobutylmethylamine (**3**)[1] previously has been reported. The synthe-

†The synthetic aspects of this work were abstracted in part from the Ph.D. dissertation presented by B.K.S., March 1972, to the Graduate School of The Ohio State University.

Scheme I



sis for *trans*-2-mercaptomethylcyclobutylamine (**4**) is summarized in Scheme I and described in detail in this section. MEA (**1**) HCl was purchased from Aldrich Chemical Co., Milwaukee, Wis., and used in these studies without further purification. All cyclobutylamines were purified and biologically evaluated *in vitro* and *in vivo* as their HCl salts. All cyclobutyl analogs were assayed for SH groups by the iodometric method of Kimball, *et al.*,[12] and were shown to contain 98–100% of the calculated SH content; *i.e.*, no disulfides were present in the synthetic materials.

***trans*-2-Carbethoxy-1-cyclobutanecarboxylic Acid (6).** To a mixture of *trans*-**5** (75.0 g, 0.52 mol) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and 23.0 g (0.52 mol) of absolute EtOH was added 2 ml of concentrated HCl. The mixture was stirred for 4 hr on a steam bath and cooled. The solution was poured with stirring into 500 ml of a saturated solution of $NaHCO_3$ and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried (Na_2SO_4), and concentrated under reduced pressure affording 15.0 g (7.8%) of the trans diester which was converted to *trans*-**5** by hydrolysis with KOH according to the method of Gelin and coworkers.[13]

The bicarbonate solution was acidified with dilute HCl and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Distillation of the combined residual oils afforded 51.5 g (57–58%) of colorless liquid: bp 104–106° (0.05 mm) [lit.[13] bp 140° (2.0 mm)].

***trans*-2-Carbethoxy-1-cyclobutanecarboxylic Acid Chloride (7).** To a chilled solution of *trans*-**6** (32.0 g, 0.18 mol) in 75 ml of dry PhH was added, dropwise with stirring, 45.7 g (0.36 mol) of oxalyl chloride. The temperature was maintained between 0 and 5° during the addition. Subsequently, the reaction mixture was stirred at 0–5° for 2 hr, allowed to warm to room temperature, and stirred for an additional 18 hr. PhH and unreacted oxalyl choride were removed under reduced pressure affording 34 g of **7** which was used in subsequent reactions without further purification.

***trans*-2-Carbethoxycyclobutylcarbinol (8).** To a suspension of 13.6 g (0.36 mol) of $NaBH_4$ in 200 ml of dry dioxane, 34.2 g (0.18 mol) of *trans*-**7** in 50 ml of dry dioxane was added dropwise with stirring. After the addition was complete, the mixture was stirred for 0.5 hr at room temperature, refluxed for 3 hr, cooled, and poured over an ice–H_2O–HCl mixture. The aqueous solution was extracted with Et_2O; the Et_2O layer was washed with H_2O, $NaHCO_3$ solution, and H_2O, dried (Na_2SO_4), and filtered. Concentration under reduced pressure and distillation of the residual oil afforded 16.5 g (57%) of a colorless liquid: bp 70–75° (0.5 mm) [lit.[13] bp 87° (1.3 mm)].

***trans*-2-Carbethoxycyclobutylcarbinol *p*-Toluenesulfonate (9).** To a well-chilled solution of *trans*-**8** (16.5 g, 0.1 mol) in 50 ml of dry pyridine was added 19.7 g (0.1 mol) of *p*-TsCl in 50 ml of dry pyridine. The reaction mixture, which immediately turned yellow, was held for 12 hr at 0–5°. The reaction mixture was poured into ice–H_2O and stirred for 1 hr. A heavy brown oil separated which was extracted with Et_2O. The Et_2O layer was washed with H_2O, dilute HCl, and H_2O, dried (Na_2SO_4), and filtered. Concentration under reduced pressure afforded 30.0 g (92%) of *trans*-**9** as a highly viscous liquid which could not be crystallized and which was used without further purification: ir (neat) 1360 (SO_2 symmetric stretch), 1190, 1180 (SO_2 stretch), 3060, 3040, 1600 (aromatic), 2870 (CH_2 stretch), 1725 cm^{-1} (C=O).

Ethyl *trans*-(2-Benzylthiomethyl)cyclobutylcarboxylate (10). To 15.0 g (0.12 mol) of benzylmercaptan was slowly added 2.3 g (0.1 g-atom) of Na metal cut in small pieces. After all the Na had dissolved, *trans*-**9** (30.0 g, 0.10 mol) in 100 ml of dry dimethylformamide was added with vigorous stirring. After the addition was complete, the reaction mixture was heated to 80–90° for 18 hr, cooled with H_2O, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Distillation of the residual oil afforded 17.5 g (70%) of trans ester **10** as a colorless liquid: bp 124–126° (0.025 mm); ir (neat) 3090, 3040, 1600 (aromatic), 2880 (CH_2 stretching), 1730 cm^{-1} (C=O). Glpc on 10% silicone gum rubber (UC-W98) on a Diatoport S (80–100 mesh) 4 ft × 0.25 in. glass column with column temperature 200°, inject port temperature 300°, detector temperature 280°, inlet pressure of 40 psi, and carrier gas (He) flow rate of 60 ml/min showed one peak at 8.0 min for *trans*-**10**.

***trans*-2-(Benzylthiomethyl)cyclobutylcarboxyhydrazide (11).** To 10.0 g of hydrazine hydrate (85%) maintained at 130–135° (oil bath) was added dropwise with stirring 25.0 g (0.10 mol) of trans ester **10**. After the addition was complete, 30 ml of EtOH was added and the mixture was kept at 130–135° for an additional 18 hr. The cooled reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with H_2O, dried (Na_2SO_4), filtered, and concentrated under reduced pressure affording 22.5 g (94%) of a highly viscous liquid which did not solidify on cooling. Crystallization was achieved by treating with boiling petroleum ether (bp 30–60°) and adding toluene dropwise to dissolve the oil. Upon very slow cooling, trans acid hydrazide **11** crystallized as white needles: mp 50–51°; ir (KBr) 3260 (NH stretching), 1600 cm^{-1} (broad, C=O). Glpc on 10% silicone gum rubber (UC-W98) on a Diatoport S (80–100 mesh) 4 ft × 0.25 in. glass column with column temperature 225°, inject port temperature 310°, detector temperature 300°, inlet pressure 40 psi, and carrier gas (He) flow rate of 60 ml/min showed one peak at 8.25 min. *Anal.* ($C_{13}H_{18}ON_2S$) C, H, N, S.

