A NEW ANTITUMOR ANTIBIOTIC, KIDAMYCIN

IV. PHARMACOKINETICS OF ACETYL-KIDAMYCIN

IWAO UMEZAWA, KANKI KOMIYAMA and HIDEO TAKESHIMA

The Kitasato Institute, Minato-ku, Tokyo, Japan

TOJU HATA

School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo, Japan

MORIHIRO KONO and NOBUO KANDA

Laboratory of Fermentation, Research Institute, Daiichi Seiyaku Co., Ltd., Edogawa-ku, Tokyo, Japan

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Acetyl-kidamycin administered intravenously to mice disappears rapidly from blood and is distributed widely in various organs, where much of it is adsorbed and/or inactivated. A large quantity of the antibiotic is found in the intestinal content indicating the possibility of excretion into the bile. Inactivation of acetyl-kidamycin with homogenates of mouse organs such as liver, intestine, kidney, *etc.* was observed *in vitro*. Tumor tissue shows low concentrations of acetyl-kidamycin but also low rates of inactivation. The inhibitory effects of acetyl-kidamycin on incorporation of ^{3}H-thymidine were correlated with organ levels of the drug, but in the case of S-180 tumor, a marked inhibitory effect was observed even with a low concentration of the antibiotic.

We have previously reported the isolation, properties and antitumor effect in animals of a new antitumor antibiotic, kidamycin, and its acetyl derivative.[1,2] Later, the blood level and organ distribution of acetyl-kidamycin, were examined and it was found that the blood level decreased rapidly *in vivo*. Experiments carried out to find the reason for this decrease are described herein. Furthermore, it is known that acetyl-kidamycin inhibits the synthesis of deoxyribonucleic acid (DNA) in HeLa cells[1] in culture. With this in mind we have attempted to compare the effect in DNA synthesis in several organs of mouse and to correlate the results with organ distribution.

Materials and Methods

(1) ^{3}H-Acetyl-kidamycin: ^{3}H-Labelled acetyl-kidamycin was prepared by exposing acetyl-kidamycin to tritium gas, with purification as reported by KANDA.[3]

(2) Tumor: Subcutaneous solid tumor of S-180 about 7 days old in *dd* mice was removed aseptically and a small piece was implanted into the left axillary region of the same strain of mice with a trocar.

(3) HeLa Cells: HeLa-S_3 cells kept in our laboratory were cultured in small square bottles for 48 hours, and treated with 0.05% trypsin before being used.

(4) Blood Level: Acetyl-kidamycin was injected into the caudal vein of *ddY* mice weighing 18~20 g in groups of 5 mice. The dose of acetyl-kidamycin administered was 100 or 200 mg/kg. Blood was drawn periodically and the antibacterial activity of acetyl-kidamycin in blood was measured by the paper disc method on an agar plate using *Lactobacillus fermenti* as the test organism.

(5) Organ-Level: Acetyl-kidamycin present in various organs was measured by the following

methods after injection of tritiated acetyl-kidamycin into mouse caudal vein.

1) Measurement of Radioactivity: After injection of ^{3}H-acetyl-kidamycin into the caudal vein of *ddY* mice, in groups of 5 mice, animals were killed by exsanguination, and the various organs were extirpated. The organs from 5 mice in a group were pooled and minced finely with scissors. To 50 mg of the minced tissue was added 1 ml of NCS solubilizer (Nuclear Chicago, Des Plaines, Illinois, U.S.A.) and after holding at 50°C overnight, 10 ml of scintillation solution (6 g of PPO per liter of toluene) was added. Radioactivity of this solution was measured in a liquid scintillation counter (Packard Model 3375 Series).

2) Measurement of Antibacterial Activity: The organs described above were made into a 20% homogenate in physiological saline with a Potter homogenizer. Antimicrobial activity of the homogenate was measured by the paper disc method on an agar plate using *L. fermenti* as the test organism.

