92. Takeo Ueda, Shigeshi Toyoshima, and Tsuneo Wachi: Researches on Chemotherapeutic Drugs against Viruses. XVI.[1] Chemotherapeutic Effect of N^1-Dodecanoyl-N^4-acetylaminonaphthalenesulfonamide-(1) on Neurotropic Viruses.

(*Pharmaceutical Institute, Keio-Gijuku University**)

These studies were undertaken as it had previously been found that N^1-dodecanoyl-N^4-acetylaminonaphthalenesulfonamide-(1), named PANS-No. 610, exerted marked *in vitro* effects on the Japanese encephalitis virus among the series of N^1-acyl-N^4-acylaminonaphthalenesulfonamides-(1) tested. In these studies PANS-No. 610 was surveyed in detail as to its activity *in vitro* and *in vivo*, and toxicity. This paper describes the chemotherapeutic properties of PANS-No. 610 which is of promise for clinical administration.

Materials and Methods

The compound is a white, odorless, and tasteless crystals; it is sparingly soluble in water but soluble in alkaline solutions. Aqueous solution of its sodium salt is so stable that its sterilization can be accomplished even by heat.

Aqueous solution of PANS-No. 610 was used. Young mice of 10～12 g. weight were employed; each received the diluted virus and solutions of the compound. The Nakayama strain of *Encephalitis japonica* and the Lansing strain, kindly given by Dr. K. Ando, Division of Bacteriology, National Institute of Health, Tokyo, were employed.

Experimental procedures were the same as those described in Part VIII.[2]

Results

Effect *in vitro* on the Nakayama Strain—Mixtures of infective brain suspension (LD_{50} corresponding to $10^{-3.5}$) in concentration of $10^{-3.5}$ were prepared in 0.1～0.01% solution of the compound, and injected intracerebrally at 22° into groups of mice one hour after the mixing of the virus and the compound solution. Results are given in Table I by the survival ratio.

Table I

Concentration of PANS-No. 610	0.1%	0.05%	0.025%	0.01%
Groups mixed with only PANS-No. 610	10/10	10/10	4/9	1/10
Groups mixed with PANS-No. 610 and serum	10/10	9/10	4/10	0/10
Control (without PANS-No. 610)	0/10	0/10	0/10	0/10

In the Table, the numerator represents the number of mice that survived and the denominator, total number injected.

It is evident from Table I that the compound exerted a remarkable effect *in vitro* on the virus, which did not decrease by the addition of serum.

Curative Effect on the Nakayama Strain—The virus dilutions of 10^{-1}～10^{-4} were inoculated intranasally into groups of mice, and three hours after the inoculation, solutions of the compound were injected intravenously into the inoculated mice with a single dose of 25～100 mg./kg. The results are given in Table II by the survival ratio and decrease of the virulence of the virus.

Table II

Virus dilution	Group treated with a single dose				Untreated group
	100 mg./kg.	75 mg./kg.	50 mg./kg.	25 mg./kg.	
10^{-1}	0/17	1/17	0/19	0/19	0/10
10^{-2}	0/19	0/20	4/20	0/19	0/10
10^{-3}	3/10	12/16	7/11	3/12	2/9
10^{-4}	9/16	8/15	6/14	8/14	2/8
LD_{50}	$10^{-3.7}$	$10^{-2.9}$	$10^{-3.1}$	10^{-4}	10^{-4}

* Shinano-machi, Shinjuku-ku, Tokyo (上田武雄, 豊島 滋, 和智恒雄).

1) Part XV: This Bulletin, 1, 375(1953)

2) T. Ueda, S. Toyoshima, T. Wachi: J. Pharm. Soc. Japan, 72, 1351(1952).

It may be said from Table II that the compound was intravenously effective on the virus with a single dose of 75 mg./kg. three hours after the inoculation of the virus.

The virus dilutions in concentration of 10^{-1}～10^{-4} were inoculated intranasally into groups of mice and at various intervals after the inoculation, solutions of the compound with a single dose of 75 mg./kg. were injected intravenously into the inoculated mice. Results are given in Table III by the survival ratio and decrease of the virulence of the virus.

