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Summary

Synthesis of 1-acetonyl-6,7-dimethoxy-3,4-dihydroisoquinoline (IV) was described. On treatment with boiling phosphoryl chloride this underwent a smooth prototropic change to form isoxazole derivative (II) in almost theoretical yield, which had been described previously. Some chemical evidence in support of the structure of (II) was also provided.

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77. Morizo Ishidate, Yoshio Sakurai, Hiroshi Imamura, and Ayako Moriwaki : Studies on Carcinostatic Substances. XXVII.*1 Anti-tumor Activity of 2-Chloroethylamine Derivatives on the *in vitro*-Cultured Yoshida Sarcoma Cells.

(*Iatrochemical Institute of Pharmacological Research Foundation**2)

This paper deals with the result of anti-tumor screening of 2-chloroethylamine derivatives on the Yoshida sarcoma cells cultured *in vitro* by the procedure described in the preceding paper.[1] Attention was called to the fact that the effect was determined chiefly by the cytomorphological observation according to a similar standard as that adopted in animal experiments using rats bearing Yoshida sarcoma. For this reason, it would be a matter of particular interest to compare the results of the two experiments, *in vitro* and *in vivo*.

From these comparisons, two remarkable findings were observed. The one is a relationship between chemical and biological reactivities of the compounds and the other, the condition for biological reduction of the N-oxide derivatives of 2-chloroethylamine.

Experimental and Results

More than 200 derivatives of 2-chloroethylamine were tested for anti-tumor activity, some of which are listed in Table I with the results indicated by minimum effective concentration (MEC).

As seen in Table I, the test of the N-oxides ended in negative results except those having more than three 2-chloroethyl groups. In order to obtain an answer to the question of why the N-oxides themselves did not exhibit any activity on the cells *in vitro*, contrary to those *in vivo*, the following experiments with the Yoshida sarcoma were carried out.

Expt. 1 : Various doses of N-methyl-bis(2-chloroethyl)amine N-oxide (HN_2-O) were given intraperitoneally to each of the rats bearing 4-day-old Yoshida sarcoma. After every 30 and 120 min., the

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*1 Part XXVI : This Bulletin, 8, 99(1960).

*2 26 Nishigahara 1-chome, Kita-ku, Tokyo (石舘守三, 桜井欽夫, 今村 博, 森脇絢子).

1) M. Ishidate, *et al.* : This Bulletin, 7, 873(1959).

Table I.