***trans*-2-(Benzylthiomethyl)cyclobutylamine Hydrochloride (14).** To a well-chilled solution of trans acid hydrazide **11** (20.0 g, 0.08 mol) in 200 ml of Et_2O was added 6.9 g (0.10 mol) of $NaNO_2$ in 25 ml of H_2O. Maintaining a temperature of 0–5°, 40 ml of 6 *N* HCl was added dropwise with stirring. After the addition, the reaction mixture was stirred for another 15 min at 0–5°. The Et_2O layer was separated and the aqueous solution was extracted with Et_2O. The Et_2O layer was washed with cold H_2O, dried (Na_2SO_4), and filtered. Ir (toluene) showed the characteristic absorption band for the azide group at 2150 cm^{-1} indicating the presence of **12**. The toluene solution containing *trans*-**12** was refluxed for 1 hr, cooled, and most of the toluene was removed under reduced pressure. The residual brown liquid showed a band at 2270 cm^{-1} (isocyanate) for **13**.

The isocyanate (*trans*-**13**) was dissolved in 200 ml of absolute Et_2O and stirred at room temperature for 0.5 hr. KOH (12.5 g, 0.22 mol) in 100 ml of EtOH–H_2O (1:1) was added and the mixture was refluxed for 18 hr. The reaction flask was cooled, diluted with H_2O, and extracted with Et_2O. The Et_2O layer was washed with H_2O, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Distillation of the residual oil afforded 7.5 g (46%) of a colorless liquid: bp 108–110° (0.005 mm); ir (neat) 3380, 3300 (NH stretching), 3090, 3070, 3040 (aromatic), 2870 (CH_2 stretch), and 1605 cm^{-1} (aromatic and NH bending). Glpc on 10% silicone gum rubber (UC-W98) on a Diatoport S (80–100 mesh) 4 ft × 0.25 in. glass column with column temperature 180°, inject port temperature 270°, detector temperature 310°, inlet pressure of 40 psi, and carrier gas (He) flow rate of 60 ml/min showed one peak at 7.0 min for the free base of *trans*-**14**. The HCl salt **14** was prepared by passing HCl gas into an Et_2O solution containing the free base of *trans*-**14**; crystallization from 2-propanol–Et_2O afforded a solid, mp 83–84°.

***trans*-2-Mercaptomethylcyclobutylamine (4) Hydrochloride.** In a 250-ml three-neck flask equipped with a stirrer, a gas inlet tube, and a Dry Ice condenser was placed 6.0 g (0.025 mol) of *trans*-**14**. (Note: this reaction fails when the free base of **14** is employed.) Dry liquid NH_3 (100 ml) was introduced into the flask. Na metal (small pieces, 1.4 g, 0.06 g-atom) was added to the solution under N_2 until a permanent blue color remained for 45 min. The reaction mixture was stirred for an additional 2 hr and the excess Na was decomposed by adding small portions of NH_4Cl. The NH_3 was evaporated; residual NH_3 was removed after adding 100 ml of dry Et_2O by warming the reaction flask gently over a hot H_2O bath. The stirred Et_2O suspension was cooled and 100 ml of dry Et_2O saturated with HCl gas was added. The contents were stirred for 1 hr, the solids were filtered and washed with dry Et_2O, and the organic salt was dissolved in anhydrous *i*-PrOH. The *i*-PrOH solution was concentrated under reduced pressure, dry Et_2O was added, and crystallization was induced at 0–5°. Three recrystallizations from *i*-PrOH–Et_2O afforded 3.5 g (90%) of analytically pure *trans*-**4** HCl as a white solid: mp 117–118°. *Anal.* ($C_5H_{12}NSCl$) C, H, N, S.

B. Biological Aspects. 1. Radiation Chemical Studies with

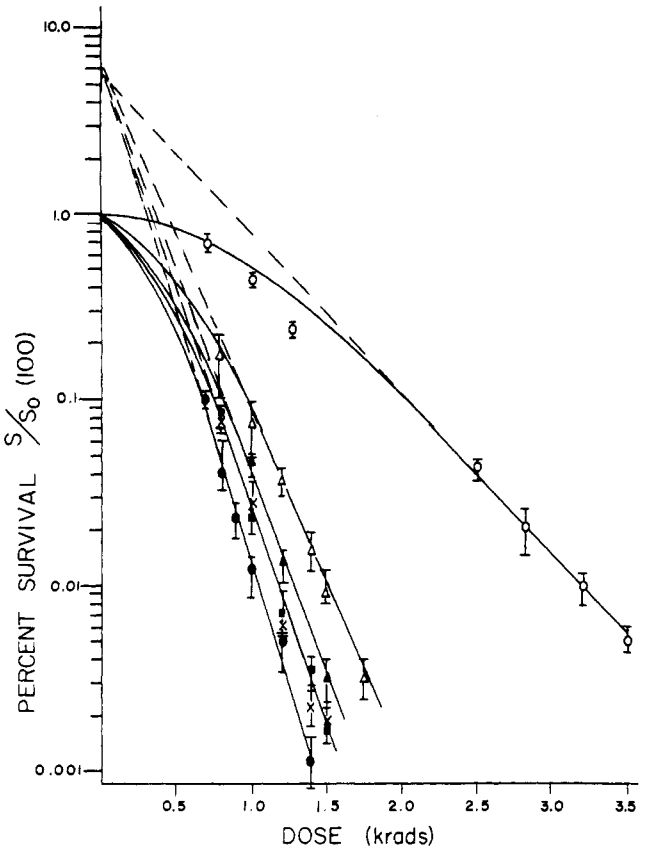


Figure 1. Dose–survival curves for Chinese hamster cells irradiated under aerobic conditions in the presence or absence of various analogs (2.5 m*M*). (–O–O–O–) no analog, cells under N_2; (–Δ–Δ–Δ–) *trans*-**3** (points shown), *trans*-**4** (points not shown), no significant difference between *trans*-**3** and *trans*-**4**; (–▲–▲–▲–) MEA (**1**) (points shown), or *trans*-**2** (points not shown), no significant difference between **1** and *trans*-**2**; (–X–X–X– and –■–■–■–) *cis*-**3** and *cis*-**2**, respectively, no significant difference; (–●–●–●–) no analog, cells under air.

