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Antibiological Actions of 2-Amino-1,4-naphthoquinone Imine

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Antibiological actions were studied on 2-amino-1,4-naphthoquinone imine-HCl (ANQI), which had been investigated *in vitro* as a potent inhibitor of DNA synthesis in Ehrlich mouse ascites tumor cells and as an interacting substance to DNA. The results are as follows:

1. Antimicrobial Action: A considerable activity was found against animal and plant microorganisms, especially *Xanthomonas oryzae*.

2. Acute Mammalian Toxicity: LD_{50} was 5.45 (4.88—6.09) mg/kg in mice (intraperitoneal injection). Only a little and transient effect was observed on behaviors of mice, and on respiration and blood pressure of dogs.

3. Antitumor Activity: ANQI exerted no therapeutic effect on Ehrlich mouse ascites tumor *in vivo*, but shortened survival days of tumor-bearing mice in its high doses.

4. Chemical Changes in Mouse Ascitic Fluid: ANQI in part was oxidized in ascitic fluid of tumor-bearing mice to 2-amino-1,4-naphthoquinone, which had been found to be not inhibitory to nucleic acid synthesis. The ineffectiveness of ANQI on the tumor *in vivo* was parlty explained from the susceptibility to biological oxidation.

As a part of the investigations on substances affecting protein or nucleic acid biosynthesis, it was previously reported that among several aminoquinone compounds 2-amino-1,4-naphthoquinone imine-HCl (ANQI) had an activity of inhibiting DNA synthesis in Ehrlich mouse ascites tumor cells *in vitro* to such a great extent as seen in the case of mitomycins.[2-4)] This inhibition was considered to be contributed to its interaction with nucleic acid, especially with DNA; for ANQI was found to bind to its purine base moieties *in vitro*,[5)] similarly to some biologically active substances such as acridine dyes,[6)] nogalamycin,[7)] ethidium bromide,[8)] chloroquine,[9)] and 4-nitroquinoline 1-oxide.[6,10)]

In view of the above facts it appeared of interest to investigate the antibacterial, antifungal, and antitumor actions of ANQI, as well as its mammalian toxicity. The experiments described below indicate that ANQI had antibacterial and antifungal activities to a considerable extent against animal and plant microorganisms, but had no antitumor effect on Ehrlich mouse ascites tumor *in vivo* in the range of its doses under LD_{50}, which was 5.45 (4.88—6.09) mg/kg in mice (intraperitoneal injection). The ineffectiveness of ANQI on the tumor may be explained from an experimental result that it was oxidized in ascitic fluid of tumor-bearing mice to form 2-amino-1,4-naphthoquinone which had been found to be not inhibitory to nucleic acid synthesis in the tumor cells *in vitro*.[2)]

1) Location: *Oshika, Shizuoka.*

2) S. Okada, *Chem. Pharm. Bull.* (Tokyo), **17**, 105 (1969).
3) S. Okada, *Chem. Pharm. Bull.* (Tokyo), **17**, 1057 (1969).
4) W. Szybalski and V. N. Iyer, "Antibiotics," Vol. I, ed. by D. Gottlieb and P.D. Shaw, Springer-Verlag, Berlin, Heidelberg, and New York, 1967, pp. 211—245.
5) S. Okada, *Chem. Pharm. Bull.* (Tokyo), **17**, 113 (1969).
6) M. Kodama, Y. Tagashira, and C. Nagata, *J. Biochem.* (Tokyo), **64**, 167 (1968).
7) B.K. Bhuyan and C.G. Smith, *Proc. Natl. Acad. Sci. U. S.*, **54**, 566 (1965).
8) M.J. Waring, *J. Mol. Biol.*, **13**, 269 (1965); M.J. Waring, *Biochim. Biophys. Acta*, **114**, 234 (1966).
9) S.N. Cohen and K.L. Yielding, *J. Biol. Chem.*, **240**, 3123 (1965); R.L. O'Brien, J.G. Olenick, and F.E. Hahn, *Proc. Natl. Acad. Sci. U. S.*, **55**, 1511 (1966).
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Experimental