Compd. No.	Compound	*in vivo* MED[a] (mg./kg.)	*in vitro* MEC[b] (m.*M*)	*in vitro* MEC[b] (mg./L.)	Ratio (MED/MEC)
320	$H\text{-}N(CH_2CH_2Cl)_2$	50	1×10^{-2}	1.8	28
24	$CH_3\text{-}N(CH_2CH_2Cl)_2$	0.05	2.5×10^{-4}	0.05	1
188	$C_2H_5\text{-}N(CH_2CH_2Cl)_2$	0.05	2.5×10^{-3}	0.5	0.1
232	$HOOC\text{-}CH_2\text{-}N(CH_2CH_2Cl)_2$	0.5	1×10^{-1}	23.5	0.02
246	$H_5C_2OOC\text{-}CH_2\text{-}N(CH_2CH_2Cl)_2$	0.1	1×10^{-2}	2.65	0.04
237	$H_2NOC\text{-}CH_2\text{-}N(CH_2CH_2Cl)_2$	0.1	2.5×10^{-3}	0.59	0.18
243	$HOOC\text{-}CH(CH_3)\text{-}N(CH_2CH_2Cl)_2$	0.05	1×10^{-2}	2.5	0.02
177	$(CH_3)_2CH\text{-}(CH_2)_2\text{-}N(CH_2CH_2Cl)_2$	0.5	5×10^{-3}	1.25	0.4
498	$CH_3\text{-}N(CH_2CHClCH_2Cl)_2$	50	5×10^{-3}	1.45	34
460	$ClCH_2CH_2NH\text{-}(CH_2)_6\text{-}NHCH_2CH_2Cl$	50	5×10^{-2}	12.5	4
220	$(C_2H_5)_2N\text{-}(CH_3)_2\text{-}N(CH_2CH_2Cl)_2$	1	2.5×10^{-2}	7.88	0.13
310	$ClCH_2CH_2(CH_3)N\text{-}(CH_2)_2\text{-}N(CH_3)CH_2CH_2Cl$	0.05	1×10^{-2}	2.86	0.01
524	$(CH_2)_3\text{-}\overset{+}{N}(CH_2CH_2Cl)_2\cdot I^-$ (O)	0.1	1×10^{-2}	3.3	0.3
539	$HOOC\text{-}CH(NH_2)(CH_2)_4\text{-}N(CH_2CH_2Cl)_2$	0.5	1×10^{-3}	0.6	0.83
27	$CH_3\text{-}N(CH_2CH_2Cl)_2$ (O)	1	—[c]		
206	cycl. $C_6H_{11}\text{-}N(CH_2CH_2Cl)_2$ (O)	5	—		
464	$CH_3O(CH_2)_2\text{-}N(CH_2CH_2Cl)_2$ (O)	1	—		
29	$N(CH_2CH_2Cl)_3$	0.01	5×10^{-4}	0.12	0.08
103	$N(CH_2CH_2Cl)_3$ (O)	0.1	2.5×10^{-3}	0.68	0.15
196	$(ClCH_2CH_2)_2N\text{-}(CH_2)_2\text{-}N(CH_2CH_2Cl)_2$	0.1	5×10^{-3}	1.38	0.07
197	$(ClCH_2CH_2)_2N\text{-}(CH_2)_2\text{-}N(CH_2CH_2Cl)_2$ (O, O)	0.5	1×10^{-2}	4.15	0.08

a) Minimum effective dose on the Yoshida sarcoma rat (Gann, **44**, 342(1953)).
b) Minimum effective concentration on the *in vitro*-cultured Yoshida sarcoma (This Bulletin, **7**, 873(1959)).
c) No effect.

Table II.

Rat No.		Dose (mg./kg.)	Contact time (min.)	Growth	Cytological effect
1	T-967	8	30	*in vitro*	+
2	T-968	4	30	〃	+
3	T-969	2	30	〃	+
4	T-967	8	120	〃	+
5	T-968	4	120	〃	+
6	T-969	2	120	〃	+
7	T-967	8		*in vivo*	+
8	T-968	4		〃	+
9	T-969	2		〃	+

tumor ascites was drawn from each rat and the tumor cells were subjected to tissue culture by the routine procedure.[2] Details of experiment are shown in Table II.

Expt. 2 : The tumor ascites was placed in several test tubes so as to make the cell population of about 10^8 cells/cc. in each tube. The serially diluted solution of HN_2-O was added to each of the test tubes and incubated for 30 min. at 37°. All samples were then centrifuged, the precipitate was

2) M. Ishidate, *et al.* : This Bulletin, **7**, 690(1959).

washed with physiological saline solution, and about 1.5×10^5 cells from each precipitate were cultured by the usual procedure. The remainder of the cells was injected into the peritoneal cavity of normal rats. The morphological observation was carried out after the routine process. The results are demonstrated in Table III.

Table III.

Contact condition (37°)					
Yoshida sarcoma (cells/cc.)	Medium	Concn. of HN_2-O (m.*M*)	Time (min.)	Growth	MEC (m.*M*)
1×10^8	Rat ascites + Physiol. saline solution (1:1)	1×10^{-1} ~ 1×10^{-4}	30	*in vitro*	1×10^{-3}
				in vivo	1×10^{-3}

Expt. 3 : The same experimental procedure as Expt. 2. was used but the cell population was decreased to 5×10^5 cells/cc. Under this contact condition, the effect of HN_2-O could not be observed even at a concentration of 1×10^{-1} m.*M*.