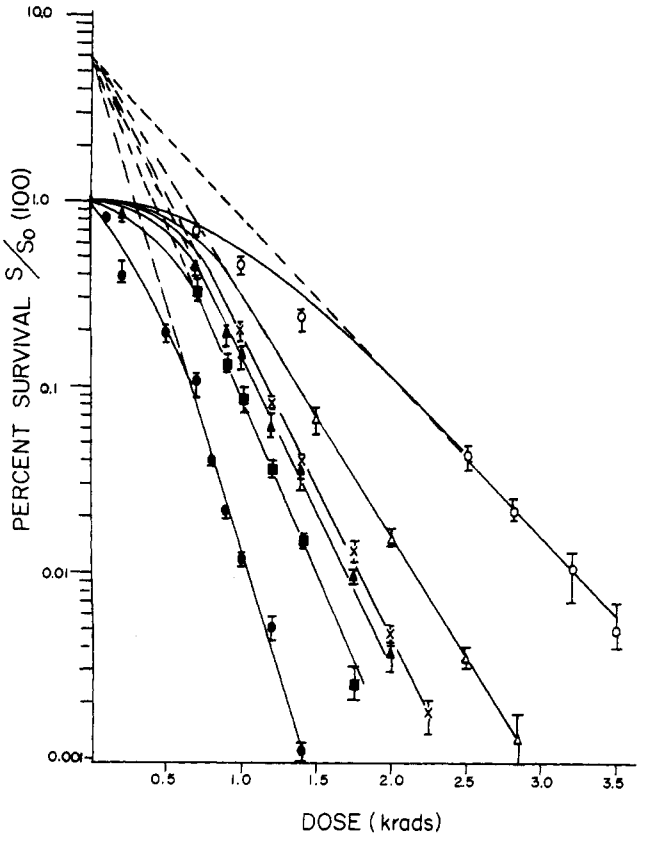


Figure 2. Dose–survival curves for Chinese hamster cells irradiated under aerobic conditions in the presence or absence of various analogs (5.0 m*M*). (–O–O–O–) no analog, cells under N_2; (–Δ–Δ–Δ–) *trans*-**3**; (–X–X–X–) *trans*-**2**; (–▲–▲–▲–) MEA (**1**); (–■–■–■–) *trans*-**4**; (–●–●–●–) no analog, cells under air.

5-Nitro-2-furfuraldehyde Semicarbazone [Nitrofurazone (NF)]. NF reacts with free radicals in DNA (generated by the indirect action of OH from the radiolysis of H_2O) resulting in acid-stable NF–DNA complexes. MEA competes with NF for these same radicals and protects the DNA from such binding.[8] The results obtained in triplicate experiments found in Table I were determined using methods identical with those reported for MEA.[7,8] The concentration of thiol analog required to effect a 50% inhibition of the reaction of 5μ*M* NF with DNA radicals is an indirect measure of the ability of the analog to compete with NF and reduce such free radicals generated in DNA.

2. Effects of Analogs in Cell Culture. The techniques described in this section were employed to obtain those data listed in Table II and displayed in Figures 1–6.

(a) Cell Culture Techniques. The Chinese hamster fibroblast cell line V-79-4 used in these experiments was grown in monolayer cultures using MEM [minimal essential medium (Eagle) with Hank's BSS] + 10% fetal calf serum (Flow Laboratory, Inc.) supplemented with 1.0 m*M* sodium pyruvate (Microbiological Assoc.), 1% nonessential amino acids (Microbiological Assoc.), 2 m*M* glutamine (Flow Laboratory, Inc.), 50 μg/ml of gentamycin sulfate and 0.05% filtered $NaHCO_3$. The pH of the media was maintained between 6.8 and 7.2; the osmolarity ranged from 285–320 mosM. Cultures were routinely maintained at 37° in a H_2O jacketed CO_2 incubator (National Corp.). Cells to be irradiated were detached from exponentially growing cultures by use of procedures given elsewhere,[10] seeded at densities between 5×10^2 and 5×10^4 cells/cm^2 in a 60-mm diameter dish (Falcon Plastics, Inc.), and incubated for 12–48 hr prior to irradiation.

(b) Irradiation Techniques. For DNA repair, as monitored by autoradiographic techniques, cells were seeded at a density of 10^4 cells/cm^2 in 100-mm diameter petri dishes in which 22 × 11 mm cover slips had been placed. The cells were permitted to attach to the cover slips at 37° for 24 hr after which the medium was

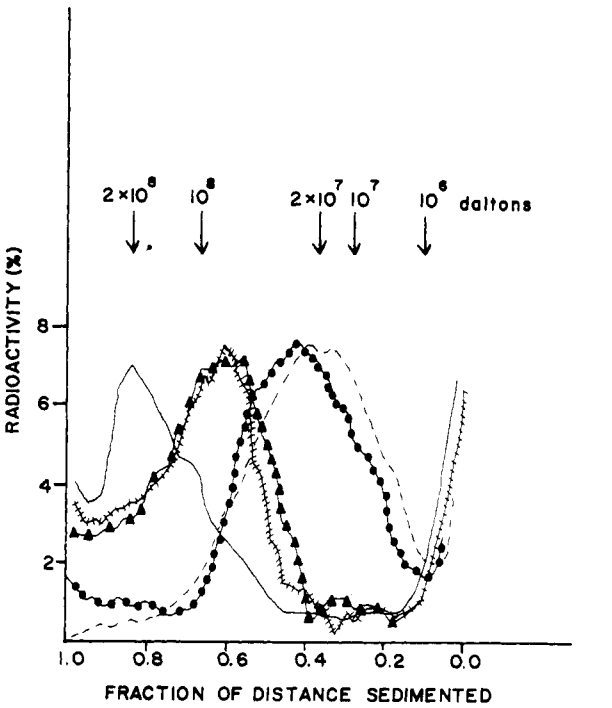


Figure 3. Sedimentation profiles for isolated DNA treated at 10 krads in the presence or absence of 5 m*M* analog. (——) no analog, 0 krad; (– – –) no analog, 10 krads; (–┼–┼–┼–) *trans*-**3** + 10 krads; (–▲–▲–▲–) MEA (**1**) + 10 krads; (–●–●–●–) *cis*-**3** + 10 krads. Sedimentation profiles were obtained by centrifugation at 35,000 rpm for 180 min on a 4-ml gradient containing 5–20% alkaline sucrose with a 0.2-ml 60% sucrose cushion layer and a 0.2-ml alkaline overlay.

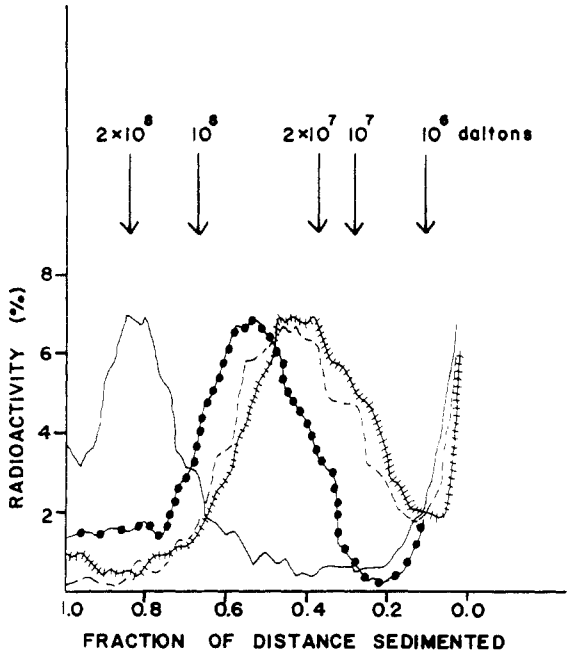


Figure 4. Sedimentation profiles for isolated DNA treated at 10 krads in the presence or absence of 5 m*M* analog. Sedimentation profile experimental conditions are the same as those described under Figure 3. (——) no analog, 0 krad; (+++++) no analog, 10 krads; (-●-●-●-) *trans*-**2** + 10 krads; (– – –) *cis*-**2** + 10 krads.

