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160. Eiichi Matsui : Studies on Carcinostatic Substances. XXI.*1 Effect of Mercapto Compounds on Experimental Leucopenia of Rat induced by Nitrogen Mustard N-Oxide.

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A frequently occurring side-effect in the treatment of cancer patients with alkylating agents is leucopenia, which is regarded as a chief cause of discontinuation of the therapy. To avoid such an unfavourable side-effect, numerous experiments have been conducted in the field of clinical investigation and one of these reported that the concomitant administration of L-cysteine with an alkylating agent could prevent the occurrence of leucopenia in patients without diminishing the anti-cancer effect of the alkylating agent.[1,2]

It has been well known that an alkylating agent combines with mercapto compounds such as L-cysteine in a neutral aqueous solution and loses either its toxicity or its effectiveness.[3,4] The above-mentioned clinical observation seemed therefore somewhat in opposition to views based on the chemical reaction.

This paper deals with the determination of chemical reactivity of various mercapto compounds with nitrogen mustards and also with their preventive action against leucopenia, when those mercapto compounds were given to rats with nitrogen mustard N-oxide (HN_2-O).

Experimental and Results

Reaction of HN_2 and HN_2-O with Mercapto Compounds—HN_2 (1 m. mol.) was dissolved in distilled water (70 cc.) and an SH-compound (4 m. mol) was added. After the mixture was adjusted to pH 6.8~7.0 by addition of a proper amount of $NaHCO_3$ solution (2%), it was filled up exactly to 100 cc. with distilled water. In the case of HN_2-O, the procedure was carried out similarly, except that the amount of SH-compound was increased to 6 m. mol. in order to cover oxidative consumption of the SH-group by the N-oxide or by the air.

The test solution was incubated at 25°, an aliquot (2 cc.) was taken out after 1, 3, 6, and 24 hr., and treated with Na-Hg to reduce the -S-S- bond appearing through oxidation. Finally a total quantity of the uncombined SH-group was determined colorimetrically with phosphotungstic acid (Folin and Trimble's reagent)[5] by the method described by Schöberl, *et al.*[6] The results are shown in Table I.

Colorimetrical determination of thiobarbituric acid by means of Folin and Trimble's reagent proved to be rather difficult and an approximate reaction rate of this compound with HN_2 or HN_2-O was determined by a color reaction described by Grote.[7]

Preparation and incubation of the mixture were carried out similarly as in the case of the other SH-compounds. The quantity of thiobarbituric acid was limited to 1 m. mol. equiv. against HN_2 and 1.5 m. mol. equiv. against HN_2-O, because the determination of the solution of higher concentration was very difficult by this color reaction.

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1) K. Shimizu, *et al.* : Surgery, **14**, 1(1952),
2) A. S. Weisberger, *et al.* : Am. J. Med. Sci., **224**, 201(1952).
3) M. Ishidate, *et al.* : Gann, **44**, 386(1953).
4) E. L. Brandt, *et al.* : Cancer, **4**, 1030(1951).
5) O. Folin, *et al.* : J. Biol. Chem., **60**, 478(1924).
6) A. Schöberl, *et al.* : Biochem. Z., **295**, 377(1938).
7) I. Grote : J. Biol. Chem., **93**, 25(1931).

Table I. Uptake of Mercapto Compounds by HN_2 or HN_2-O (in mol. equiv.)

SH-compound	Reagent	1 (hr.)	3 (hrs.)	6 (hrs.)	24 (hrs.)
L-Cysteine hydrochloride	HN_2	0.9	1.9	1.9	2.0
	HN_2-O	0.2	0.4	0.6	1.1
L-Cysteine methyl ester·HCl	HN_2	1.0	1.4	1.6	—[a]
	HN_2-O	0.1	0.2	0.9	—[a]
Thioglycolic acid	HN_2	0.9	1.5	1.6	1.7
	HN_2-O	0.3	0.5	0.7	1.0
2-Mercaptoethylamine·HCl	HN_2	0.5	1.6	1.9	2.0
	HN_2-O	0.3	0.4	0.7	0.8
2-Mercaptoethanesulfonic acid[b]	HN_2	0.8	1.7	2.0	2.1
	HN_2-O	0.1	0.3	0.7	0.9
Sodium thiosulfate	HN_2		1.9 (2 hrs.)		
	HN_2-O		0.8 (2 hrs.)		

