J. RADIAT. RES., 36, 31–37 (1995)

2-Mercaptopropionylglycine Affords Enhanced Radioprotection After a Liposome Encapsulation

R. N. SHARAN*, A. ALAM[§], SANGHAMITRA CHAKRABORTY, JYOTI R. SAIKIA, and P. N. SRIVASTAVA[#]

Radiation & Molecular Biology Unit and [§]Immunology Laboratory
Department of Biochemistry, School of Life Sciences
North-Eastern Hill University, Umshing SHILLONG 793 022, India
(*Received, November 4, 1994*)
(*Revision received, January 9, 1995*)
(*Accepted, January 17, 1995*)

2-mercaptopropionylglycine (MPG)/Liposome/Drug delivery system/Radioprotection/Cell viability/Acetylcholine esterase

Use of radioprotective drugs in radiotherapy is desirable to protect normal tissues. 2-mercaptopropionylglycine (MPG) has shown promising results in experimental radioprotection. In this report, a liposome drug delivery system for MPG has been used in Swiss albino mice exposed to 1 to 8 Gy whole body Gamma-irradiation to test whether or not this modality enhances the MPG afforded radioprotection. A statistically significant, dose dependent enhancement of protection by liposome encapsulated MPG (LEM) was observed. LEM, as compared to free MPG, improved the viabilities of spleen and bone marrow cells by factors between 1.11 and 2.23 for different doses of radiation.

INTRODUCTION

2-mercaptopropionylglycine (MPG), an aminothiol, has been extensively used in experimental radioprotection *in vivo* and *in vitro*[1–3] because it affords moderate radioprotection at an effective dose, 20 mg kg b.w.$^{-1}$, far below its toxic dose, 2100 mg kg b.w.$^{-1}$. However, MPG showed radiosensitization effect in cases of Gamma-induced microsomal lipid peroxidation[4], catalase radiolysis[5], and *in vitro* human-lymphocyte-DNA damage[6,7]. Two main reasons could explain this undersirable reversal of effect of MPG. At first, free MPG is distributed to various tissues depending on the physiological conditions[8]. Thus, the quantity of MPG available in a particular tissue may be other than optimum for protection. Secondly, MPG metabolism to its oxide, disulfide and other derivatives after its administration[8–10] may render MPG less effective radioprotector. It has been demonstrated that MPG caused radiosensitization

* Corresponding author
# Nuclear Science Center, JNU Campus, New Delhi 110 067, India.

of microsomal lipid peroxidation[4], and of catalase radiolysis[5] due to MPG/Fe complex formation. Therefore, it is desirable to design modalities wherein MPG, or other similar drugs, may continue to be a radioprotector, possibly with an enhanced efficiency.

In this report, a liposome drug delivery system has been tested for MPG to see whether or not this modality increase radioprotection afforded by MPG to spleen, bone marrow and liver of Swiss albino mice at its non-toxic effective dose.

MATERIALS AND METHODS

Chemicals: MPG (Tiopronine) was obtained from Santen Pharmaceuticals Co., Japan.; dipalmitoyl phosphatidyl choline (DPPC), dicetylphosphate (DCP), cholesterol, trypan blue and acetylcholine from Sigma Chemical Co., USA; dithionitrobenzoic acid (DTNB) from SRL, India and Sepharose CL-4B from Pharmacia Fine Chemicals, Sweden. All other reagents and chemicals were of analytical grade.

Animals and Gamma-irradiation: Female Swiss albino mice (6–8 weeks old), randomly inbred colony housed at $21 \pm 2^{\circ}C$ with water and pellet dry feed *ad libitum*, were acutely irradiated at doses of 1, 2, 4, 6, and 8 Gy of Gamma-rays (^{60}Co Gamma source; 23.65 Gy min^{-1}) using Gamma Chamber 900 (BARC, Bombay, India).

Preparation of liposomes: Liposomes were prepared by a reverse phase evaporation method reported earlier[11,12]. Briefly, 5, 2.5 and 1 mg of DPPC, Cholesterol and DCP, respectively, were dissolved in 0.25 ml of chloroform by vortexing. To this lipid solution, 1 ml of 5 mM aqueous solution of MPG was added in alipuots of 0.2 ml while vortexing. Separation of liposome encapsulated MPG (LEM) from free MPG was done by Sepharose CL-4B column chromatography or by centrifugation[12].