changed to MEM (modified as above) + 10% dialyzed fetal calf serum containing 2×10^{-3} *M* hydroxyurea (this compound inhibits its normal DNA replication but permits unscheduled DNA synthesis and strand break rejoining to take place).[14] Five minutes prior to irradiation various concentrations of radioprotectants and 2 μCi/ml of [^{3}H]-TdR with a specific activity of 50.1 Ci/m*M* (Swartz-Mann, Inc.) were added to the media. Cells were maintained at room temperature (21°) until the completion of the radiation treatment (25 min). They were then placed in a 5% CO_2 environment at 37° and, depending upon the experiment, maintained for varying durations of time (0–12 hr) prior to sampling. Cells were irradiated under either aerobic or anaerobic conditions. Irradiation under anaerobic conditions was performed in an air-tight chamber flushed for 5 min prior to irradiation with N_2 thus inducing acute hypoxia. The radiation source, quality, and dose rate for cultures irradiated under these conditions have been described previously.[11] The media and method used in clone formation have also been described elsewhere.[15]

(c) Radioautography (Autoradiographic Analysis). At the end of the incorporation period cover slips were washed with a nonradioactive balanced salt solution, fixed in Carnoy's solution for 10 min and prepared for radioautography by standard procedures.[16] The cover slips were dipped into twofold diluted, Ilford No. 4 emulsion and maintained at 4° for 6–7 days. Slides were developed in D19, fixed, and stained in Harris hemotoxylin with an eosin counter stain. Approximately 400 cells were counted in each sample at a magnification of 200-fold and the number of grains in each cell was determined.

Table I. Reactivity of MEA and Its Stereoisomeric Cyclobutyl Analogs and Homologs with Radicals in DNA

Compd	Concentration,[a] μ*M* (rel effect)[b]		
	Expt 1	Expt 2	Expt 3
MEA (1)[c]	18[d] (1.0)[b]	12[d](1.0)[b]	8[d](1.0)[b]
trans-2	4 (4.5)	4 (3.0)	1.5 (5.3)
cis-2	10 (1.8)	7 (1.7)	5.6 (1.4)
trans-3	3 (6.0)	3.8 (3.2)	1.1 (7.3)
cis-3	4 (4.5)	3.5 (3.4)	1.7 (4.6)
trans-4		5 (2.4)	

[a]Concentration of mercaptoamine required to effect a 50% inhibition of the reaction of 5 μ*M* nitrofurazone with DNA radicals. [b][MEA]$_{50\%\ \text{protection}}$/[analog]$_{50\%\ \text{protection}}$ = relative effect. [c]All mercaptoamines were studied as their HCl salts. [d]The standard error in these measurements is ±20%.

Table II. Toxicity Evaluation for MEA (1) and Its Stereoisomeric Cyclobutyl Analogs (*trans*- and *cis*-**2**) and Homologs (*trans*- and *cis*-**3** and *trans*-**4**) in the Chinese Hamster Fibroblast Line V-79-4

Compd	Concn. m*M*	Cloning efficiencies (% of clone)[a]
MEA (1)	15.0	94.8 ± 3.2[b]
	10.0	97.1 ± 1.2
	5.0	95.5 ± 3.1
	2.5	98.1 ± 2.3
trans-2	15.0	89.3 ± 1.7
	10.0	93.1 ± 0.6
	5.0	98.7 ± 1.3
	2.5	99.1 ± 1.1
cis-2	15.0	3.1 ± 2.7
	10.0	17.9 ± 1.1
	5.0	31.2 ± 6.1
	2.5	42.3 ± 1.2
trans-3	15.0	79.9 ± 1.7
	10.0	85.4 ± 1.2
	5.0	92.3 ± 0.6
	2.5	98.8 ± 1.1
cis-3	15.0	7.3 ± 3.4
	10.0	19.7 ± 2.6
	5.0	39.8 ± 1.7
	2.5	84.7 ± 1.3
trans-4	15.0	67.6 ± 4.2
	10.0	89.9 ± 2.1
	5.0	96.6 ± 3.1
	2.5	98.2 ± 3.1

[a]Cloning efficiencies of cultures treated with various radioprotectants for 60 min prior to cloning at a density of 10 cells/cm^2. Cloning efficiencies are expressed as the number of colonies containing at least 150 cells/colony at the end of a 5-day incubation period. [b]Each value is the mean ± standard deviation with a sample number of ten plates and normalized with respect to the control values.

(d) Toxicity and Dose-Survival Relationships. The toxicity of each compound was determined by clone formation. Fibroblast cultures at a cell density of 1×10^4/cm^2 were treated for 60 min with varying concentrations of radioprotectants, washed twice with Hank's salt, and trypsinized at 4° with 20 μg/ml of 3X crystalline trypsin (Worthington Chemicals, Inc.) in Hank's salt solution minus Ca^{2+} and Mg^{2+} with 0.5% lactoalbumin (Worthington Chemicals) and 2% tris(hydroxymethyl)aminomethane at an initial pH of 7.9. Cells were subsequently seeded at a density of 500, 250, and 125 cells/60-mm diameter dish under MEM-H, 2 m*M* glutamine, fourfold concentration of nonessential amino acids, twofold concentration of vitamins, 1 m*M* sodium pyruvate, 0.22% $NaHCO_3$ and 1% bovine serum albumin (type H-7) (Reheis Chemicals Corp.), and 10% fetal calf serum. These cultures were then incubated at 37° in a 5% CO_2–95% air atmosphere for 5 days after which the cultures were fixed with 2% phosphate buffered formalin and stained with a hematoxylin-eosin stain. Dose-survival relationships with and without concurrent γ irradiation were determined by the technique described above.