The solution was filtered before colorimetrical determination to remove turbidity, if any.

a) A solid precipitate disturbed the determination.

b) After addition of Folin's reagent, 60-min. incubation at 25° is recommended to stabilize color intensity.

After incubation, solid $NaHCO_3$ (200 mg.) and Grote's reagent (0.5 cc.) were added to an aliquot (2 cc.). The mixture was incubated at 25° for 10 min. and diluted exactly to 10 cc. with distilled water. This solution was colorimetrically determined instantly at 675 mμ. The reaction mixture turned turbid during incubation and, in order to avoid possible error owing to turbidity, the absorbancy at 675 mμ of a control solution consisting of the same components but without the Grote's reagent was deducted from that of the test solution. The results are shown in Table II.

Table II. Decrease of Absorbancy of the Test Solution

(hr.)	control	HN_2	HN_2-O	(hr.)	control	HN_2	HN_2-O
1	0.7	0.8	1.0	6	0.7	0.5	0.8
3	0.7	0.7	0.9	24	0.8	0.2	0.5

From these data it can be said that thiobarbituric acid also combined with alkylating agents *in vitro* forming a thiuronium-type compound, although the data in the table showed only approximate values.

2,3-Dimercapto-1-propanol (BAL) reacted promptly with these reagents but the quantitative determination appeared difficult because of polymerization to insoluble substances by alkylation and oxidation due to its bifunctional reactivity.

Induction of Leucopenia in a Rat by HN_2-O—Iwata, *et al.*[8] had reported induction of leucopenia in a rat by a single dose of HN_2-O. The method was modified by the author in order to produce a constantly repeatable leucopenia by the smaller but repeated doses of HN_2-O. The procedure was as follows:

Female Wistar rats of 80~100 g. in body weight were fed under a constant condition for 2 weeks. Rats of abnormal physical condition, if any, were excluded. The blood was taken out 4 times successively every 5th day from their tail vein and the leucocytes were counted by the usual method.

Rats showing a deviation of leucocyte count of more than 4000 through this period were excluded from the valid number of rats. It seemed preferable not to give any food or water to animals

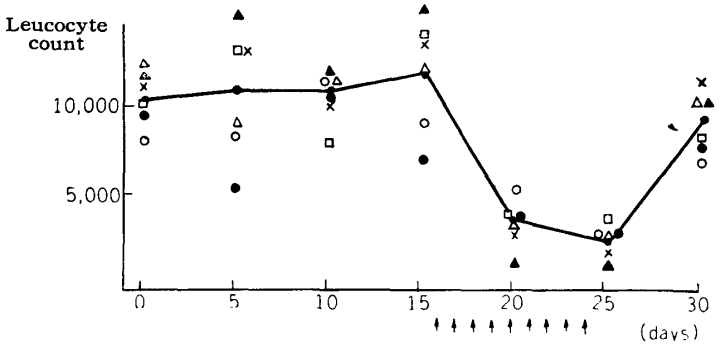

Fig. 1.
Depression of Leucocyte Number by HN_2-O (10 mg./kg. × 9)
•—•—• Average number of six cases
↑ Injection

8) H. Iwata, *et al.*: Nippon Yakurigaku Kaishi, **50**, 169(1954).

for 24 hr. before examination of the blood. These rats were then divided into groups, each of which consisted of six animals. They were given HN_2-O (10 mg./kg.) subcutaneously on one side of the back once a day for 4 successive days. On the 5th day, the rate of decrease in leucocyte number reached a constant 70%, but recovered almost to the initial number 5~7 days after discontinuing the injection. A curve showing the variation of leucocyte number in this experiment is demonstrated in Fig. 1.

Anti-leucopenic Action of Mercapto Compounds—The compounds tested are listed in Table III. Some of them were prepared by the author in accordance with the literatures cited.

Table III.