Determination of MPG concentration: The concentration of free MPG as well as LEM was calculated by the assay of -SH group and has been described elsewhere[11]. Briefly, 0.1 ml of test sample was added to 2.9 ml of N_2 flushed assay mixture containing 10 mM DTNB in 100 mM PBS (pH 7.9) and 0.1 mM EDTA, mixed and absorption was immediately read at 412 nm. Cystein was used as a standard.

Administration of free MPG or liposome encapsulated MPG (LEM): Mice were intraperitoneally injected with 0.408 mg equivalent of MPG either as 0.5 ml of 5 mM aqueous solution of MPG or 1 ml of LEM 30 min prior to Gamma-irradiation. The control animals were irradiated without MPG.

Cell viability test: The viabilities of spleen cells (SC) and bone marrow cells (BMC) were calculated by dye exclusion technique as described earlier[13]. Immediately after irradiation, animals were killed by cervical dislocation and spleen and bone marrow cell suspensions were prepared in minumum essential medium. Viable and dead cells were counted on a Burker chamber under a Zena light microscope after 5 min incubation of cells with 1% trypan blue at 37°C. Each data point represents a minimum of 3 independent experiments, each with 3–5 replicates.

Calculation of viability modification factor (VMF): Dividing % viabilities of cells after irradia-

tion in the presence of either free MPG or LEM by that of radiation alone gave this factor:

$$\frac{\%\text{ viability in the presence of either MPG or LEM after} \times \text{Gy Gamma-irradiation}}{\%\text{ viability after} \times \text{Gy Gamma-irradiation}}$$

Student's t-test was applied to calculate the significance of differences.

Assay of liver acetylcholine esterase (AchE): Immediately after irradiation, animals were killed, livers removed and homogenized in 0.2 M sucrose solution. The whole homogenate was centrifuged at 2,000 *Xg* for 30 min at 40°C. Resulting supernatant was used for the assay of AchE following the method of Ott *et al.*[14] with minor modifications. The assay mixture in 3 ml contained 1 mM acetylcholine, 0.125 mM DTNB, 0.05% Triton X100 and 0.5 ml enzyme preparation. The reaction was followed on a spectrophotometer at 412 nm and increase in absorption min^{-1} was calculated. The activity of enzyme has been expressed as a specific unit which consumes 1 μmole of substrate at room temperature min^{-1} mg $protein^{-1}$. For controls, 6 mice were used in six independent experiments while the number of mice in MPG and LEM groups were 3 and 4 respectively.

RESULTS AND DISCUSSION

The biodegradable nature of liposome and its high potential of encapsulating target molecules[15,16], were main reasons for the selection of this drug delivery system. Reverse phase evaporation method of encapsulation of MPG into liposomes, used in this report, is simple and has been shown to be reproducible[11,12]. The molar ratio of DPPC:Cholesterol:DCP in the liposomes was 1:0.9:0.25 and the lipid/MPG ratio was 5.21. The percent entrapment of MPG