TABLE III

Virus diln.	Treated group: Interval after the inoculation (hrs.) 0	3	6	12	18	24	30	36	42	48	60	72	84	96	120	Untreated group
10^{-1}	0/9	0/8	0/9	2/10	1/10	1/9	0/10	1/9	0/10	1/8	1/10	0/7	1/10	0/10	0/10	0/10
10^{-2}	4/9	2/10	4/10	2/10	3/9	7/19	1/10	3/10	4/9	7/14	1/9	7/19	2/6	6/19	4/7	2/22
10^{-3}	3/9	7/10	8/10	6/8	4/10	9/19	7/10	9/10	10/10	6/14	4/8	8/16	3/7	7/19	5/9	2/7
10^{-4}	5/10	7/12	9/9	8/9	7/9	16/18	6/9	8/9	6/10	10/15	7/7	12/17	8/11	12/18	6/10	10/18
LD_{50}	$<10^{-4}$	$10^{-2.9}$	$10^{-2.2}$	$10^{-2.5}$	$10^{-3.0}$	$10^{-2.8}$	$10^{-2.8}$	$10^{-2.5}$	$10^{-2.4}$	$10^{-2.9}$	$10^{-2.7}$	$10^{-2.9}$	$10^{-3.0}$	$10^{-3.0}$	$10^{-2.9}$	$10^{-3.4}$

It may be said from Table III that the compound was effective on the virus within 120 hours after the inoculation, i.e. just before and after the symptoms of encephalitis would occur in mice, and it may be supposed that the effectiveness of the drug might be improved if more doses were employed by a more direct route of administration.

Protective Effect on the Nakayama Strain—The compound was administered orally into groups of mice with a single or more doses of 2～5 g./kg., and then at various intervals after the administration, dilutions of the virus in concentration of 10^{-1}～10^{-4} were inoculated intravenously into the treated mice. Results are given in Table IV by the survival ratio and decrease of the virulence of the virus.

TABLE IV

Dose of PANS-No. 106	Interval before inoculation	Virus dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	LD_{50}
1×5 g./kg.	0	17/18	10/17	10/18	—	$10^{-1.9}$
2×3 g./kg.	0	5/13	4/13	8/10	—	$10^{-2.1}$
3×2 g./kg.	0	5/9	7/10	9/13	—	$10^{-1.5}$
Untreated	—	0/6	1/6	4/6	—	$10^{-3.6}$
3×2 g./kg.	1 day	—	3/12	8/15	9/15	$10^{-3.1}$
3×2 g./kg.	2 days	—	2/9	7/12	13/16	$10^{-2.9}$
3×2 g./kg.	3 days	—	3/10	6/12	13/15	$10^{-2.9}$
3×2 g./kg.	4 days	—	3/9	7/11	9/11	$10^{-2.7}$
Untreated	—	—	0/6	2/6	5/7	$10^{-3.5}$

It may be said from Table IV that the oral administration of the compound gave considerable protection against the virus.

Effect *in vitro* on the Lansing Virus—The virus dilutions in concentration of 10^{-2}(corresponding to LD_{50} 10^{-4}) were prepared in 0.3～0.01% solutions of the compound and injected intracerebrally into groups of mice at 22°, one hour after the mixing of the virus and the compound. Results are given in Table V by survival ratio.

TABLE V

Concentration of PANS-No. 610	0.3%	0.1%	0.05%	0.01%
Group mixed with PANS-No. 610	4/6	7/10	0/10	0/9
Control (without PANS-No. 610)	0/8	0/10	0/10	0/10

It is shown by Table V that the compound exerted a remarkable effect *in vitro* on the virus in concentration of more than 0.1%.

Curative Effect on the Lansing Virus—The virus dilutions in concentration of 10^{-1}～10^{-3} were inoculated intranasally into groups of mice, and one hour after the inoculation, solutions of the

compound were injected intravenously into the inoculated mice with a single dose of 25~100 mg./kg. The results are given in Table VI by survival ratio.

TABLE VI

Virus dilution	Group treated with a single dose				Untreated group
	100 mg./kg.	75 mg./kg.	50 mg./kg.	25 mg./kg.	
10^{-1}	1/20	1/20	1/20	1/20	0/6
10^{-2}	1/20	1/19	1/20	1/20	1/10
10^{-3}	3/20	3/20	5/20	6/20	2/6

It may be said from Table VI that the compound exerted a weak curative effect on the virus.