No.	SH-compound	m.p. (°C)	b.p. (°C/mm.Hg)	Recrystn. solvent	Analysis (%) Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
1	L-Cysteine·HCl[a]	176~177 (decomp.)		H_2O			8.89			8.83
2	D-Cysteine·HCl[b]	178~179 (〃)		〃						
3	D,L-Cysteine·HCl[b]	174~176 (〃)		〃						
4	L-Cysteine methyl ester·HCl[c]	138~140 (〃)		MeOH-Et_2O	27.41	5.75	7.99	27.62	5.60	7.93
5	2-Mercaptoethylamine·HCl[d]	70~72		Dehyd. EtOH	21.15	7.05	12.34	21.17	6.71	12.02
6	Thioglycolic acid[a]		100/10							
7	2-Mercaptopropionic acid[e]		109/10		33.96	5.70		33.96	5.51	
8	2-Mercaptoethanesulfonic acid[f]		[g]		16.89	4.25		16.40	4.43	
9	2,3-Dimercapto-1-propanol (BAL)[h]		104/6							
10	Thiobarbituric acid[i]	>250		H_2O	33.33	2.80	19.44	33.42	3.06	19.17
11	*dl-cis*-2-Aminocyclohexanethiol·HCl[j]	245~247		MeOH						
12	*dl-trans*-2-Aminocyclohexanethiol·HCl[j]	225								
13	2-Aminoethylisothiuronium bromide·HBr (AET)[k]	186~188								
14	$Na_2S_2O_3 \cdot 5H_2O$[l]									

a) Commercial product; purified in the laboratory.
b) Supplied by Yoshitomi Pharm. Co. Ltd.
c) H. J. Prebluda: C. A., **37**, 900(1943).
d) S. Gabriel: Ber., **22**, 1137(1889); **24**, 112(1891).
e) E. Biilmann: Ann., **348**, 125(1906).
f) C. H. Schramm, *et al.*: J. Am. Chem. Soc., **77**, 6231(1955).
g) Obtained from its guanidine salt through ion exchange resin (Amberlite IR-120).
h) Supplied by Daiichi Seiyaku Co. Ltd.
i) A. Michael: J. prakt. Chem., [2] **35**, 456(1887); **49**, 38(1894).
j) Supplied by Prof. T. Taguchi, University of Kyushu.
k) Supplied by Takeda Pharm. Ind., Ltd.
l) Merck product.

Animal experiments were carried out as follows: A neutral aqueous solution of L-cysteine (500 mg./kg., 4.1×10^{-3}mole) was injected subcutaneously on one side of the back of the rat. After 5~10 min., HN_2-O (10 mg./kg.) was administered on the other side of the back. These injections were repeated once a day for 4 days. On the 5th day, the leucocyte number of the blood sample from every rat was counted after Thoma-Zeiss. Usually, 2~6 rats were used for one test compound. Rate of depression was calculated in accordance with the following formula.

$$\text{Rate of depression} = \frac{A - A'}{A} \times 100$$

A: Average initial leucocyte number.
A': Average leucocyte number on the 5th day of experiment.

In these experiments, SH-compounds were compared at the same molar equivalent dosage as to their SH-group content, because their anti-leucopenic action was considered to be due to chemical reaction between mercapto and 2-chloroethylamino group of the two reagents. A single dose in the

above experiment and the maximum tolerance dose on rats of each test compound are shown in Table IV.

Table IV. Single Dose in Treatment, Maximum Tolerance Dose (MTD), and Rate of Depression of Leucocyte Number

SH-Compd. No.	Single dose mg./kg.	Single dose 10^{-3} mole	MTD (i.p.) mg./kg.	Anti-leucopenic action (average depression, %)
1 (a)	500	4.1	>1000	15
1 (b)	150	1.2	>1000	55
2	450	4.1	>1000	55
3	450	4.1	>1000	60
4	490	4.1	1000	55
5	97	1.2	200	75
6	78	1.2	100	60
7	70	0.6[a)]	90	75
8	405	4.1	2500	75
9	50	0.6[b)]	105[c)]	90
10	411	4.1	1000	85
11	200	1.5	220	55
12	250	1.9	300	80
13	250	2.8	300	90
14	710	4.1	>1000	55
HN_2-O alone	10			70

a) Dose over 70 mg./kg. could not be tested because of its toxicity.
b) Dose equivalent to 1.2×10^{-3} mole of monomercapto compound.
c) Rat, intramuscular. Data from C. Stock, *et al.*: Science, **102**, 601(1945).