(6) Thymidin Incorporation into DNA: Mice were given intravenous injections of ^{3}H-thymidine-6T (^{3}H-TdR), 10 μci/20 g of body weight, 60 minutes before sacrifice. The animals were killed by cervical dislocation. The tissues pooled from 3 mice in a group were quickly chilled in iced Petri dish for immediate extraction. DNA was extracted by a slight modification of the ORLOV and ORLOVA procedure.[4]

Each pooled tissue sample was heated in 3 ml of 1 N NaOH in a boiling water bath for 5 minutes and then chilled in an ice-bath. To each sample was added 1.5 ml of an ice-cooled solution of 20% acetic acid saturated with NaCl. After standing in an ice-bath for 60 minutes, samples were centrifuged and 3.5 ml of the supernatants were mixed with 7 ml of cold 95% EtOH. After standing in an ice-bath overnight, the precipitates were collected by centrifugation, washed twice with 5% trichloro-acetic acid, twice with cold 95% ethanol, and twice with solution of ethanol - diethylether (2: 1) at 50°C for 10 minutes.

(7) DNA Measurement: Precipitates were hydrolized in 2 ml of 0.5 N perchloric acid at 70°C for 15 minutes. The hydrolysate was used for determination of DNA by the diphenylamine method and for liquid scintillation counting. A Packard Tri-carb liquid scintillation spectrometer (mode 3375 series) was used for counting, and counts were corrected for quenching. The specific activity of DNA was expressed as cpm/mcg DNA. All the experiments were done in duplicate.

Results and Discussion

1. Blood Level

From the antibacterial activity against *Lactobacillus fermenti*, it was found that the blood level of intravenously injected acetyl-kidamycin decreased rapidly, as shown in Fig. 1. After administration of 100 mg/kg of acetyl-kidamycin, the amount of the antibiotic in blood decreased to 8.3 mcg/ml after 15 minutes, and to 2.5 mcg/ml after 2 hours, with no antibiotic detected thereafter. With a dose of 200 mg/kg, the blood level decreased to 20 mcg/ml and 6 mcg/ml after 1 hour and 2 hours respectively, but increased slightly after 3~4 hours, and a trace was detected after 5 hours.

2. Distribution of Acetyl-kidamycin in Organs

(1) Detection by Radioactivity:

After injection of 200 mg/kg of ^{3}H-acetyl-kidamycin into the caudal vein of mice, organs were extirpated 30 minutes, and 3, 5, 24 and 48 hours later and radioactivity in each organ was measured. As shown in Table 1, ^{3}H-acetyl-kidamycin was found to be distributed widely in various organs, though the amount varied with each organ. The radioactivity was the highest in the liver after 30 minutes of injection, followed by the lung, kidney, small intestine, intestinal content (considered to have been excreted in bile), lymph node, thymus gland, tumor, brain, testis and bone marrow. This radioactivity decreased with time but a considerable count was found in the liver, lung, and kidney