TABLE VII

Interval after the inoculation (hrs.)	Treated group										Untreated group
	0	12	24	36	48	60	72	84	96	120	
Virus diln.											
10^{-1}	1/10	2/10	0/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10	0/10
10^{-2}	2/10	2/10	1/10	2/9	1/9	0/10	1/10	0/10	2/10	2/10	1/10
10^{-3}	2/7	5/10	3/9	1/9	5/10	3/10	5/10	5/10	5/10	8/10	1/6
LD_{50}	—	—	—	—	—	—	—	—	—	$10^{-2.5}$	$10^{-3.3}$

The virus dilutions in concentration of 10^{-1}~10^{-3} were inoculated intranasally into groups of mice and at various intervals after the inoculation, solutions of the compound with a single dose of 75 mg./kg. were injected intravenously into the inoculated mice. Results are given in Table VII by survival ratio.

Table VII shows that the compound exerted only a weak curative effect on the virus throughout 5 days after the infection.

Toxicity of the Compound—The toxic effect of a single injection of the compound was determined in mice. Results are given in Table VIII by maximum tolerative dose.

Table VIII shows that the compound possessed a comparatively low toxicity.

TABLE VIII

Toxicity of PANS-No. 610*

Route of administration	M.T.D. (mg./kg.)
Intravenous	125
Intraperitoneal	75
Subcutaneous	400
Oral	more than 10 g.

* This compound has been observed to exert hemolytic action and affinity with venous track. However, it has been shown by Dr. Uchiyama and Dr. Yokota that the compound hardly showed any unfavorable clinical side-reaction by careful intravenous administration.

Discussion and Conclusion

Ever since several compounds of the 3-phenylazo-4-aminonaphthalenesulfonic acid and the 3-phenylazo-4-aminonaphthalenesulfonamide series had been found to exert considerable effects against neurotropic viruses such as the Japanese encephalitis virus and Col. SK virus, effective compound against so-called small viruses has not been claimed at all.

As described in previous papers[2,3], it was hitherto observed that 3-(*o*-methylphenylazo)-4-aminonaphthalenesulfonic acid (PANS-No. 326), 3-(*p*-octylphenylazo)-4-aminonaphthalenesulfonic acid (PANS-No. 315), and *p*-(3-phenylazo-4-aminonaphthalenesulfonamido)-benzoic acid (PANS-No. 325) exerted effects *in vitro* on the neurotropic viruses, far stronger than PAN-No. 25, but almost equal effects *in vivo* to PAN-No. 25.

PANS-No. 325 and PANS-No. 326 were observed by clinical tests to possess remarkable prophylactic effect against the Japanese encephalitis virus[4] but not curative effect stronger than PAN-No. 25.

3) T. Ueda, S. Toyoshima, T. Ito: This Bulletin, 1, 271(1953).
4) Unpublished: This chemoprophylactic test was carried out by Dr. Ando, *et al.* in the Toyama District, Japan.

It might be supposed that antiviral compounds possessing azo structure are not beneficial in neurotropy, cerebrotropy, and penetration into cells because they might be inactivated en route to the brain by combining with proteinous components in the host. On the contrary, PANS-No. 610, without azo structure, was selected as the curatively effective one on *Encephalitis japonica*. This compound was found, as described in the experimental part, to possess a remarkable effect *in vitro* and *in vivo* against the virus, with a comparatively low toxicity.

Chemoprophylactic effect of this compound can be anticipated since, as described in the experimental part, the compound was observed to show a considerable protective effect against the virus. According to clinical tests carried out with PAN-No. 25, PANS-No. 325, and PANS-No. 326, it was concluded that the protective effect was qualitatively parallel with *in vitro* effects.

PANS-No. 610 was observed to be more effective *in vitro* than the above three. From these findings it may be said that this compound is of promise for protective administration.

Chemotherapeutic effect of the compound was observed: It was noteworthy that the compound was effective *in vivo* just before and after the symptoms caused by the virus would occur. In this regard, the compound was predominant over other azo compounds hitherto taken up. This property was assumed to be favorable for clinical administration.

In fact, the chemotherapeutic effect of this compound was confirmed clinically by Dr. Uchiyama, Director of the Komagome Hospital, Tokyo. The clinical findings with this compound will be reported in detail in a medical journal of Japan in the near future.