Expt. 4 : A mixture of 0.5 cc. each of the tumor cell suspensions in Medium-I,*3 which were respectively at a population of 2×10^8, 2×10^7, 1×10^7, and 2×10^6 cells/cc., and 0.5 cc. of HN_2-O solution in physiological saline solution of a specified concentration was incubated at 37° for 10 or 30 min. After centrifugation, about 5×10^4 cells from each cell precipitate was cultured by the usual procedure. The results are demonstrated in Table IV.

Table IV.

Contact time (min.)	Yoshida sarcoma (cells/cc.)	MEC (m.*M*)	Contact time (min.)	Yoshida sarcoma (cells/cc.)	MEC (m.*M*)
10	1×10^6	5×10^{-2}	30	1×10^6	2.5×10^{-2}
10	5×10^6	2.5×10^{-2}	30	5×10^6	2.5×10^{-3}
10	1×10^7	2.5×10^{-2}	30	1×10^7	1×10^{-3}
10	1×10^8	1×10^{-2}	30	1×10^8	5×10^{-4}

Expt. 5 : A mixture of 0.5 cc. each of the tumor cell suspension in Medium-I (5×10^6 cells/cc.) and 0.5 cc. of HN_2-O solution of a specified concentration, was divided into 5 groups, each of which was incubated at 37° respectively for 15, 30, 60, 120, and 240 min. After incubation, a portion of each precipitate was transferred into the usual medium*4 and incubated. The results are given in Table V.

Table V.

Contact time (min.)	15	30	60	120	240
MEC (m.*M*)	2.5×10^{-2}	2.5×10^{-3}	5×10^{-4}	2.5×10^{-4}	2.5×10^{-4}

Expt. 6 : In case of the cell population of 2.5×10^6 cells/cc., pH of the contact solution (37°, 30 min.) was varied from 6.4 to 7.8 by using a phosphate buffer. The MEC in the contact solutions at the respective pH values are shown in Table VI.

Table VI.

pH	6.4	6.7	7.2	7.4	7.8
MEC (m.*M*)	5×10^{-3}	1×10^{-2}	1×10^{-2}	1×10^{-2}	2.5×10^{-2}

Expt. 7 : One cc. of the diluted cell suspension in Medium-I (1.5×10^5 cells/cc.) was mixed with 1 cc. of HN_2-O solution of a specified concentration and incubated at 37° for 30 min. After centrifugation, precipitate was transferred to the usual medium and incubated. The results are shown in Table VII.

In such a short contact experiment, cytological effect was observed at concentrations higher than 2.5×10^{-1} m.*M*, at which the cells treated could not be saved from complete destruction by cytolysis, if they were kept in contact with this agent for 24 hr. or more in the usual screening procedure.

*3 Horse serum 96%, physiological saline solution 4%, containing potassium penicillin G 200 units/cc., dihydrostreptomycin sulfate 200 γ/cc.

*4 A mixture (1:1) of Medium-I and Earle's solution.

Table VII.

Concn. of HN_2-O (m.M)	Concn. of HN_2-O (γ/cc.)	Cytological effect	Concn. of HN_2-O (m.M)	Concn. of HN_2-O (γ/cc.)	Cytological effect
1	210	+	5×10^{-2}	10.5	−
5×10^{-1}	105	+	2.5×10^{-2}	5.25	−
2.5×10^{-1}	52.5	+	1×10^{-2}	2.1	−
1×10^{-1}	21	−			

Discussion

All the compounds listed in Table I, which were found effective on the tumor cells *in vitro*, were divided into 3 groups. As seen in Table I, the compounds which had ratios $\left(\frac{\text{MED}}{\text{MEC}}\right)$ much greater than 1, proved to be chemically inert by determining their thiosulfate consumption. It was believed that such a compound needed longer contact with the tumor cells than the rapid reacting compound in order to show the biological effect. This might be the reason for the efficacy of such compound appearing far less in animal experiment than in *in vitro* screening because the compound would be promptly catabolized *in vivo* or excreted from the animal body before its action is completed. In the *in vitro* test, it was supposed that a concentration of the less reactive agent remained unchanged from the initial concentration for a longer period.