Fig. 1. Blood levels of acetyl-kidamycin in mice

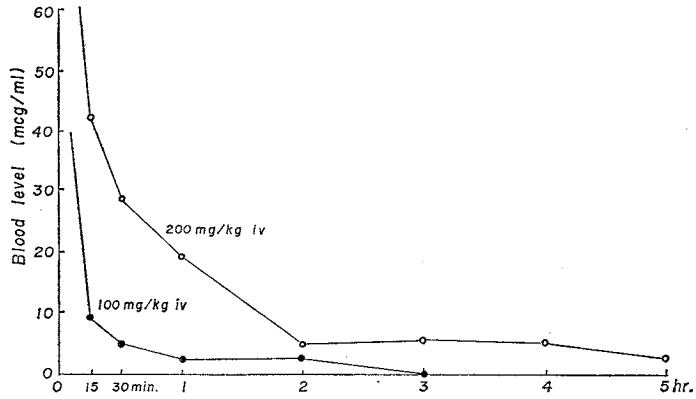


Table 1. Organ levels of ^{3}H-acetyl-kidamycin in mice

Organ	mcg/g wet tissue				
	30 min.	3 hr.	5 hr.	24 hr.	48 hr.
Brain	34 (0)	25 (0)	29 (0)	16 (0)	16
Thymus	79 (0)	61 (0)	58 (0)	35 (0)	40
Heart	232 (143)	186 (114)	143 (117)	69 (0)	52
Lung	392 (334)	360 (260)	325 (315)	186 (0)	142
Liver	>600 (287)	600 (198)	550 (295)	476 (0)	340
Stomach	69 (0)	58 (0)	59 (0)	31 (0)	28
Small intestine	328 (0)	192 (0)	154 (0)	51 (0)	50
Large intestine	139 (0)	106 (0)	78 (0)	14 (0)	40
Kidney	360 (174)	325 (170)	292 (182)	172 (0)	135
Spleen	195 (120)	216 (135)	234 (130)	244 (0)	245
Testis	28 (0)	26 (0)	28 (0)	18 (0)	20
Abdominal memb.	— (0)	54 (0)	49 (0)	26 (0)	30
Muscle	73 (0)	49 (0)	52 (0)	29 (0)	33
Skin	— (0)	44 (0)	50 (0)	25 (0)	37
Tumor	52 (0)	55 (0)	57 (0)	— (0)	—
Lymph node	107 (0)	87 (0)	96 (0)	53 (0)	68
Intestinal contents	550 (–)	258 (–)	149 (–)	66 (–)	20
Bone marrow	27 (0)	28 (0)	28 (0)	26 (0)	15
Urine	>600 (141)	>600 (155)	— (78)	4 (0)	2

Number in parenthesis indicates antibacterial activity.

even after 24~48 hours. On the other hand, the radioactivity in the spleen increased, though slightly, with time from that immediately after the injection, and a decrease was not observed even at 48 hours. Considerable radioactivity was detected in urine 30 minutes after the injection.

(2) Distribution Detected through Antibacterial Activity:

The assay of active material, using the same samples as in the above, is also shown in Table 1. Antibacterial activity was found in the lung, liver, kidney, heart and spleen, and the values were approximately the same until 5 hours after the injection. In other organs, antibacterial activity was

not detected even 30 minutes after the injection. These results indicate that antibacterial activity disappears more rapidly than radioactivity.

Since it was found that intravenous injection of acetyl-kidamycin in mice resulted in rapid loss from the blood and that antibacterial activity was extremely low in some organs in spite of high radioactivity, it was considered that the antibacterial activity of acetyl-kidamycin might be inactivated by organ homogenates, and the following experiments were carried out.

3. Effect of Temperature on the Antimicrobial Activity of Acetyl-kidamycin in Organ Homogenate

Various organs of mice and sarcoma-180 solid tumor were prepared as 20% homogenates, a quan-

Fig. 2. Inactivation of acetyl-kidamycin added to various organ homogenates at 37°C