Materials——ANQI and 2-amino-1,4-naphthoquinone were synthesized in the authors' laboratory according to the procedure of Fieser.[11]

Tritiated ANQI was prepared from tritiated 2,4-dinitro-1-naphthol which had been obtained by exposing the dinitro compound to tritium gas according to the method of Wilzbach.[12] The reason for the tritiation of 2,4-dinitro-1-naphthol instead of ANQI itself was to avoid the formation of some reduced products of ANQI. The details of the procedure were as follows: A sample (1 g) of 2,4-dinitro-1-naphthol was tritiated by exposing to 1.5 Ci of carrier-free tritium gas[13] in a Wilzbach's apparatus modified in the authors' laboratory.[14] Then, the labile ^3H of the tritiated compound was replaced by ^1H of water by repeating five times dissolution in water (*ca.* 1 liter) and removement of the water *in vacuo*. The residual 2,4-dinitro-1-naphthol-U-^3H was recrystallized from water, and was reduced to 2,4-diamino-1-naphthol-diHCl-U-^3H by $Na_2S_2O_4$ and HCl. Tritiated ANQI was obtained by oxidizing this diamine compound with $FeCl_3$, and was purified radiochemically through paper chromatography with *n*-butanol–acetic acid–water (4:1:2). Finally, this ANQI-U-^3H was recrystallized from diluted HCl, and its radiochemical purity was confirmed by radio-paper chromatography with the solvent system mentioned above ($Rf=0.83$). Its specific radioactivity was determined to be 0.235 mCi/mmole by a liquid scintillation counter.

Mice——Male mice of strain dd/Y weighing 19.5—20.5 g were used.

Antimicrobial Test for Animal Microorganisms——The dilution method using nutrient agar was applied. Incubation was carried out at 37° for 17 or 40 hr.

Antimicrobial Test for Plant Microorganisms——The dilution method was also applied using modified Misato's medium, which consisted of yeast extract (2 g), soluble starch (5 g), and sucrose (10 g) in 1 liter of 3% agar. The plates were incubated at 28° for 72 hr.

Acute Toxicity in Mice——ANQI was dissolved in 0.5 ml of physiological saline and injected intraperitoneally into mice, then their toxic behaviors were observed for 2 hr and the subsequent period, and on the basis of the mortality 24 hr after the injection the value of LD_{50} was determined according to the method of van der Waerden.[15]

Effect on Respiration and Blood Pressure of Dogs——ANQI solution was injected intravenously into two dogs anesthesized with pentobarbital, and the changes in their respiration and blood pressure were recorded on a kymographion by the usual method.

Antitumor Activity——Approximately 2×10^6 cells of Ehrlich ascites tumor were transplanted intraperitoneally into mice. Then, ANQI in 0.5 ml of physiological saline was injected also intraperitoneally into each mouse once a day for 7 days starting 24 hr after the transplantation, and each survival period was observed.

Chemical Changes of ANQI in Mouse Ascitic Fluid——ANQI-U-^3H (20 micromoles) was dissolved in 0.5 ml of the ice-cold supernatant of ascitic fluid from a few mice which had been inoculated with Ehrlich ascites tumor cells 7 days in advance. This mixture was incubated immediately at 37° and was paper-chromatographed with *n*-butanol–acetic acid–water (4:1:2) at several incubation times. ANQI, and other compounds formed during the incubation on the paper chromatograms were detected under visible and ultraviolet light, and the radioactivities distributed on the papers were determined in a windowless 2π-gas-flow counter by cutting the paper strips into small pieces.

Results and Discussion

Antimicrobial Action on Animal Microorganisms

As represented in Table I, ANQI showed a wide antimicrobial spectrum over Gram-positive and -negative bacteria, and fungi. The minimum inhibitory concentrations against all the tested strains of microorganisms were concentrated in a narrow range, 1.56—12.5 μg/ml.

