SOME CHEMICAL AND BIOLOGICAL CHARACTERISTICS OF SHOWDOMYCIN

Yoiti Titani and Yuji Tsuruta

Shionogi Research Laboratory, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka, 553 Japan

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The chemical properties of showdomycin, a maleimide type antibiotic, were very similar to, but its biological properties rather different from, those of N-ethylmaleimide. Another maleimide derivative, citraconimide, showed very low reactivity both in chemical and biological reactions compared with the above two compounds. The results of quantitative analyses of cellular sulfhydryl content of *Escherichia coli* did not indicate a perfect correspondence between the elimination of cellular sulfhydryl and the inhibition of cell growth by these maleimide type compounds.

In spite of many similarities between showdomycin (SHM) and N-ethylmaleimide (NEM) with respect to radiosensitization,[1] radiomimetics sensitization (synergism with alkylating agents)[2] and mode of reaction with thiols,[3] the chemical structure and U. V. absorption spectrum of SHM are more nearly like those of citraconimide (CI),[4] which has a high antimitotic activity *in vitro*,[5] than those of NEM. Chemical modification of SHM at the sugar moiety affects its antimicrobial activity but not its radiosensitizing effect on *Escherichia coli* B/r under anoxic conditions.[6] This report describes a study in which the chemical and biological characteristics of SHM are compared with those of NEM and CI.
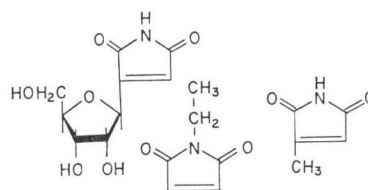
Reaction Rates

1. Reaction with sulfhydryl compounds at pH 6.8

The second order rate constants for the reactions of SHM, NEM and CI with some sulfhydryl compounds at pH 6.8 (M/15 phosphate buffer) were measured by the method of Karlin and Winnik;[7] the results are summarized in Table 1.

As shown in Table 1, SHM and NEM have almost the same rate constants in their reaction with cysteine and MEA, but the rate constant for CI is less than 1/100 of these values.

2. Reaction with cysteine at pH 6.3 and pH 8.0

We tried to measure the rate constant of

Showdomycin N-Ethylmaleimide Citraconimide

Table 1. Rate constants k ($M^{-1} sec^{-1}$) for the reactions of SHM, NEM and CI with cysteine and β-mercaptoethylamine (MEA) at pH 6.8

Compound	Rate constant k($M^{-1}sec^{-1}$)*		Initial concentration
	with cysteine	with MEA	
SHM	990 ± 60	1130 ± 40	5×10^{-5}M
NEM	1230 ± 60	1370 ± 40	2.5×10^{-5}M
CI	4.5 ± 1.7	—	1×10^{-3}M

* Mean value ± SEM.

the reaction of SHM and NEM with cysteine in slightly acidic and slightly alkaline buffer solutions. This was not possible at pH 8.0 since both compounds decomposed rapidly without reaction with cysteine and their U.V. absorption at 300 nm disappeared.

Table 2 shows that the reaction rates at pH 6.3 are markedly slower than those meas-

Table 2. Rate constants k ($M^{-1}sec^{-1}$) for the reaction of SHM and NEM with cysteine at pH 6.3

Compound	Rate constant* k ($M^{-1}sec^{-1}$)	Initial concentration
SHM	160 ± 20	$5\times10^{-5}M$
NEM	130 ± 20	$2.5\times10^{-5}M$

* Mean value $\pm$ SEM.

Fig. 1. Thermal decomposition reaction at 37°C of SHM, NEM and CI in $M/15$ phosphate buffer at pH 6.8 (a) and 6.0 (b). Amount of compound remaining was traced by absorbance at 300 nm
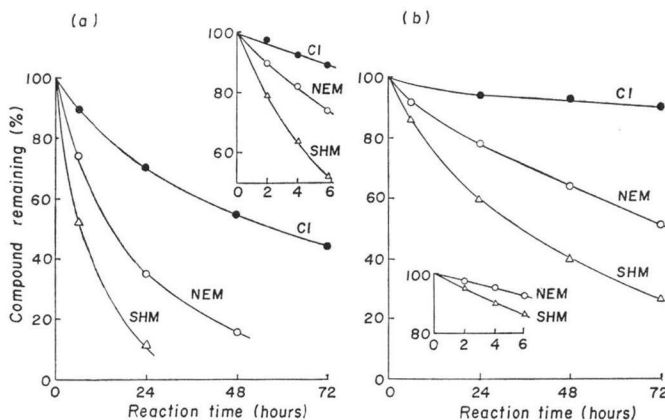
ured by the same method at pH 6.8, though there is still no significant difference between the constants for SHM and NEM.