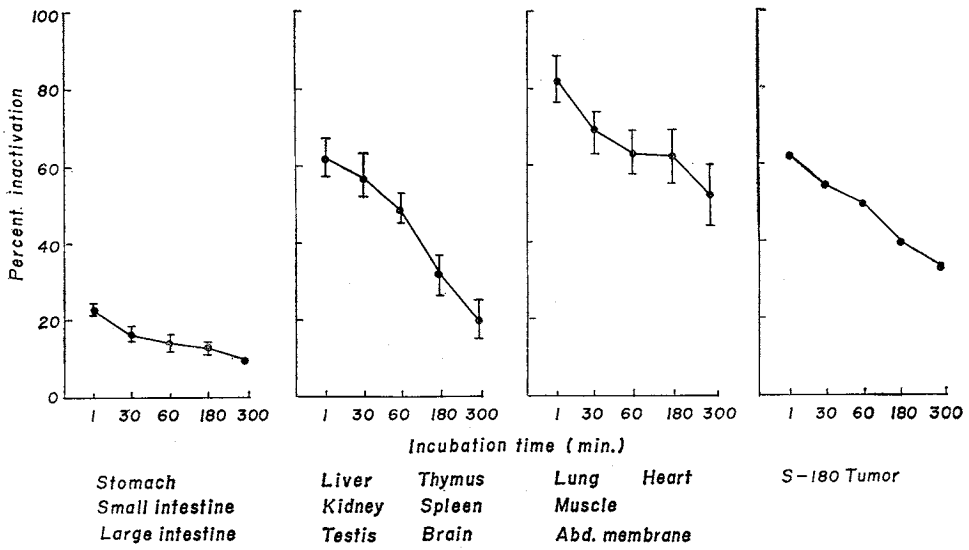
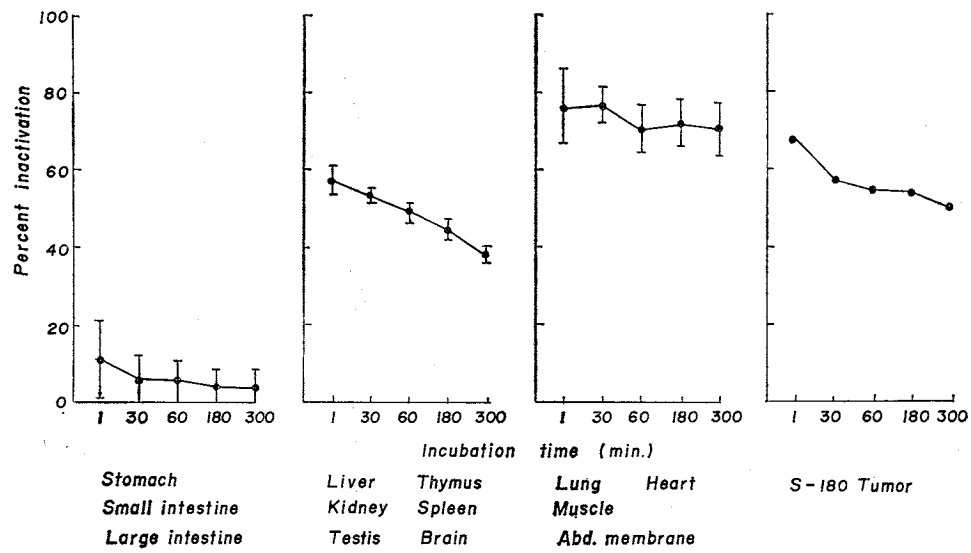


Fig. 3. Inactivation of acetyl-kidamycin added to various organ homogenate at 5°C



tity of acetyl-kidamycin was added to each homogenate to make the final concentration of the antibiotic to 200 mcg/ml, and the mixtures were incubated at 37°C or 5°C for 30 minutes, and 1, 3 and 5 hours. Antibacterial activity of acetyl-kidamycin was examined, and from the results, the organs can be classified into 3 groups as shown in Figs. 2 and 3.

Comparison of the effect of temperature 1 minute after the addition of acetyl-kidamycin showed that 77% of the activity was lost at 37°C and 88% at 5°C with stomach or small or large intestine. With liver, kidney, testis, thymus, spleen and brain, the loss was 38% at 37°C and 24% at 5°C. With lung, muscle, abdominal membrane and heart, the loss was 37% at 37°C and 32% at 5°C. In the tumor, 37% of the activity was lost at 37°C and 32% at 5°C. These results indicate that, more than 20% of the activity is lost immediately after the addition of acetyl-kidamycin and suggest that acetyl-kidamycin is rapidly adsorbed by organs. The reason for greater inactivation in some organs is not clear. Antibacterial activity of the homogenates decreased with time, more rapidly at 37°C than at 5°C, which may be due to the effect of inactivating enzyme(s) present in the organs.