11) L.F. Fieser, "Experiments in Organic Chemistry," 3rd ed., D.C. Heath & Co., Boston, 1957, (reprinted by Maruzen Co., Ltd., Tokyo, 1958), pp. 234—238.
12) K.E. Wilzbach, *J. Am. Chem. Soc.*, **79**, 1013 (1957).
13) Product of The Radiochemical Centre.
14) S. Okada and O. Tamemasa, *Radioisotopes*, **14**, 42 (1965).
15) B.L. van der Waerden, *Arch. Exptl. Pathol. Pharmakol.*, **195**, 389 (1940).

Table I. Antimicrobial Spectrum of ANQI on Animal Microorganisms

Microorganisms	Growth Concentration of ANQI (μg/ml) 25	12.5	6.25	3.12	1.56	0.78
Staphylococcus aureus 209P	−	−	−	±	+	+
Staphylococcus aureus ActMf	−	−	−	−	+	+
Staphylococcus aureus 193	−	−	−	±	+	+
Staphylococcus aureus 52–34	−	−	−	−	+	+
Staphylococcus aureus Smith	−	−	−	−	+	+
Staphylococcus aureus Terajima	−	−	−	−	±	+
Sarcina lutea PCI 1001	−	−	−	−	±	+
Micrococcus floavus 16	−	−	−	−	−	+
Bacillus subtilis PCI 219	−	−	−	−	+	+
Bacillus subtilis NRRL B-558	−	−	−	±	+	+
Bacillus anthracis	−	−	−	−	+	+
Bacillus cereus ATCC 10702	−	−	+	+	+	+
Mycobacterium phlei[a]	−	−	−	−	+	+
Mycobacterium phlei 607[a]	−	−	−	−	−	±
Escherichia coli NIHJ	−	−	−	±	+	+
Shigella flexneri laEw8	−	−	−	−	±	+
Salmonella enteritidis	−	−	−	±	±	+
Klebsiella pneumoniae PCI 602	−	−	−	−	±	+
Pseudomonas aeruginosa A3	−	−	+	+	+	+
Candida albicans 3147	−	−	±	±	+	+

−: no growth ∓: little growth ±: a little growth +: growth
a) incubated at 37° for 40 hr (others; for 17 hr).

Antimicrobial Action on Plant Microorganisms

The potent activity against *Xanthomonas oryzae* was noted in the antimicrobial spectrum shown in Table II, that is, the minimum inhibitory concentration was 1 μg/ml, and was comparable to that of phenylmercury acetate.

Table II. Antimicrobial Spectrum of ANQI on Plant Microorganisms

Microorganisms	Growth Concentration of ANQI (μg/ml) 1000	100	10	1	0.1
Cochliobolus miyabeanus	−	−	+	+	+
Diaporthe citri	−	−	−	+	+
Corticium rolfsii	−	+	+	+	+
Gloeosporium laeticolor	−	−	−	+	+
Piricularia oryzae	−	−	−	+	+
Cladosporium carpophilum	−	−	−	+	+
Alternaria kikuchiana	−	−	−	+	+
Fusarium oxysporum f. *niveum*	−	−	+	+	+
Xanthomonas oryzae	−	−	−	−	+
Xanthomonas citri	−	−	−	+	+
Corynebacterium sepedonicum	−	−	+	+	+

−: no growth +: growth

The disease of rice plant by *Xanthomonas oryzae* (Ine-shirahagarebyo) is one of the most serious ones in Japan, particularly in Kyushu and Shikoku, and yet have not been found any of the agricultural chemicals specific for this disease. Some organomercury compounds, and antibiotics such as chloramphenicol and cellocidine were found to be effective, but the former is not applied practically from the viewpoint of its residual toxicity to mammals,

and the latter is not sufficiently effective for complete therapy. ANQI, therefore, is considered to be valuable to investigate furthermore as a drug for this plant disease, in spite of its high acute toxicity to mammals as stated later.