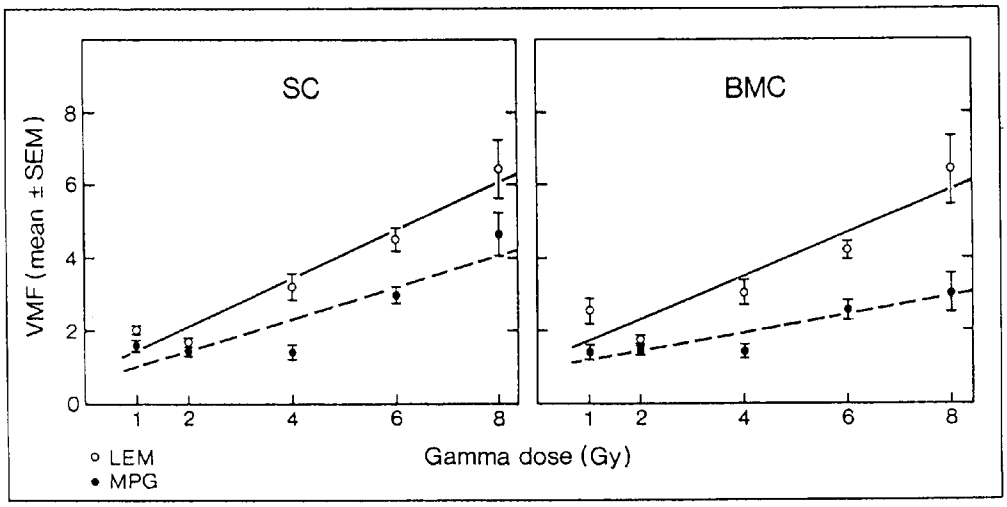

Fig. 1. Viability modification factor (VMF) for spleen cells (SC) and bone marrow cells (BMC) at different doses of whole body Gamma-irradiation. Free MPG and LEM were intraperitoneally injected to Swiss albino mice at the same dose level 30 min prior to irradiation.

was found to be 50±2.9. Administration of 0.5 ml of 5 mM MPG aqueous solution or 1 ml of LEM (0.408 mg MPG equivalent) delivered 20 mg MPG kg b.w.$^{-1}$ in each mouse (average weight=20 g) which is the effective radioprotective dose of MPG[2–7].

The viability modification factor (VMF) for spleen and bone marrow cells after various doses of whole body Gamma-irradiation in the absence (control) or the presence of equal amount of MPG (0.408 mg) administered as such (free MPG), or as LEM 30 min prior to irradiation showed a radiation dose dependent increase (Fig. 1). The numerical data on viability, with statistical evaluations, are given in Table 1. The LEM group displayed higher viability over the respective free MPG group by factors between 1.11 and 2.23 (for SC) and between 1.18 and 2.07 (for BMC) for different doses of radiation (Table 1, Fig. 1). For 4 & 8 Gy Gamma doses, the release of liver enzyme AchE into supernatant, signifying radiation induced membrane damage, was significantly reduced by MPG in both free MPG and LEM groups; the latter showing a higher tendency of protection (Fig. 2). The dose of MPG used in these experiments was far below its toxic dose, therefore, it is assumed that the drug did not influence the metabolism or the survival of the treated animals. Because the assays were performed for all groups, including the controls, identically, the immediate postirradiation protection of MPG, reported here, is likely to represent the normal situation of MPG affordable radioprotection.

Upon administration, free MPG (a) migrates to different tissues, consequently limiting the quantities of MPG available to SC, BMC and liver, and (b) is subjected to normal metabolic alterations[8–10]. The behaviour of LEM is likely to differ from the MPG on both these criteria because liposomes preferentially migrate to tissues rich in reticuloendothelial cells and fenstrated capillaries, viz. liver, spleen, bone marrow etc.[15] and the encapsulation delays metabolic

Table 1. Spleen cell (SC) and bone marrow cell (BMC) viabilities as a function of Gamma dose in the absence (control) and presence of equal quantity of free MPG (MPG), or liposome encapsulated MPG (LEM)

Tissue	Treatment groups	% viable cells (mean±SD)/dose of radiation				
		1 Gy	2 Gy	4 Gy	6 Gy	8 Gy
SC	Control	29.12 ±5.3(9)*	35.80 ±9.1(9)	19.31 ±6.7(11)	16.05 ±2.8(9)	11.98 ±5.3(16)
	MPG	47.35 ±8.1(9)	54.59 ±8.2(9)	27.70 ±9.5(11)	48.02 ±8.0(9)	55.73 ±13.8(17)
	LEM	59.73♣ ±2.8(9)	60.69◆ ±4.0(9)	61.86♥ ±9.8(11)	72.04♥ ±8.7(9)	77.14♠ ±18.1(19)
BMC	Control	24.73 ±9.8(9)	35.09 ±6.6(9)	19.54 ±6.8(11)	15.00 ±1.9(9)	11.45 ±4.7(19)
	MPG	35.25 ±6.2(9)	54.02 ±1.5(9)	29.30 ±6.2(11)	40.02 ±9.7(9)	36.20 ±10.6(9)
	LEM	63.74♥ ±4.9(9)	63.57♥ ±3.1(9)	60.78♥ ±8.4(11)	65.65♥ ±4.5(9)	74.63♥ ±7.9(11)

Significantly higher viability as compared to respective MPG groups: ♥($p \leq 0.0001$), ♣($p \leq 0.002$), ◆($p \leq 0.1$) and ♠($p \leq 0.001$)

* Numbers in paranthese indicates number of mice used.