(e) Alkaline Sucrose Sedimentation. Methods used for the determination of single strand breaks have been previously reported.[11,17]

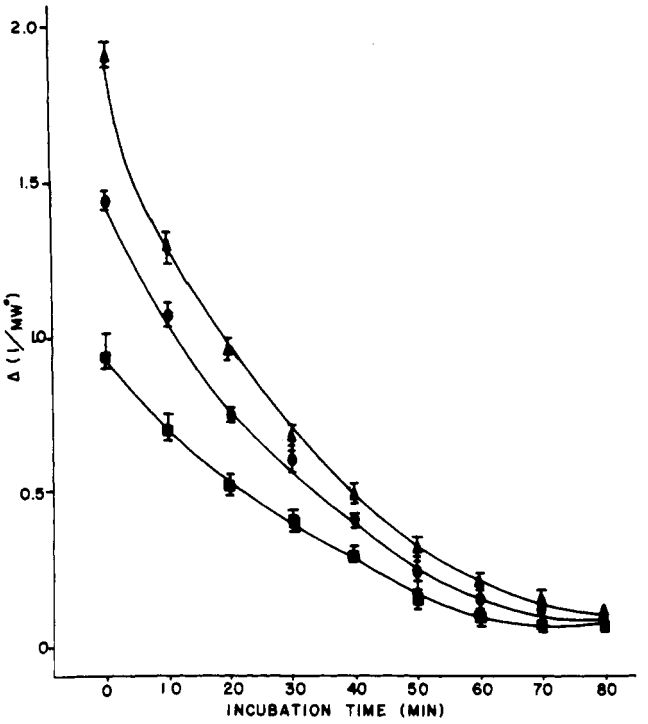


Figure 5. Rejoining of single strand breaks in DNA induced by 20 krads γ irradiation followed by incubation at 37° for varying times. (–▲–▲–▲–) absence of mercaptoalkylamines; (–●–●–●–) 5 m*M* *cis*-**3**; (–■–■–■–) 5 m*M* *trans*-**3**.

3. Determination of Radiation Inactivation Rates. Data listed in Table III were obtained using techniques previously described.[18] For these investigations the Chinese hamster cell line V79-379A was employed.

Results

All stereoisomeric cyclobutyl analogs (**2**–**4**) studied compete more favorably for radiation induced radicals in isolated DNA than does the parent compound MEA (**1**) when assessed for their ability to prevent NF bonding to the biopolymer (Table I). Analogs *trans*-**2**, -**3**, and -**4** and *cis*-**3** are equally active in this system; the μM concentration of analog required to effect a 50% inhibition of NF bonding with DNA is equal to or less than the μM concentration of NF used in these studies. Thus, all of these analogs donate H atoms more readily to radicals in DNA than either *cis*-**2** or MEA. The major stereoselective difference was observed between *cis*- and *trans*-**2**; the latter isomer is approximately 2–3 times as effective as the former.

MEA (**1**) and all cyclobutyl analogs were evaluated for their toxicity in the Chinese hamster fibroblast line V-79-4 at four different concentrations (2.5, 5, 10, 15 m*M*). The results summarized in Table II show that *cis*-**2** has a TD_{50} of approximately 2.5 m*M* and is the most toxic of all cyclobutyl analogs tested. Furthermore, both cis isomers (**2** and **3**) were significantly more toxic than their respective trans isomers at all concentrations ($p < 0.001$). At concentrations up to 10 m*M* all trans isomers, like MEA (**1**), demonstrated little toxicity. At a concentration of 15 m*M* *trans*-**3** and **4** were significantly more toxic than either *trans*-**2** or MEA (**1**) ($p < 0.001$).

Utilizing concentrations exhibiting the least toxicity, radiation inactivation rates ($k = 1/D_0$ rad^{-1}) for aerobically irradiated Chinese hamster fibroblast cultures (cell line = V79-379A) were determined for each compound at concentrations between 0 and 5.0 m*M* (Table III). No significant modification of inactivation rates or survival was observed for MEA (**1**) or any of its cyclobutyl analogs at concentrations of 0.5 m*M* or less. At a concentration of 1.0 m*M* MEA (**1**) shows an apparent, but not significant, reduction in the inactivation rate constant. At this same concentration *cis*-**2** exhibited a toxicity level incompatible with analysis by this method. Radioprotection was not reported if compounds in a parallel toxicity control (20-min exposure at 21°) reduced plating efficiences to ≤90%. Cyclobutyl analog *cis*-**3**, which is less toxic than *cis*-**2**, produced no significant modification of the inactivation rate. Similarly, *trans*-**2** exhibited

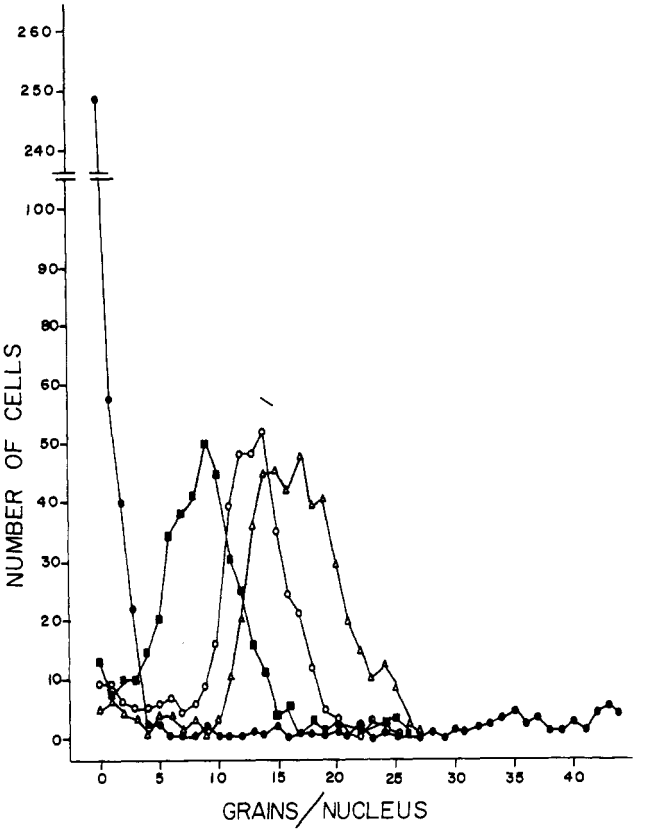


Figure 6. Autographic analysis of the effect of MEA (**1**, 5.0 m*M*) and *trans*-**3** (**5.0 m*M***) after 2 krads γ radiation in the Chinese hamster fibroblast cell line V-79-4. Each profile represents approximately 400 cells analyzed. (–●–●–●–) 0 krad; (–Δ–Δ–Δ–) 2 krads; (–○–○–○–) MEA (**1**) + 2 krads; (–■–■–■–) *trans*-**3** + 2 krads.