It is noteworthy that L-cysteine exhibited a distinct anti-leucopenic action at a dosage of 500 mg./kg. and a slight but recognizable effect even at 150 mg./kg. All test compounds were therefore compared as to their effect with L-cysteine at a dosage level equivalent to 500 or 150 mg./kg. of L-cysteine, according to their toxicity on experimental animals. The results are also demonstrated in Table IV.

Concerning the compounds showing only slight effectiveness, the leucocyte count of each case is given in Fig. 2.

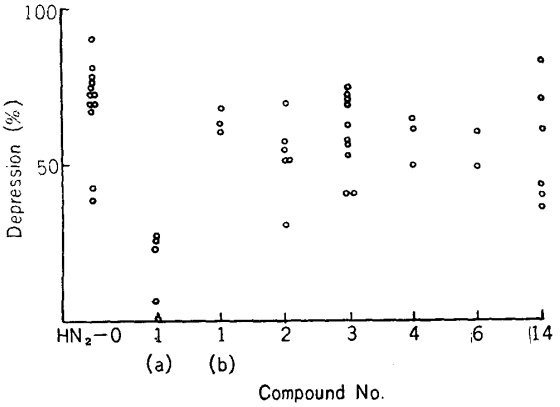

Fig. 2.
Rate of Decrease of Leucocyte Number of Rat induced by HN_2-O

From these results, it was seen that every SH-compound listed above could be S-alkylated *in vitro* in a neutral aqueous solution either by HN_2 or HN_2-O, although some part of SH-group was simultaneously consumed by oxidation prior to alkylation in case of the N-oxide. In spite of the fact that there were several SH-compounds which showed similar reaction velocity with HN_2 or HN_2-O, the real prevention of the experimental rat leucopenia could only be observed with L-cysteine. Even D-cysteine exhibited an activity far less than that of its optical antipode.

Discussion

The protective action of L-cysteine against leucopenia induced by HN_2 seems to be due to a direct chemical reaction of the two agents, which results in a complete inactiva-

tion of the latter. At the same time, it was proved that the various SH-compounds, e.g. 2-mercaptoethylamine, which reacts with HN_2 *in vitro* as easily as with L-cysteine, could not always be expected to be equally anti-leucopenic *in vivo*.

Maisin, *et al.*[9] insisted that 2-mercaptoethylamine reduces the mortality rate of mice irradiated by X-rays, but it did not prevent leucopenia by alkylating agents. This seems to show some difference between the damages induced by HN_2 and X-ray irradiation.

This suggests the neccessity of a definite molecular structure of SH-compounds in order for it to manifest protective activity *in vivo* against leucopenia.

It might be a matter of particular interest that D- and D,L-cysteine, and the methyl ester of L-cysteine were found to be only slightly or hardly effective in these experiments.

Weisberger, *et al.* reported[10] in 1952 about the inhibiting action of mercapto and its related compounds, including L-cysteine, 2-mercaptoethylamine, thioglycolic acid, etc., against leucopenia of rabbit induced by HN_2 and concluded that L-cysteine alone was effective. They, however, injected the test compounds only once just prior to the injection of HN_2 at a dosage level near the maximum tolerance dosage and no attention was paid as to the molecular equivalency of the SH-concentration. They also reported[11] about a comparison of the effect of L- and D-cysteine against leucopenia induced by HN_2.

This seems to suggest other factors which are indispensable in displaying anti-leucopenic effect. One such factor might be the affinity of the compound to the hematopoietic tissue depending on its chemical structure.

Accordingly, there may be some hope of finding a new SH-compound which can depress leucopenic action of HN_2-O without lessening its anti-tumor activity by further investigation of SH-compounds having various chemical structures.