Acute Toxicity

As shown in Table III, the value of LD_{50} obtained in mice 24 hr after intraperitoneal injection of ANQI was 5.45 (4.88—6.09) mg/kg. Since the administration in doses under the lethal caused only a weak and transient depression in spontaneous motility and in responses to exterocetive stimuli, the action of ANQI on the central nervous systems in mice was considered to be weak. The lethal action was revealed immediately after the injection, and mice survived 30 min thereafter were no more mortal at least for a few months. Such an

Table III. Acute Toxicity of ANQI in Mice

Dose of ANQI injected *i.p.* (mg/kg)	Mortality (Survived/Administered)	Toxic behavior
1.48	7/7	transient depression in spontaneous motility and in responses to exterocetive stimuli
2.22	7/7	
3.33	6/7	locomotive ataxia (all mice), followed by clonic and tonic convulsion (dead mice)
5.00	5/7	
7.50	1/7	
11.25	0/7	
16.88	0/7	

LD_{50}=5.45 (4.88—6.09) mg/kg

acuteness in toxicity suggests that the lethal action of ANQI was attributable to a biological action other than the inhibition of nucleic acid and protein synthesis which was revealed by this compound in Ehrlich mouse ascites tumor cells *in vitro*.[2,3]

On the other hand, ANQI caused a weak and transient hypertonia being followed a weak hypotonia, and a little stimulation of respiration in dogs (Table IV). These actions were, however, so weak that ANQI was thought to be not so effective to the respiratory and circulatory systems.

Table IV. Effect of ANQI on Respiration and Blood Pressure of Dogs

Dose of ANQI injected *i.v.* (mg/kg)	Stimulation of respiration	Transient hypertonia (mmHg)	Hypotonia Press. (mmHg)	Hypotonia Lasting time (min)	Effect of anti-Adr.[a]	Effect of anti-A. ch.[b]	Effect of anti-His.[c]
0.25	±	21	10	15	—	—	—
0.5	±	18	30	30	—	—	—
1.0	+	54	40	60	—	—	—

a) adrenalin *b*) acetylcholine *c*) histamine
—: ineffective ±: hardly effective +: effective

Antitumor Activity

Fig. 1 presents the effect on Ehrlich mouse ascites tumor of ANQI given in doses of 4, 2, 1, and 0.5 mg/kg/day, once a day for 7 days starting 24 hr after the inoculation. ANQI exerted no therapeutic effect on the tumor, but shortened survival days of mice in its high doses, *i. e.* more than 2 mg/kg/day.

ANQI was expected to have been effective on the tumor, for it had highly inhibited DNA synthesis in the tumor cells *in vitro*.[2,3] The ineffectiveness on the tumor is probably due to its instability *in vivo*, therefore, the following experiment was performed.

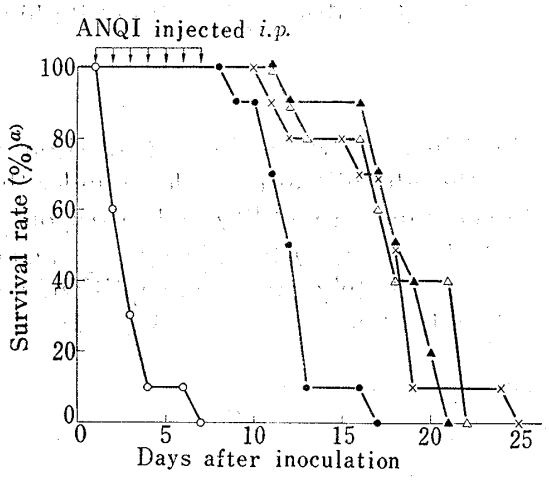

Fig. 1. Effect of ANQI on Ehrlich Mouse Ascites Tumor

a) from 10 mice in each group

	dose (mg/kg/day)	average of survival days
—○—	4	2.6
—●—	2	11.1
—△—	1	17.3
—▲—	0.5	17.3
—×—	0	16.6
	(control)	