Table III. Radiation Inactivation Rates ($k = 1/D_0$ rad^{-1}) for Air-Saturated Chinese Hamster Fibroblast Cells V79-379A Irradiated in the Presence of Various Concentrations of Mercaptoamines

	Concentration of analog in medium, m*M*						
Compd	0	0.1	0.25	0.5	1.0	2.5	5
MEA (**1**)[a]	5.71[b]	5.6	5.7	5.4	5.3	4.9	4.6
trans-**2**	5.7	5.7	5.7	5.7	5.5	4.5	3.8
cis-**2**	5.7	5.7	5.7	5.7	*c*		
trans-**3**	5.7	6.0	6.0	5.7	4.8	3.94	3.05
cis-**3**	5.7	5.5	5.7	5.6	5.6	4.3	
trans-**4**	5.7	5.7	5.7	5.6	4.8	4.5	4.06

[a] All mercaptoamines were biologically evaluated as their HCl salts. [b] Numbers in this table represent radiation inactivation rates (k) × 10^3 for each indicated concentration of analog. Each k represents an average of three independent determinations. The standard error for each determination is ≤5%. [c] indicates cell toxicity; cells were exposed to all drugs for 20 min at 21° in parallel drug controls and a reduction of plating efficiency to ≤90% defined a toxic response.

no significant reduction, while *trans*-3 and -4 were the only compounds to exhibit a significant effect at these concentrations. While MEA (1) at a concentration of 2.5 m*M* exhibits a barely significant reduction, all cyclobutyl analogs, at this same concentration, exhibited a significant reduction with *trans*-3 being the most effective. At the highest concentration tested (5 m*M*) all *cis*-cyclobutyl analogs were too toxic to be assessed, while MEA (1) and all trans analogs showed little toxicity but significant radioprotective activity. Although all trans analogs are equal to or are more potent than MEA (1), *trans*-3 exhibits significantly greater radioprotective activity than any other analogs tested. At those concentrations where the cis isomers could be assessed no significant stereoselective differences in activity were observed. However, there is an apparent increase in effectiveness with increasing concentration with *trans*-3 that is greater than the effect observed for *cis*-3.

Radiation inactivation rate data obtained for MEA (1) and related cyclobutyl analogs (2–4) may be compared with dose–survival curves obtained with 2.5 (Figure 1) and 5.0 m*M* (Figure 2) mercaptoalkylamine. At either dosage neither MEA nor its analogs proved to be as effective as irradiation under N_2. At a concentration of 2.5 m*M* *trans*-3 and -4 proved to be the most effective radioprotective agents. MEA (1) and *trans*-2 were the next most effective radioprotectants, whereas *cis*-2 and -3 showed an apparent, but nonsignificant radioprotective effect. At a 5.0 m*M* concentration *trans*-3 under air was approximately two-thirds as effective as irradiation under N_2 alone in preventing radiation induced lethality. Cyclobutyl analog *trans*-3 was the most potent of all compounds assessed. Owing to their toxicity none of the cis analogs were evaluated at 5.0 m*M* concentrations. All trans isomers (2–4) were effective as radioprotectants at this concentration. Both *trans*-2 and -3 were more effective than MEA. MEA (1) seems to be slightly more effective than *trans*-4.

Ionizing radiation produces several forms of damage to DNA (single and double strand breaks, point mutations, and γ-endonuclease sensitive lesions).[19] These lesions may be repaired by as many as three repair systems (prereplication, strand break, and post replication repair).[20] Each of these repair systems may have several subcomponents.[21-25] Autoradiography following γ irradiation can be used to measure incorporation of radiolabeled bases into cellular DNA. In the absence of DNA synthesis this incorporation is thought to represent repair synthesis in the damaged regions of the DNA.[26] Recently, it has been estimated that the size of the repaired regions is approximately four bases.[27] Single strand break repair does not involve insertion of new bases[20,22,28] and can be readily measured by sedimentation of denatured DNA in an alkaline sucrose gradient.[28]

Figures 3 and 4 illustrate the change in molecular weight after a radiation dose of 10 krads in the presence and absence of MEA (1) and selected analogs at a concentration of 5 m*M*. The average molecular weight of DNA derived from nonirradiated cells was approximately 2×10^8 daltons. A dose of 10 krads, in the absence of any radioprotectants, decreased the average molecular weight tenfold (*i.e.*, 2×10^7 daltons). In the presence of MEA (1) the average molecular weight was decreased approximately fivefold (*i.e.*, to 5×10^7 daltons). Analogs *cis*-2 (Figure 4) and *cis*-3 (Figure 3) showed no significant alteration in the number of breaks obtained, whereas MEA and *trans*-3 (Figure 3) showed approximately equal protection against strand break induction. In the presence of these compounds the average molecular weight of the DNA was decreased by approximately one-half following 10 krads of γ radiation. As was the case with *trans*-3, the *trans*-2 analog (Figure 4) was more effective than either of the cis analogs. This is significant with regard to the fact that in this study all cells were subjected to lethal doses of ionizing radiation. Thus, the cytotoxic effect of the cis compounds would not be expected to influence the DNA repair processes owing to the rapidity of this repair system; DNA single strand break rejoining in mammalian cells is completed in <2 hr even after a lethal dose of irradiation. Further, if cells are held at 37° for various times after aerobic γ irradiation (20 krads, 0°), a rapid increase in the DNA molecular weight is observed (Figure 5) indicating that strand breaks are rejoined. The kinetics of strand break rejoining show that 50% of these lesions disappear within 20 min and that by 90 min less than 5% remain. The presence of the *trans*-3 analog reduced the initial number of breaks by 50% at a concentration of 5 m*M* whereas the *cis*-3 analog was less effective reducing the initial number of single strand breaks by only 25%. Both stereoisomers appear to reduce the effective rate of single strand break rejoining in the latter but not initial phase of the process. While the effect appears to be small it may be noted that the time to complete rejoining regardless of the initial number of breaks is similar in the presence or absence of radioprotectant.

As with single strand repair, replication repair (excision of γ-endonuclease sensitive sites) is assumed to be completed very shortly after γ irradiation (within 6 hr). Autoradiography measures the incorporation of labeled bases, presumably, into the repair regions of the DNA. Figure 6 represents a profile of this form of repair in cells irradiated in the absence and presence of MEA and *trans*-3. In the absence of irradiation and analogs, less than 5% of the cells demonstrate more than four grains per cell. Following a dose of 2 krads the average number of grains per nuclei is 18, thus demonstrating that a significant number of new bases have been incorporated into the DNA. In the presence of 5 m*M* MEA (1) the average nuclei showed 13 grains after a dose of 2 krads. Again, *trans*-3 proved to be more effective than MEA (1) with the average number of grains per nucleus being approximately 9. It is, therefore, suggested by these data that the initial lesion is prevented from occurring and thus fewer endonuclease sensitive sites are induced. An alternate hypothesis would be that *trans*-3 or MEA causes a stereoselective block which prevents the γ-endonuclease from recognizing the site of its incising function.

The comparative radioprotective activity in mice of all mercaptocyclobutylamine analogs synthesized is found in Table IV. The relative order of activity can only be approximated but by ip injection appears to be *cis*-2 ≥ *cis*-3 ≥ *trans*-4 ≥ *trans*-2 ≥ *trans*-3. By oral administration, *cis*-3 ≥ *cis*-2 ≥ *trans*-2 ≥ *trans*-3 > *trans*-4. Clearly, the cis isomers are among the most active compounds.