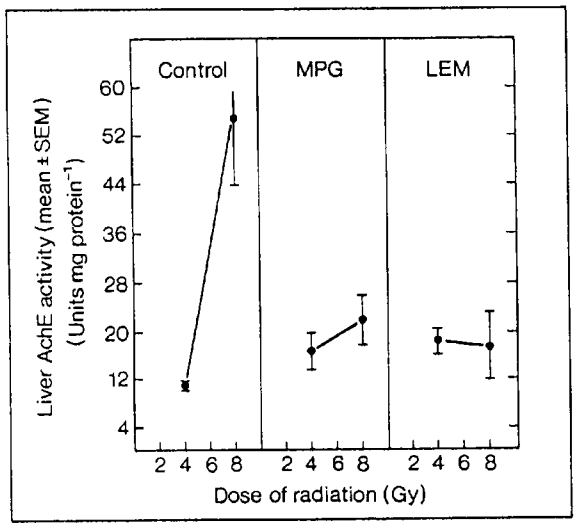

Fig. 2. Liver Acetylcholine esterase (AchE) released in the supernatant fraction after 4 and 8 Gy whole body Gamma-irradiation. Control group did not receive MPG while free MPG and LEM were intraperitoneally injected to Swiss albino mice at the same dose level 30 min prior to irradiation.

alterations of the entrapped drug[15,17]. Therefore, LEM afforded significantly higher radioprotection than free MPG, to SC and BMC (Fig. 1, Table 1) with liver AchE showing supporting trend (Fig. 2). The relative contribution of the two factors in LEM radioprotection has not been ascertained and is subject of further investigations.

LEM, as compared to MPG, afforded greater protection for all doses of radiation-statistically highly significant ($p \leq 0.001$) in case of BMC and at lower levels of statistical probability (p between 0.1 and 0.0001) in case of SC (see Table 1). BMC and SC exhibited higher viabilities with increasing doses of radiation in LEM treated groups but not in case of free MPG. Since MPG and LEM were administered to mice 30 min prior to irradiation, a part of the free MPG could be metabolically altered[8–10] making them less effective radioprotector. Thus, free MPG afforbable viability was nearly the same for different doses of radiation. In case of LEM, on the other hand, the metabolic alterations of MPG was delayed[15,17] and MPG remained longer in its protective form. Furthermoer, the encapsulation of MPG also prevents circumstantial interaction of MPG with Fe which has been shown to be the cause of its radiosensitizing effect[4,5]. To explain LEM induced increasing viability with higher doses of radiation, it may be reasonable to assume that release of MPG from liposomes into the cells is enhanced by radiation as liposomes are essentially like biomembranes. Thus, increasingly more MPG leaked out from liposomes into the cells at higher doses of radiation and could offer increased protection (higher viability). A relatively poor viability in 1 Gy controls is attributed to differences in batch of randomly inbred mice.

In conclusion, we have found that liposome encapsulation of MPG not only prevented undesirable radiosensitization effects of MPG reported earlier[4–7] but noticeably enhanced protective effects of MPG in our experimental conditions. These results agree with the earlier

observations of Papahadjopoulos *et al.*[17] that liposome encapsulation increased efficacy of antitumour drugs in mice. In an attempt to explain the reasons for it, Gabizon[18] has reported that metabolic alterations rather than quantitative differences caused liposome encapsulated DOX, an antitumour drug, to be more effective as compared to its free form. Whatever may be the reason, the efficiency of radioprotection afforded by MPG was significantly enhanced by liposome encapsulation. Thus, our results open up a new possibility of use of liposome drug delivery system for enhanced radiomodulation. Use of liposome drug delivery system offers an additional advantage of tissue targeting[15]. Liposomes may be directed to selective tissues with the help of specific antibody (against an antigen of the target tissues) tagged onto the liposome. Thus, the liposome vehicle can deliver calculated amounts of MPG to specific tissues in relation to the dose and protocol of irradiation and avoid a possible radiosensitization reported earlier[4–7]. Furthermore, liposome vehicle can be successfully used for other drugs in radiotherapy, whose use is presently limited due to high toxicity or metabolism problems. More work is underway in this laboratory on these lines.