If an SH-compound should be able to increase the practical chemotherapeutic index of HN_2-O, viz. a difference between the effective dosage on tumor and the toxic dosage on the bone marrow, a time between the injections of two drugs would have a definite meaning. The following experiments were carried out with L-cysteine and HN_2-O, a preliminary report of which has been published by the author in 1956.[12]

From the results, it seemed most favorable to give L-cysteine 30 min. after HN_2-O injection, as shown in Table V, in order to obtain the highest chemotherapeutic index with this combination of the drugs.

Table V. Effect of preventing Leucopenia and Antimitotic Effect against the Yoshida Sarcoma by Concomitant Treatment with HN_2-O and L-Cysteine

Time after HN_2-O inj. L-Cysteine inj. (min.)[a]	15	30	45	60
Decrease in leucocyte count[b]	+	+	−	−
Anti-tumor effect[c]	±	+	+	+

a) HN_2-O (10 mg./kg.) and L-cysteine (500 mg./kg.) for a single dose. Both were injected subcutaneously on different sides of back of each rat.
b) + Decrease rate of leucocyte count less than 30% of the initial.
c) + Anti-mitotic effect corresponding to 5~10 mg./kg. of HN_2-O.

Applying this administration condition, 24 Yoshida sarcoma rats were treated and both prolongation of their lives and decrease in leucocyte number were observed. Results are demonstrated in Fig. 3.

9) J. H. Maisin, *et al.*: Nature, **171**, 971(1953).
10) A. S. Weisberger, *et al.*: J. Clin. Invest., **31**, 217(1952).
11) A. S. Weisberger, *et al.*: J. Lab. Clin. Med., **43**, 246(1954).
12) Y. Sakurai, *et al.*: Gann, **47**, 337(1956).

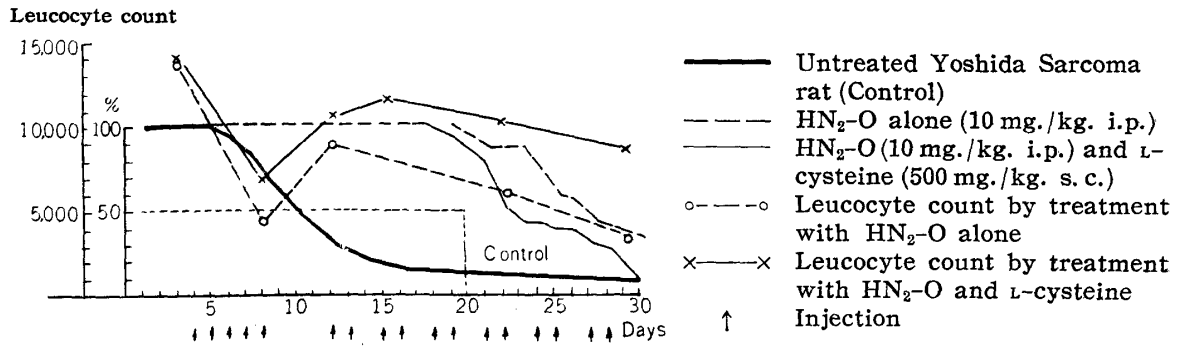

Fig. 3. Yoshida Sarcoma Rats by Dual Treatment with HN_2-O and L-Cysteine with Injection Interval of 30 min.

The injections began on the 4th day after implantation of the tumor and each leucocyte number, dotted on the diagram, showed an average value of all treated rats.

It was shown that administration of L-cysteine 30 min. after HN_2-O injection could decrease the leucopenic tendency of the rats to some extent, without accompanying a remarkable loss in its anti-tumor effectiveness.

Although the results were not completely satisfactory, it can be said, that the results give hope to the search of a more potent and practical agent among the SH-compounds which protects the bone marrow from the damage caused by HN_2-O.

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Summary

Mercapto compounds were tested as to their chemical reactivity with nitrogen mustard (HN_2) and its N-oxide (HN_2-O) *in vitro*, and as to their preventive action *in vivo* against rat leucopenia induced by the same agents. Of these compounds, L-cysteine alone was found to show remarkable activity *in vivo*, while D-cysteine and L-cysteine methyl ester were proved to be only slightly effective.

Administration of L-cysteine 30 min. after HN_2-O injection prevented to some extent the leucopenia of Yoshida sarcoma rats without being deprived of its anti-tumor effect.

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