Discussion

Ionizing radiation produces immediate chemical alteration in irradiated tissues; the initial chemical changes result in metabolic derangements which during subsequent days may lead to cellular damage and death.[30] One assumption made is that such damage is due to the destruction or modification of a limited number of specific molecules referred to as target molecules. While the identity of such molecules is not known, nucleic acids and proteins are likely candidates;[31] for years radiation-induced damage to DNA has been regarded as one of the most important factors leading to the death of irradiated mammalian cells.[32-35] Furthermore, depression of DNA synthesis is a prominent biochemical effect of irradiation.[36-41]

Since MEA (1) is known to increase the survival time for mice given a lethal dose of whole body X-radiation[3,42-44]

Table IV. Protection of Mice against γ Radiation by Stereoisomeric Mercaptocyclobutylamine Analogs and Homologs of MEA (1)[a]

Compd[b]	LD_{50}, mg/kg (route of admin)[c]	Drug dose, mg/kg	Time interval, min[d]	% survival (30 day)[e]
trans-2	180 (ip)	50	15	0
		100	15	40
	500 (oral)	150	30	0
		300	30	50
cis-2	200 (ip)	37	15	0
		50	15	60
		75	15	70
		100	15	90
	500 (oral)	150	30	0
		300	30	60
trans-3	280 (ip)	50	15	0
		100	15	20
	750 (oral)	175	30	10
		350	30	10
cis-3	225 (ip)	50	15	30
		100	15	70
	450 (oral)	125	30	20
		250	30	70
trans-4	250 (ip)	37.5	15	0
		75	15	20[f]
		150	15	50[g]
	500 (oral)	200	30	0
		300	30	0

[a] The data found in this table were supplied by M. M. Grenan, Department of Biology, Division of Medicinal Chemistry, Walter Reed Army Institute of Research. The methods employed to obtain these data are similar to the ones reported in ref 4, except that a cesium-137 γ irradiator was employed. All mice (Walter Reed strain ICR females, 9–10 weeks old, weighing 22–33 g at the time of the test) were given a radiation dose of 849 rads. [b] All analogs were administered as their water-soluble HCl salts. [c] When administered ip all analogs were dissolved in NaCl–H_2O. When administered orally all analogs were dissolved in H_2O. [d] Administration prior to radiation. [e] Ten mice were employed at each dose level. In all experiments ten mice were also employed as controls. All controls (no drug) showed 0% survival. [f] Repeat experiment at 75 mg/kg ip gave 10% survival. [g] Repeat experiments at 150 mg/kg ip gave 60% survival.

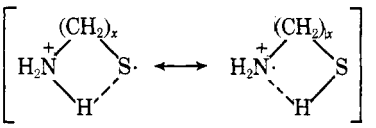
and reduce markedly the radiosensitivity of bacteria when added to cell suspension prior to irradiation,[45] it appeared to us that cyclobutyl analogs **2–4** would exhibit stereoselective biological properties and, therefore, be useful as probes for investigating the mechanism of mercaptoalkylamine radioprotection. Since the pK_a's (Table V) for all of these compounds fall within a relatively narrow range (pK_1 = 7.8–9.2) we rationalized that selective differences in biological activity should mainly reflect differences in configuration and for homologs of **2** (namely **3** and **4**) some differences in partition coefficient. It also should be stressed that any differences in pK_a observed for the individual geometrical isomers of **2** or **3** are negligibly small and fall within the experimental error of the method employed.[46]

Table V. Mercaptoalkylamine pK_a's[a]

Compd	pK_1	pK_2
MEA (1)	8.6[b]	10.75[b]
trans-2	7.8	9.7
cis-2	7.8	10.3
trans-3	8.8	10.8
cis-3	8.7	11.2
trans-4	9.2	10.9

[a] Calculated according to ref 45 using a computer program written by Dr. Clark Dehne, Professor of Chemistry, Capital University; visiting research associate, The Ohio State University, Columbus, Ohio. [b] Taken from ref 47.

Three suggested[48] modes of action for thiol analogs involve (1) target molecule protection by interception of free radicals from water or organic radicals (radical scavenging), (2) target molecule repair by H transfer, and (3) target molecule radioprotectant interaction resulting in decreased intrinsic radiosensitivity of the target molecule. With regard to radical scavenging, Doherty and coworkers[44] suggested that facile formation of cyclic resonance stabilized radical **1a** of MEA accounts for the good radioprotective activity of this compound. The somewhat greater radioprotective activity observed for 3-mercaptopropylamine (MPA) was rationalized on the basis of increased stability of six-membered resonance stabilized cyclic radical **1b**. This interesting hypothesis does not find support in our results. Both



1a, $x = 2$
b, $x = 3$

of the conformationally restricted analogs most closely related to MEA, namely *cis*- and *trans*-**2**, are active *in vivo* and show relatively small differences in radioprotective activity in mice (Table IV). Under conditions similar to the one employed for the *in vivo* biological evaluation of the cyclobutyl analogs, ip injection of MEA (150 mg/kg) afforded 80% 30-day survival of mice after a radiation dose of 1000*R* [^{60}Co γ irradiation (dose rate 100–500/min)].[4] Although ip injection of *cis*-**2** compares most favorably with MEA (**1**) and appears to be nearly twice as potent as *trans*-**2**, radiochemical studies (Table I) *in vitro* argue against cis cyclic intermediates. When *cis*- or *trans*-**2** were assessed for their ability to react with radicals in DNA (Table I), *trans*-**2** was found to be about twice as effective as *cis*-**2**. *trans*-**2** has a juxtaposition of SH and NH_2 groups too distant to intramolecularly hydrogen bond in either of its possible flip conformations (Drieding molecular models). Thus, these data argue against the necessity for cis cyclic intermediates.[44]

There are several examples in the literature which show that functional groups substituted 1,2-trans in cyclobutanes undergo reaction with nucleophiles at a faster rate than the corresponding cis isomer, presumably because trans functions are more exposed to attack.[2,49,50] Similarly, *trans*-**2** more readily reacts with radicals in DNA (by a hydrogen donating mechanism)[51] than does *cis*-**2** because the SH group is less sterically hindered in the former isomer. However, all of these results cannot be explained on steric grounds alone. Insertion of a $-CH_2-$ function between the cyclobutyl ring and the NH_2 group in *cis*-**2** affords *cis*-**3** which is equally as active as *trans*-**2** or **4** (Table I). Increased reactivity of *cis*-**3** [and, for that matter, all cyclo-

butyl analogs over MEA (1)] with radicals in DNA may be found in the increased lipophilic properties of the inserted $-CH_2-$ function and the cyclobutane ring per se over the ethylene function in MEA (1). Increased lipophilic character would lead to enhanced hydrophobic binding to DNA and consequently increased competition with NF for DNA radicals.