Thermal Decomposition Reaction

The decomposition rate of these three compounds at 37°C in $M/15$ phosphate buffer at different pH values was traced by measuring absorbance at 300 nm; the results are illustrated in Fig. 1. The initial concentration of each compound was adjusted to 1 mM. Whereas the rate of decomposition of all three compounds was considerably retarded in acidic (pH 6.0) solution (Fig. 1b), the rate was too fast to measure at pH 8.0.

Fig. 2. SHM, NEM and CI remaining at different pH ranges after 24 hours at 37°C, measured by absorbance at 300nm. (Initial concentration 1 mM, pH 1: 0.1 N HCl, pH 2.2~8: Na_2HPO_4-citric acid buffer)

Fig. 2 shows the amount of compound remaining undecomposed after 24 hours in solutions of different pH values. No significant decomposition was observed at low pH values. The results with SHM are not incompatible with the pH dependence of its antimicrobial activity.[15] Using the curves in Fig. 1 for decomposition over the first 6 or 24 hours, the rate constants of the decomposition, calculated as a second order reaction, are as given in Table 3.
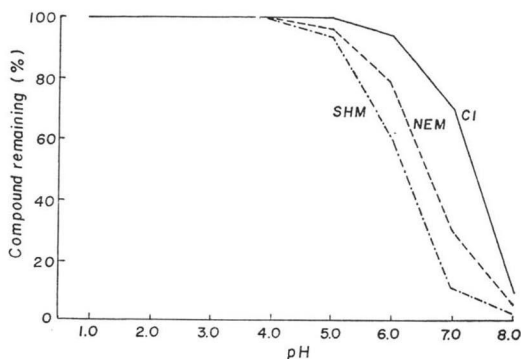
Table 3. Rate constants k ($M^{-1}sec^{-1}$) for thermal decomposition reaction of SHM, NEM and CI at 37°C

Compound (1 mM)	k ($M^{-1}sec^{-1}$)	
	at pH 6.8	at pH 6.0
SHM	42.7×10^{-4}	7.5×10^{-4}
NEM	16.3×10^{-4}	4.0×10^{-4}
CI	5.7×10^{-4}	$(0.7 \times 10^{-4})*$

* A plot at 24 hours was used for calculation.

Table 4. Radiosensitizing effects of SHM, NEM, and CI on *E. coli* B/r under anoxic conditions. DMF was obtained by the method described previously.[6]

Compound	Concentration	DMF (Anoxic)
SHM	1 mM	2.5 ± 0.2
NEM	1 mM	2.5 ± 0.2
CI	1 mM	1.0 ± 0.2
	10 mM	2.3 ± 0.2

The values show that, unlike the reaction with sulfhydryl compounds, SHM decomposed much faster than the other compounds at both acidic and at alkaline pHs. However, for each of the three compounds the absolute value of the decomposition rate constant was very much smaller than that for the reaction with sulfhydryl compounds.

Radiosensitization of *E. coli* B/r under Anoxic Conditions

The radiosensitizing effect of SHM, NEM and CI was measured under anoxic conditions by the method described previously.[6]

As shown in Table 4, the radiosensitizing effect of CI was unexpectedly lower than the others. While SHM and NEM showed significant effects at 1 mM, a concentration of 10 mM of CI was necessary and no effect was shown at 1 mM.

## Induction of SHM-resistant Mutant

When *E. coli* B/r was grown in glucose-salts medium containing increasing concentrations of SHM, it became resistant to this antibiotic. Whereas B/r-S4, resistant to 4 μg/ml of SHM, was initially isolated, a highly resistant mutant B/r-S 200 was finally isolated from a culture containing 200 μg/ml of SHM. B/r-S 200 grows normally in the presence of 200 μg/ml of SHM but is slightly inhibited by 500 μg/ml in glucose-salts medium at 37°C. On the contrary, in spite of repeated trials, no NEM resistant cells could be isolated by this method.

Growth Inhibition

As reported previously, 1.5 μg/ml of SHM completely inhibits growth of *E. coli* cells at 24 hours,[6] though 300 μg/ml is necessary to kill all of the cells. However, cells grown in a culture medium to which 100 μg/ml of SHM was added initially did not acquire any resistance to SHM. On the other hand, 4 μg/ml of NEM completely kills the cells. These curious observations led us to examine in detail growth inhibition by these compounds by a novel method to clarify the difference between SHM and NEM.