On the other hand, the compounds having ratios $\left(\frac{\text{MED}}{\text{MEC}}\right)$ much smaller than 1 showed very high velocity of chemical reactions and therefore could not be sufficiently active *in vitro*, because their concentration would be promptly diminished in the medium by hydrolysis. However, in animal experiment, the concentration could temporarily reach a high level at the site of injection before it was equalized through the whole ascites, because the compounds were usually given intraperitoneally to the rats bearing ascites tumor.

For this reason, in case of such reactive agents as N,N-bis(2-chloroethyl)-DL-alanine, the initial high concentration at the moment of injection was enough to cause damage to the tumor cells just existing at the site of injection, even though the high concentration lasted only for a short period.

Ratio $\left(\frac{\text{MED}}{\text{MEC}}\right)$ of N-methyl-bis(2-chloroethyl)amine was 1 and most of the promising candidate compounds as practical antitumor chemotherapeutics were to be found in the compounds having ratio $\left(\frac{\text{MED}}{\text{MEC}}\right)$ of nearly 1, such analysis of the results of experiments both *in vivo* and *in vitro* seemed very useful to guide the synthetic research of alkylating agents.

It had already been reported that the N-oxides transformed readily into the corresponding 1,2-oxazetidinium derivatives in a neutral medium, the latter of which were proved to be reduced more easily than the former. By this reduction succeeding the transformation, the bifunctional N-oxides yielded the monofunctional tertiary 2-chloroethylamines which were biologically inactive. Therefore, the bifunctional N-oxide cannot be effective unless it is reduced before transformation.

On the contrary, the N-oxides having three or four 2-chloroethyl groups like Nos. 130 and 197 remained bifunctional even after reduction of the transformed intermediates and appeared always active in the routine procedure as shown in Table I. The experiments (Expt. 1~7) were carried out to determine the conditions for the bifunctional N-oxides to be biologically reduced before transformation and display their anti-tumor activity against tumor cells *in vitro*.

To this end, the experimental process was divided into two parts: One, by way of contact of the cells with the agent and the other, the culture of these affected cells by the usual means at small cell population as 5×10^4 cells/cc. Throughout the experiments, HN_2–O was used as typical of the N-oxides. Since HN_2–O appeared effective when it was brought in contact with the tumor cells for 30 or 120 min., either in the peritoneal cavity of rat (Expt. 1) or in a test tube with the ascites containing large cell population as 10^8 cells/cc. (Expt. 2), the most important factor for activating HN_2–O seemed to be the cell population. As seen in Expt. 3, HN_2–O did not appear effective at a small cell population even if a similar contact medium as in case of Expt. 2 was used.

Expt. 4 demonstrated a definite relationship between MEC and cell population and, at the population of 10^8 cells/cc., MEC of HN_2–O reached almost the same level as that of HN_2, which seemed to indicate that nearly the whole amount of the N-oxide in the contact solution was reduced, i.e. activated, by the cells during 30 min. of incubation at 37°.

In the routine screening procedure, contact of the cells with the reagent and succeeding growth of the affected cells were carried out at one stretch and therefore it was unavoidable to limit the cell population to below 5×10^4 cells/cc., because the cell growth was markedly retarded or ceased at a higher population even when they were not affected by any anti-tumor agents. This is the reason for ineffectiveness of the N-oxide in the routine screening procedure. However, the fact that HN_2–O could exhibit its effect on the tumor cells of small population *in vitro* at very high concentrations, as shown in Expt. 7, indicated that the activation also proceeds under such conditions though its velocity might be extremely low.

In conclusion, the activation of the N-oxide in this experiment depended chiefly on the cell population and the length of contact time, and slightly on pH of the contact medium, as seen in Expt. 6. Further detailed experiments on activation of N-oxides are now being conducted.

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Summary

More than 200 derivatives of 2-chloroethylamine were tested as to their anti-tumor effect on the *in vitro*-cultured Yoshida sarcoma cells. The results were discussed, especially in connection with conditions of activation of N-methyl-bis(2-chloroethyl)amine N-oxide by the tumor cells.

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