The toxicity observed for analogs *cis*-2 and *cis*-3 (Tables II and III) precluded thorough comparative radioprotective evaluation of cis and trans isomers in Chinese hamster fibroblast cells. Previously, Vergroesen, Budke, and Vos,[52] working with an established heteroploid cell line derived from human kidney tissue, showed that thiol compounds with a pK value lower than 10 were very toxic at concentrations between 0.1 and 2.0 mM; the toxicity could be prevented by lowering the pH of the medium to 6.3. The greater toxicity observed for *cis*-2 or -3, however, cannot be explained on the basis of their pK_a's. Geometrical isomers, *cis*- and *trans*-2, have virtually identical pK_a's which differ from the pK_a's of *cis*- and *trans*-3 by approximately 1 pK_a unit and *trans*-4 by 1.4 pK_a units (Table V). These data indicate a stereoselective difference in activity at the cellular level and may, as proposed by Vergroesen, Budke, and Vos,[52] be caused by an irreversible intracellular reaction of a specific oxidation product of these compounds with some cellular constituent. Unless these analogs are taken into the cell by some stereoselective process these data argue against the proposal that the degree of radioprotection is only dependent upon the intracellular thiolate ion concentration.[52] The greater activity of *trans*-2, -3, and -4 (5 mM concentration) over MEA (1) might be due to increased thiolate ion concentration owing to increased absorption of the more lipophilic cyclobutanes. However, *trans*-3 and -4 have significantly different radioprotective activities (Table III) but similar pK_a's and predicted lipid solubilities.

Furthermore, at the 0.1 mM concentration, *trans*-3 shows a small but significantly better degree of radioprotective activity than does *cis*-3. As the toxic level for *cis*-3 is approached, its radioprotective activity approaches the activity observed for *trans*-3 (*cf.* Table III; 2.5 mM concentration of drugs). On steric grounds *trans*-2 and -3 would be expected to undergo reaction with cellular components to a similar extent since in both analogs the SH groups are bonded directly to the cyclobutane ring and have a similar steric environment. In fact, *trans*-2 and -3 are the most potent radioprotective agents at the 5 mM concentration and *trans*-4, which contains a primary SH function, also exhibits good protection. Although *trans*-4 would be expected to undergo reaction with cellular components more readily, since this compound contains the sterically less hindered primary SH group, it may also undergo metabolic degradation more rapidly and hence its activity relative to *trans*-2 and -3 is somewhat decreased.

The relative order of radioprotective activity observed in Chinese hamster cells at the 2.5 mM concentration is *trans*-3 or -4 > MEA (1) or *trans*-2 > *cis*-3 or -2 (Figure 1). At concentrations of 5.0 mM (Figure 2) the cis isomers were too toxic to be evaluated and the order of decreasing radioprotection is *trans*-3 > MEA (1) or *trans*-2 > *trans*-4. These data also cannot be interpreted on the basis of steric effects alone; *i.e.*, the SH group in *trans*-4 is expected to be less sterically hindered to reaction with SH groups in proteins and radicals in DNA than is the SH group in *trans*-2 or -3, or any of the cis isomers. The increased radioprotective activity of *trans*-3 over MEA (1) is likely due to increased lipophilicity and, therefore, enhanced absorption. Since *trans*-4 shows a relatively small increase in radioprotective activity upon doubling the concentration, it would seem that the difference in potency between this analog and *trans*-3, which contains a secondary SH group, is related to the possibility that the former undergoes metabolic degradation more rapidly than the latter.

Stereoselectivity is also observed when these compounds are assessed for their ability to prevent against γ radiation induced single strand breaks in DNA. The relative protective activity follows the order *trans*-3 $\simeq$ MEA (1) $\gg$ *cis*-3 (Figure 3). Similarly, *trans*-2 shows moderate protection while *cis*-2 is inactive (Figure 4). Autoradiographic analysis also shows *trans*-3 to be a more effective radioprotectant than MEA (1) (Figure 6). Although these studies illustrate the stereoselective action of the cyclobutyl isomers it should be realized that the radiation doses employed in these studies are considerably greater than the biologically effective doses normally employed in cellular or animal studies owing to the lack of experimental techniques available for determination of DNA damage at lower doses. The lack of any correlation between the ability of these isomers to prevent DNA single strand breaks and protect mice against the lethal effects of ionizing radiation is in agreement with the work of Alexander and coworkers[53] who showed that variations of radiosensitivity in marine lymphoma and *M. radiodurans* are independent of the magnitude of the primary lesion in DNA.

An explanation for the fact that the cis isomers, 2 or 3, are relatively more effective than their corresponding trans isomers in mice may ultimately be found in their differential absorption, distribution, and metabolism. An analogous situation appears to exist in the study of hypoxic cell radiosensitizers. These compounds are believed to effect their radiosensitizing activity by an ability to oxidize transient free radical species in cellular targets.[7,8] Some chemicals, which are extremely effective as sensitizers in chemical and cellular systems *in vitro*, are ineffective as radiosensitizers in animal systems owing to their rapid chemical or biochemical inactivation.[54] The stereochemical protection of the active group in various drugs becomes as important a criterion for the design of a clinical compound as does the optimization of the chemical process producing the effect.

Presently, our studies seem to be consistent with the following. (1) The mechanism of radioprotection by mercaptoalkylamines is independent of the enzymatic repair mechanism for single strand breaks. This is not to say that mercaptoalkylamines may not be involved in the modification of other repair processes (base alteration or point mutation damage). These interpretations are supported by cell cycle analyses[55] where it has been shown that cells subjected to lethal doses of ionizing radiation do not die immediately, but most often only after several divisions; *i.e.*, cell death may be attributed to γ radiation induced alteration in the secondary, but not primary, structure of DNA. Furthermore, it should be emphasized that while MEA (1) in high concentrations (over 5 mM) is reported[56] to inhibit DNA synthesis of nonirradiated cells and slow the rejoining of radiation-induced single strand breaks at the 5 mM concentration, neither MEA (1) nor *cis*- or *trans*-3 at the 5 mM concentration significantly influenced rejoining of single strand breaks in our studies. (2) It is a reasonable assumption that radioprotection by these agents is related to the prevention of damage leading to alterations of genetic information by reduction of transient radicals either in the cytoplasma or on DNA. Further, direct enzyme protection, perhaps through metal chelation,[57] or *via* disulfide bond formation and other chemical–biochemical processes may be involved. However, as pointed out by Birzu and coworkers[58] the mechanism of chemical radioprotection is influenced by the number of free mole-

cules (unmetabolized by cells) rather than the total amount of protective molecules stored; comparative metabolic studies must be carried out with MEA and these analogs before further conclusions can be reached. Cis isomers may be more active *in vivo* because they are extremely poor substrates owing to steric crowding of the SH function. Consequently, they would remain in target tissue longer than trans isomers and be expected to have enhanced radioprotective activity *in vivo*. (3) Since there is a good correlation between the protection of radiation-induced lesions in cellular DNA and of the inactivation of cellular proliferative capacity of cells treated *in vitro* these compounds may effect radioprotection of animals by reducing the DNA damage in the rapidly proliferating cell compartments of the animal. The results are consistent with a mode of action involving chemical repair of transient radicals and protection against DNA and critical enzymatic sites.

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