Table 2. Recovery of acetyl-kidamycin from mouse organ

Organ	within 5 min.*		60 min.	
	Sup.	Ext.	Sup.	Ext.
Lung	22.7%	25.5%	12.5%	14.9%
Liver	18.3	20.7	14.1	13.7
Kidney	17.5	24.3	12	17.9
Small intestine	13.7	0	0	0
Spleen	14.3	7.7	3.2	0
Tumor	16.5	7.1	16.2	9.9
Saline	100.0	80.0	100.0	85.0

Acetyl kidamycin was added to homogenate to make the final concentration of 200 mcg/ml.
* Homogenates were immediately centrifuged after the addition of the agent.

In order to examine the relationship between the loss of antibacterial activity and adsorption, the following experiment was carried out. Lung, liver, kidney, small intestine, spleen, and tumor of mice were prepared as 20% homogenates, a quantity of acetyl-kidamycin was added at 37°C, shaken vigorously for 1 minute, and centrifuged at 3,000 rpm for 15 minutes. Antibacterial activity of the supernatant was examined and, at the same time, the precipitate was treated with an equal volume of chloroform, mixed thoroughly for 3 minutes, and centrifuged to obtain a chloroform extract solution. This extraction procedure was repeated twice and the activity of acetyl-kidamycin was measured in the combined chloroform layer. As shown in Table 2, only 14~23% of the activity was detected in the centrifuged supernatant of each organ immediately after the addition of the antibiotic.

Activity of acetyl-kidamycin that transferred from the precipitate to the chloroform layer was 26~32% with lung, liver and kidneys, 9% with spleen and tumor, and none with small intestine. The recovery of acetyl-kidamycin, after leaving the mixture at 37°C for 60 minutes after the addition of the antibiotic was examined in the same manner, and was 12~18% in both the supernatant and the extract from the lung, liver and kidney, and only 3.2% in the supernatant from the spleen, none from its extract. The antibiotic was not detected with the small intestine, either in the supernatant or the extract.

The total recovery rate from both supernatant and precipitate, was 55, 44 and 49% from lung, liver and kidney, respectively, while the values were 30, 30 and 33% when the homogenate were left at 37°C for 60 minutes. The recovery was 24% immediately after and 3.2% after 60 minutes in the spleen, and was 13.7% immediately with the small intestine, and none after 60 minutes.

The antibacterial activity extracted into chloroform corresponds to the amount of acetyl-kidamycin

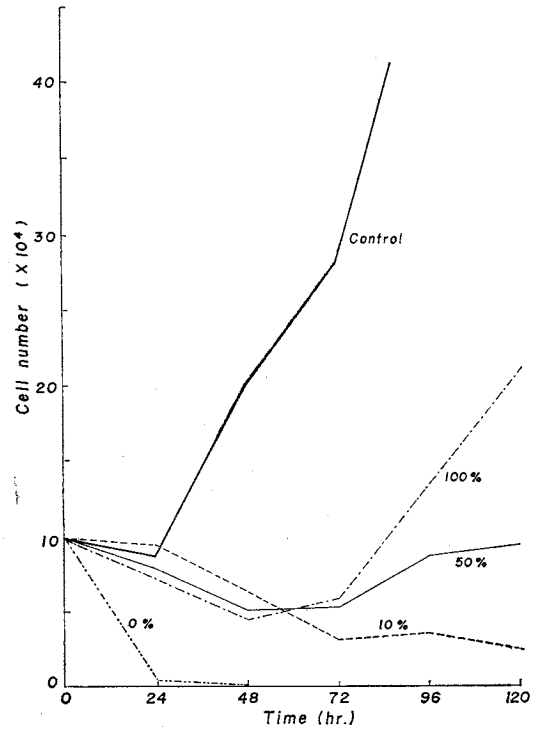
adsorbed in each organ. This amount was approximately 30% in the lung, kidney and liver, and 9% in the tumor. It is clear from this result that the antibiotic is inactivated in the small intestine by factors other than adsorption, and that the adsorbed antibiotic is further inactivated with passage of time.