E. coli B/r cells harvested from overnight culture in 0.8 % nutrient broth (Difco) were washed twice with M/15 phosphate buffer, resuspended in the same phosphate buffer in the presence of test compound, and incubated for 30 minutes at 37°C. The cells were then collected by centrifugation, resuspended in 0.8 % nutrient broth, and incubated at 37°C with continuous shaking. The growth rate, measured by change of absorbance at 650 nm, was

Fig. 3. Change of absorbance at 650 nm of *E. coli* B/r cells incubated in 0.8% nutrient broth at 37°C with continuous shaking after pretreatment with SHM (10^{-3}M, 10^{-4}M, 10^{-5}M, none) or NEM (10^{-3}M, 10^{-4}M, 10^{-5}M).
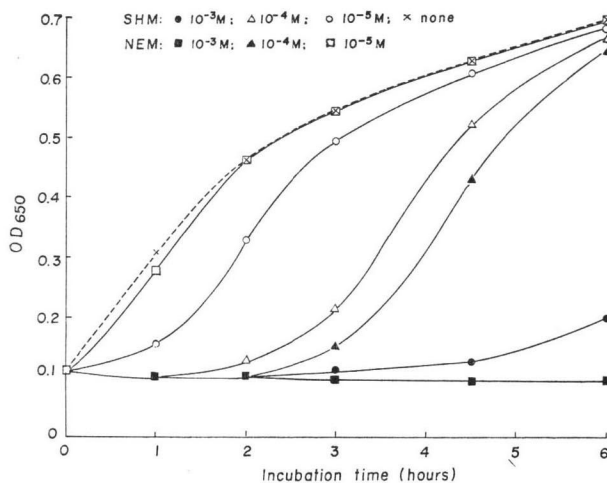
found to be dependent upon the pre-treatment with test compounds.

As shown in Fig. 3, cells not treated with the reagents started to grow as soon as incubation was begun, but there was a delay in the increase of absorbance when the cells were pre-treated with the reagents. However, the growth rates for these pre-treated cells seem to be almost the same as that of non-treated cells. At the same time as the absorbance was measured, cell samples were taken and viable cells counted after 24-hour incubation on nutrient agar plates. At 0 time there was no significant difference in the viable cell count (more than 80% was viable) according to pre-treatment with SHM or NEM, except that 10^{-3}M NEM treatment decreased the viable cell count about 1/10 of the control value. And in all cases the viable cell count remained unchanged during the lag period where no increase of absorbance was observed.

Reaction with Cellular Sulfhydryl Groups

1. Method for measuring total cellular sulfhydryl content

The colorimetric method for determining low concentration of tissue sulfhydryl content described by ELLMAN[8] was modified for bacterial cells as follows:

(1) Early stationary phase cells were collected by centrifugation.

(2) Cells were resuspended in phosphate buffer to titre of about 3×10^{10} cells/ml and incubated for 1 hour at 37°C to stabilize the cellular sulfhydryl content.

(3) Samples of 10 ml containing 3×10^9 cells/ml were prepared from the stabilized cell suspension and incubated for 30 minutes with various concentrations of reagents in phosphate buffer at 37°C.

Table 5. Cellular sulfhydryl content of *E. coli* B/r and B/r-S200

Strain	Cellular sulfhydryl content (mole/cell)	
	Total	Acid soluble fraction
B/r	$8.95 \pm 0.75 \times 10^{-18}$*	$1.20 \pm 0.20 \times 10^{-18}$*
B/r-S200	$8.83 \pm 0.52 \times 10^{-18}$**	$1.98 \pm 0.15 \times 10^{-18}$*

* Mean of 9 experimental values+S.D.
** Mean of 5 experimental values+S.D.

(4) Cells were collected on a membrane filter and resuspend in 5 ml of 0.1 M tris-HCl buffer (pH 8.1).

(5) Then 100 μl of lysozyme solution (1 mg/ml) and 100 μl of EDTA solution (10^{-2}M) were added to this suspension.

(6) The suspension was treated with two cycles of freeze and thaw with dry-ice acetone mixture, and finally incubated at 37°C for about 10 minutes.

(7) After the incubation, 2 ml of the tris-HCl buffer and 1 ml of 1 % sodium laurylsulfate solution were added to the suspension and mixed in gently with a stainless rod.

(8) The clear lysate obtained (8.2 ml; absorbance at 650 nm was 0.01 ± 0.003) was

Fig. 4. Change of cellular sulfhydryl content in *E. coli* B/r cells after incubation with 1 mM of SHM, NEM or SHM-triacetate at 37°C for 30 minutes.

divided into two fractions, and 50 μl of DTNB (4 mg/ml) was added to one fraction, which was then allowed to stand for 1 hour. The sulfhydryl content of this fraction was then determined by absorbance at 412 mμ, using the other fraction as a control.

2. Method for measuring cellular sulfhydryl content in acid soluble fraction

(1) One ml of stabilized cell suspension (1.5×10^{11} cells/ml) and 10 % TCA solution were mixed and allowed to stand for 10 minutes.

(2) The mixture was centrifuged and the supernatant was neutralized with solid sodium bicarbonate and its sulfhydryl content determined by the method described above.