ACKNOWLEDGEMENT

The authors wish to thank Prof. T. Sugahara and Santen Pharmaceuticals Limited, Japan, for a gift of MPG and Drs. F. Schneeweiss and H. P. Peterson for constructive criticism of the manuscript. Financial support for this work came from CSIR (RNS & SC) and UGC (AA & JRS).

REFERENCES

1. Devi, P. U. and Prasanna, P. G. S. (1990) Radioprotective effect of combination of WR-2721 and mercaptopropionylglycine on mouse bone marrow chromosomes, Radiat. Res. **124**: 165–170.
2. Sugahara, T. and Srivastava, P. N. (1976) MPG (2-mercaptopropionyl glycine)—a review on its protective action against ionizing radiations. In "Modification of Radiosensitivity of Biological Systems", pp. 77–78, IAEA, Vienna.
3. Sugahara, T. (1972) Chemical radiation protection by 2-mercaptopropionyl glycine. In "Advances in Antimicrobial and Antineoplastic Chemotherapy", Ed. Hejzler and Miroslav, pp. 825–826, University Park Press, Baltimore.
4. Ayene, S. I. and Srivastava, P. N. (1985) Radioprotective effect of 2-mercaptopropionyl glycine in radiation-induced microsomal lipid peroxidation, Int. J. Radiat. Biol. **48**: 197–205.
5. Wary, K. K. and Sharan, R. N. (1988) Effect of the radioprotector 2-mercaptopropionyl glycine (MPG) on the radiation inactivation of catalase *in vitro*, J. Radiat. Res. **29**: 104–109.
6. Wary, K. K., Laltanpuia and Sharan, R. N. (1989) 2-mercaptopropionylglycine (MPG) induced protection of DNA damage in gamma-irradiated human lymphocytes: A preliminary report, Radiosensitization Newslett. **8**: 2–4.
7. Sharan, R. N. (1990) MPG concentration dependent sensitization of gamma induced DNA strand breaks in human lymphocytes *in vitro*, Radiosensitization Newslett. **9**: 8–9.
8. Toshioka, N., Mita, I. and Chiba, T. (1970) Absorption, distribution, metabolism and excretion of ^{35}S-Thiola in rats. In "Proc. International Symposium on Thiola". Tokyo, pp. 1–8.

9. Chiba, t. (1973) Studies on thiol and disulfide compounds 1. Absorption, distribution, metabolism and excretion of ^{32}S-2-mercaptopropionyl glycine, Yakugaku Zasshi **93**: 112.
10. Carlsson, S. M., Denneberd, T., Emanuelsson, B.-M., Kagedal, B. and Lindgran, S. (1990) Pharmacokinetics of intravenous 2-mercaptopripionylglycine in man, Eur. J. Clin. Pharmacol. **38**: 499–503.
11. Sharan, R. N., Alam, A., Saikia, J. R., Chakraborty, S. and Srivastava. P. N. (1992) Liposome mediated delivery of 2-mercaptopropionyl glycine: Entrapment of MPG in liposome. Radiosensitization Newslett. **11**: 16–17.
12. Alam, A., Bhuri, S. R. K., Mavila, A. K. and Singh, V. (1992) Design of liposome to improve encapsulation efficiency of gelonin and its effects on immune reactivity and ribosome inactivating property, Molec. Cell Biochem. **112**: 107–114.
13. Wary, K. K. and Sharan, R. N. (1988) Aqueous extract of betal-nut of North-East India induces DNA strand breaks and enhances cell proliferation *in vitro*, J. Cancer Res. Clin. Oncol. **114**: 579–582.
14. Ott, P., Jenny, B. and Brodbeck, U. (1975) Multiple molecular forms of purified human erythrocyte acetylcholine esterase, Eur. J. Biochem. **57**: 469–480.
15. Gregoriadis, G. (1988) In, "Liposome as Drug Carrier" , pp. 1–863, Wiley, New York.
16. Alen, T. M. and Cleland, L. S. (1980) Serum induced leakage of liposome contents, Biochim. Biophys. Acta. **597**: 418–426.
17. Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S. K., Lee, K.-D., Woodle, M. C., Redemann, C. and Martin, F. J. (1991) Sterically stabilized liposomes: Improvement in pharmacokinetics and antitumor therapeutic efficacy, Proc. Natl. Acad. Sci. (USA) **88**: 11460–11464.
18. Gabizon, A. A. (1992) Selective tumour localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes, Cancer Res. **52**: 891–896.