4. Effect of Serum on Acetyl-Kidamycin

Since above experiments have shown that acetyl-kidamycin was easily bound to high-molecular substances like protein, effect of such binding with high-molecular substance on the activity was examined.

HeLa cells were mixed with 0.6 mcg/ml acetyl-kidamycin in minimum essential medium (MEM) containing 100, 50, 10 or 0% of calf serum, shaken, and centrifuged to collect the cells. The cells were washed twice with fresh MEM, suspended in growth medium to make the final concentration 10^6 cells/ml, and the cells were incubated at 37°C. Growth of the cells was examined periodically after 24, 48, 72, 96 and 120 hours. As shown in Fig. 4, acetyl-kidamycin applied to HeLa cells in MEM not containing any serum completely inhibited their growth at a concentration of 0.6 mcg/ml, and the anti-HeLa activity of acetyl-kidamycin tended to decrease with increasing amount of the serum.

Fig. 4. Effect of serum on anti-HeLa activity of acetyl-kidamycin



5. Inhibition of Acetyl-Kidamycin Thymidine Incorporation into DNA

^{3}H-TdR was given to mice to establish a relationship between tissue concentration of acetyl-kidamycin and the ability of the agent to inhibit the incorporation of ^{3}H-TdR into DNA. Five hours after a dose of acetyl-kidamycin the incorporation moderately inhibited in kidney, lung, spleen, liver and S-180 tumor (Table 3). By 24 hours, the inhibition was marked in spleen, S-180 tumor and liver, however at 48 hours, spleen was the only organ in which thymidine incorporation into DNA was markedly inhibited. At 72 hours, all organs shown in Table 3 had recovered with respect to the uptake of ^{3}H-TdR into DNA.

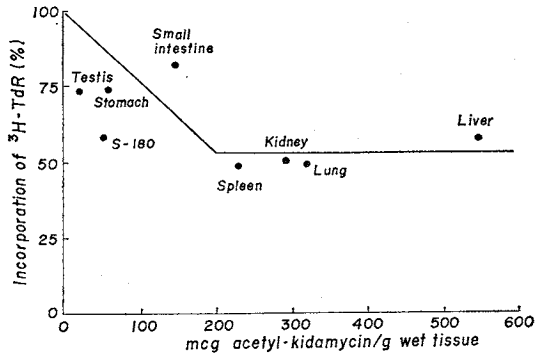
Table 3. Effect of acetyl-kidamycin on the incorporation of ^{3}H-TdR into DNA (Percent of control)
Acetyl-kidamycin, 100 mg/kg, was administered i.v.

Organ	Hour after injection of acetyl-kidamycin			
	5	24	48	72
Lung	50.0	71.4	109.1	151.6
Liver	58.6	45.0	74.8	87.8
Kidney	51.0	84.0	90.6	89.2
Small intestine	82.5	89.8	80.7	81.6
Spleen	48.6	39.4	28.4	90.4
Stomach	73.9	95.0	96.0	136.2
Testis	72.8	80.4	89.9	99.2
Tumor	58.6	44.9	97.1	108.0

Fig. 5 shows the relationship between early acetyl-kidamycin concentrations in organs and TdR

incorporation. From these results, moderate inhibition of TdR incorporation was shown when the concentration of acetyl-kidamycin exceeded approximately 200 mcg/g. This may correlate directly with the spleen toxicity observed in mouse (unpublished data). This toxicity can be clearly seen in that there is a greater accumulation and a slower removal of the agent from the spleen. It is interesting that acetyl-kidamycin in S-180 tumor was small in amount compared to liver and kidney whereas the rate of ^{3}H-TdR incorporation into DNA of tumor was almost the same as that of liver. Studies on the susceptibility of tumor to acetyl-kidamycin are now in progress.

Fig. 5. Values for effects on ^{3}H-TdR incorporation at 5 hours are from Table 3. Concentrations of ^{3}H-acetyl-kidamycin at 5 hours after the injection are taken from Table 1



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