The cellular sulfhydryl contents measured by our method for *E. coli* B/r and B/r-S 200 strains are shown in Table 5.

Use of pronase to liquify the lysate is not recommended because the sulfhydryl content of samples treated with pronase was sometimes $10\sim50$ % lower than that of untreated samples.

3. Reaction of SHM, NEM, and SHM-triacetate with cellular sulfhydryl groups

Suspension of *E. coli* B/r cells containing 3×10^8 cells/ml were incubated with 1 mM of SHM, NEM or SHM-triacetate for 30 minutes at 37°C. After incubation, cells were collected and the cellular sulfhydryl content measured by the method described above.

As shown in Fig. 4, the rate of reaction of SHM with cellular sulfhydryl groups is somewhat slower than that of NEM, though the reaction reaches a stationary state within 30 minutes with either compound. The percentage of sulfhydryl groups retained after 90-minute incubation was 45 % in the SHM reaction and only 30 % in the NEM reaction. It should be mentioned that SHM-triacetate, which does not inhibit growth of *E. coli* at this concentration,[6] reacted similarly as NEM on the cellular sulfhydryl groups.
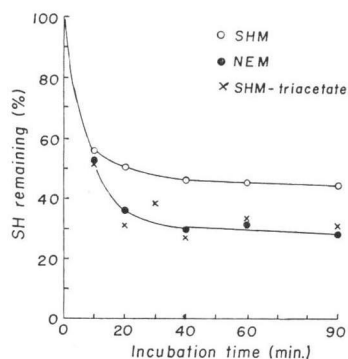
Discussion

Although the U. V. spectrum of SHM is similar to that of CI, the mode of its reaction with sulfhydryl compounds and of its thermal decomposition reaction are analogous to those of NEM rather than those of CI. This fact clearly indicates that a side chain at the C-C double bond of maleimide influences the reactivity of the double bond, a ribose moiety having a small and a methyl moiety a marked retarding effect on the reactivity. Stabilization of the C-C double bond both in SHM and NEM could be established by acidifying the solution.

This fact suggest that in radiosensitization SHM may have an electron affinic property as proposed for NEM.[12]

Studies on the specificity of the double bond side chain would give information regarding chemical modification of SHM, but it should be mentioned here that CI was more effective than NEM in antimitotic activity on tissue culture of chick fibroblast.[5] It is not known, however, whether such difference of cytotoxic activity on bacteria and on cultured cells depends on the existence of a cell wall in bacteria or on some biological activating systems of the methyl group in cultured cells.

Under anoxic conditions SHM and NEM had quite similar radiosensitizing effects on *E. coli* B/r cells, while a 10 times higher concentration was needed for CI to exhibit similar activity. There are two possible explanations for this; (1) a low rate of incorporation into the cell and (2) a low rate of reaction with cellular sulfhydryl groups, though our present results suggest that it is the low reactivity of the C-C double bond of CI, hence its low reaction rate with cellular sulfhydryl groups.

Induction of an SHM-resistant mutant is thought to depend on the mechanism of incorporation of the antibiotic into the bacterial cell.[10] SHM-resistant *E. coli* B/r was as sensitive as the parent strain to NEM,[9] as reported for K-12 strain of *E. coli*.[11]

Growth inhibition by SHM and NEM seems to be temporary and cells start to grow after a certain delay period as shown in Fig. 3. Considering to the results of viable cell count it is likely that this delay in increase in absorbance is due to a delay in the initiation of growth and not to a retardation of growth rate. The effect is very obvious for SHM but, because of high toxicity, not so clear for NEM. It may be that this phenomenon is a characteristic peculiar to the maleimide structure of the compounds. The temporary inhibition of cell growth by NEM has been reported previously by BRIDGES.[13] He observed that the appearance of viable colonies of *E. coli* was delayed up to 6 days when cells were treated with NEM at 10^{-2}M. This same effect may be concluded from our results of delayed initiation of growth, though the mechanism is not yet known.

An attempt to explain the growth inhibiting effect of SHM as being due to a decrease in the cellular sulfhydryl content of *E. coli* B/r cells was not successful. Striking evidence contrary to this was shown by SHM-triacetate, which though it reacted with cellular sulfhydryl faster and more completely than SHM (to almost the same extent as NEM) as shown in Fig. 4, has an M. I. C. 100 times higher than SHM.[6] This fact suggests that elimination of cellular sulfhydryl groups may be a necessary, but not the only factor for the appearance of antimicrobial activity on *E. coli* by these maleimide compounds. Another possible factor for this may include the differences in cellular uptake of these compounds.

Although the HMB (*p*-hydroxymercuribenzoate) bound sulfhydryl groups in bacteria have been measured accurately by neutron activation analyses,[14] our simple method could measure the total sulfhydryl groups in *E. coli* cells as accurately as the neutron activation analysis.

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