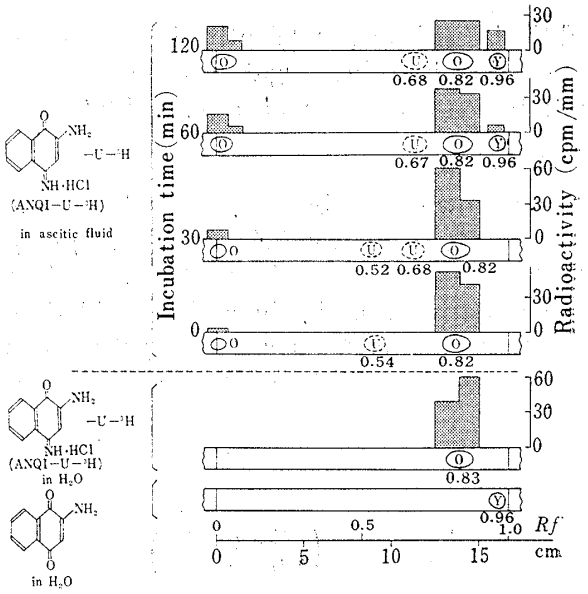

Fig. 2. Chemical Changes of ANQI in Ehrlich Mouse Ascitic Fluid

concentration of ANQI-U-^{3}H in ascitic fluid: 4×10^{-2} M
application on a paper: about 1000 cpm aliquot
paper: Toyo No. 51, ascending
solvent: *n*-BuOH–AcOH–H_2O (4:1:2)
color in spot: Y—yellow, O—orange, U—ultraviolte absorption

Chemical Changes in Ascitic Fluid of Tumor-bearing Mice

When ANQI was incubated in ascitic fluid of tumor-bearing mice, the amount of ANQI decreased with time (Fig. 2). After the incubation at 37° for 2 hr about a half amount of ANQI initially applied was detected as two visible spots other than ANQI itself. The *Rf* value of one yellow spot was 0.96, which agreed with that of 2-amino-1,4-naphthoquinone having no inhibitory action on nucleic acid biosynthesis.[2] Although the chemical nature of another spot remained at the origin was not identified, this was assumed to be ANQI and/or its derivative(s) bound to some macromolecules such as proteins. The assay data for radioactivity showed that the amounts of ANQI, 2-amino-1,4-naphthoquinone, and the substance(s) remained at the origin were approximately in ratio of 5:2:3 after the incubation for 2 hr. This means that a considerable portion, 20 or 50% of initial, of ANQI was changed to the substance(s) ineffective on the synthesis of nucleic acid. On the other hand, the substances corresponded to the other two spots detected under ultraviolet lamp at *Rf*=0.52—0.54 and 0.67—0.68 were probably not originated from ANQI, for they showed no radioactivity.

Therefore, the ineffectiveness of ANQI on Ehrlich mouse ascites tumor and the transientness of its mammalian toxicity may be explained at least partially from its susceptibility to biological oxidation.

When compared the correlation between biological action and chemical structure of ANQI with that in the case of such antibiotics as actinomycins, mitomycins, porfiromycins, and streptonigrin, all of which has aminoquinone moiety in the molecule, the action of ANQI is analogous to actinomycins, and contrary to mitomycins, porfiromycins, and streptonigrin, in view of that the chemical structures of the former have been known to be active in themselves,[16] while that of the latters become active only when reduced enzymatically.[17] Since

16) E. Reich and I.H. Goldberg, "Progress in Nucleic Acid Research and Molecular Biology," Vol. 3, ed. by J.N. Davidson and W.E. Cohn, Academic Press, New York and London, 1964, pp. 184—234; E. Reich, A. Cerami, and D.C. Ward, "Antibiotics," Vol. I, ed. by D. Gottlieb and P.D. Shaw, Springer-Verlag, Berlin, Heidelberg, and New York, 1967, pp. 714—725.

17) V.N. Iyer and W. Szybalski, *Proc. Natl. Acad. Sci. U. S.*, **50**, 355 (1963); V.N. Iyer and W. Szybalski, *Science*, **145**, 55 (1964); J.R. White and H.L. White, *Science*, **145**, 1312 (1964).

the interaction with nucleic acid was found to occur in the form of ANQI itself,[2,5] the biologically active form is considered to be ANQI itself, not some reduced forms such as 2,4-diamino-1-naphthol which is extremely unstable in air.[11]

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18) Present name: Kumiai Chemical Industry Co., Ltd.