Therefore, it may be claimed that PANS-No. 610 is the first compound which has been confirmed to have chemotherapeutic effect on patients suffering from the so-called small virus such as the Japanese encephalitis virus. It should be noted that these findings have made clear the relationship between clinical tests and animal tests with small virus and a chemical compound for the first time. However, it is questionable at the present stage whether the drug is effective by direct action on the virus or indirect action such as that of excitation or secretion of specific organs.

It is therefore not timely to discuss the mode of action of the compound on the virus in this paper. However, according to observations with the results of animal tests, the antiviral effect of the compound might be supposed to be associated with its specific properties such as those of neurotropy, cerebrotropy, and power of penetration into cells in host, related to its surfactant properties.

As described above, it may be concluded that the compound is of promise for clinical use against Japanese encephalitis.

PANS-No. 610 was observed to exert only a weak effect *in vitro* and *in vivo* on the Lansing virus. From this, it may be said that the compound is not of promise for clinical use against poliomyelitis.

Some promising compounds showing effects on the Lansing virus, stronger than PANS-No. 610, have been found. Further work on these will be reported in the future.

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Summary

(1) PANS-No. 610 shows a remarkable effect on the Japanese encephalitis virus *in vitro* and *in vivo*. It is effective even at a longer interval after inoculation of the virus.

(2) The compound shows a weak effect *in vitro* and *in vivo* on the Lansing virus.

(3) The compound possesses a comparatively low toxicity.

(4) It is assumed that the effect of this compound on the Japanese encephalitis virus

might be caused by its neurotropy, cerebrotropy, and power of penetration into cells of host, related to its surfactant properties.

(5) The compound is of promise for the treatment of diseases caused by the Japanese encephalitis virus.

(6) It may be claimed that this is the only compound which has been confirmed to have chemotherapeutic effect on patients suffering from the so-called small viruses such as the Japanese encephalitis virus.

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93. Takeo Ueda, Isao Nakata, and Shigeshi Toyoshima: Researches on Quaternary Ammonium Salts as Chemotherapeutic Drugs. I. Syntheses of Trialkyl-*p*-alkylanilinium Salts.

(*Pharmaceutical Institute, Keio-Gijuku University**)

A number of long-chain quaternary ammonium salts have been reported concerning their germicidal activity since their high potency and usefulness in this field was pointed out by Domagk in 1935.[1] It had already been known that some quaternary nitrogen compounds, pyridinium salts, quinolinium salts, dyestuffs, and so on, were effective against microbes but the study of the utilization of quaternary ammonium salts as germicides was greatly stimulated by his improvement of the germicidal activity in attaching long-chain aliphatic residues to the quaternary nitrogen atom. It was also found that lower members in the series of alcohols and alkylphenols were useful as germicides but attempts to test higher members as to their activity were fruitless because of their low solubility in germicidal concentration. Fortunately, quaternary ammonium salts, even though of high molecular weight, in general, were found to be remarkably soluble in water.

Among those quaternary nitrogen compounds, the effective ones were found to possess long-chain alkyl groups and in general, surface active properties, though their germicidal activity did not run parallel with their surface active properties.

As reported in previous papers,[2] 3-alkylphenylazo-4-hydroxynaphthalenesulfonic acids were observed to be more effective against the Japanese encephalitis virus than other compounds in this series, and their virucidal activity was shown to increase with lengthening of alkyl group and then reach the optimum with the octyl group. It might be deduced thereby that alkylphenyl residue should be an important factor in increasing the activity of the compounds of this type. In order to examine whether this assumption was in point or not, some quaternary ammonium salts were synthesized by converting N,N-dimethyl-*p*-alkylanilines.

N,N-Dimethyl-*p*-alkylaniline was prepared by various methods, for instance, by alkylation of *p*-alkylaniline, by condensation of dimethylaniline with alcohol in the presence of catalysts,[3] and so on. However, these methods were not always suitable in laboratories because of low yields or of difficulty in the purification of resulting substances. N,N-

* Shinano-machi, Shinjuku-ku, Tokyo (上田武雄, 中田 公, 豊島 滋).

1) G. Damagk: Med. Wochshr., **61**, 829(1935).
2) T. Ito, S. Toyoshima, M. Taniguchi, T. Ueda: This Bulletin, **1**, 275(1953).
3) W. Cule Davies, F. L. Hulbert: J. Soc. Chem. Ind., **57**, 349(1938), *et seq.*