STUDIES ON ANTI-TUMOR AGENTS. V

Studies on the Absorption, Distribution and Excretion of TC-17 in Experimental Animals

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INTRODUCTION

It has been found[1,2] that TC-17, 4,4′-bis(2-*o*-oxyanilino-4-*m*-sulfoanilino-1,3,5-triazyl-6) stilbene disulfonate-2,2′, is a new compound with a marked tumor-inhibiting activity, in spite of its mild cytocidal activity on the tumor cells. And it is one of the remarkable characteristics that the marked tumor-inhibiting activity of TC-17 with pretreatment is maintained for one or two weeks after the last injection.

Therefore, it appears of interest to investigate the absorption, distribution and excretion of TC-17. The present report describes the results of studies on physiological disposition of TC-17.

MATERIALS AND METHODS

Animal Experiments :

Male mice (ICR-JCL, 6-weeks old, 24～27 g body weight) bearing EHRLICH carcinoma, normal male rats (Wistar, 7 weeks old, 200～250 g body weight) and male rabbits (hybrid, 2.0～2.5 kg body weight) bearing BROWN-PEARCE carcinoma were used to investigate the absorption, tissue distribution and excretion of TC-17.

Mice : Twenty mice bearing EHRLICH carcinoma (solid forms) were used and TC-17 was administered in a dose of 75 mg/kg/day intraperitoneally for 7 successive days. Five mice of each group were decapitated at 4, 8, 15 and 22 days after the first injection. Blood was collected and centrifuged. Serum was diluted with 19 volumes of physiological saline. The tissues were removed and homogenized with 19 volumes of KREBS-RINGER phosphate solution, and then 1 ml of the prepared serum solution or homogenates was used for assay of TC-17.

Rats : Three rats of each group receiving the intraperitoneal injection of TC-17, 100 mg/kg were decaptitated at 1, 2, 5, 10, 20 and 30 days after the drug injection. Lymphatic fluid was collected by means of a polyethylene cannula into the thorasic duct of ether anesthetized rats before the decapitation. Urine was collected from animals kept in metabolic cages. Each one ml of 5% serum, lymphatic fluid and urine solution diluted with physiological saline, or tissue homogenates prepared in the same way as mice, was used for assay of TC-17.

Rabbits : TC-17 was administered intravenously in a dose of 50 mg/kg into rabbits bearing BROWN-PEARCE carcinoma. Each group consisted of three rabbits and the animals were killed by air injection at 1, 3, 5, 10 and 20 days after the injection of TC-17. Urine was collected from rabbits kept in metabolic cages. Five milliliters of blood were obtained by cardial puncture before air injection and immediately after this procedure, the tissues were removed. One milliliter of the diluted serum solution or the tissue homogenates was assayed for TC-17. Tissue distribution on 30 th day after the injection of TC-17 was determined in normal rabbits, because the life span of rabbits implanted BROWN-PEARCE carcinoma was about 20 days.

Estimation of TC-17 in Biological Materials :

One milliliter of biological materials (5% serum, lymph and urine solution diluted with physiological saline, and 5% tissue homogenates prepared with KREBS-RINGER phosphate solution) mixed with 4.0 ml of dimethyl-formamide was shaken for 5 min. in a centrifuge tube, centrifuged at 3,000 rpm for 10 min. and the supernatant was transferred to the 10 mm square cuvett, and the fluorescence intensity was determined in Hitachi MPF-2A spectrophotofluorometer at the activation were length of 370 mμ and the fluorescence wave length of 410 mμ. The recovery of known amounts of TC-17 added to serum or lymphatic fluid in 60 μg/ml was 95±10%.

Recoveries were not fully obtained from brain, liver, heart, kidney, spleen, skin and lung (70～90%). Urine gave variable blanks from animal to animal and therefore gave unreliable values in the

excretion studies.

Binding to Serum Protein :

One milliliter of rabbit serum was incubated with each 1.0 ml of 0.2, 0.1 and 0.02% TC-17 physiological saline solution (pH 7.0) at 37°C for 30 min. The original concentrations of TC-17 in the incubated mixtures were 1,000, 500 and 100 μg/ml respectively.

Gel filtration on Sephadex G-25 has been used for the separation of bound TC-17 from a mixture of intact TC-17 and bound TC-17. In this experiment, two columns of Sephadex G-25 were prepared. One was used with pH 7.0 and another with pH 8.6. Gel filtration of each 1.0 ml of the incubated mixtures was carried out to pass the column (column size, 10 mm×250 mm) with 0.1 M phosphate buffer solution of pH 7.0 or 8.6 at flow rates of 30 ml/h. Effluent was separated each 4.0 ml by a fraction collector. Forty milliliters (fraction No. 1-10) of a moving zone containing serum proteins were used to measure the binding capacity of TC-17 towards serum proteins. One milliliter of each fraction of effluent was mixed with 4.0 ml of dimethylformamide, shaken, centrifuged, and the fluorescence of supernatant was determined at the activation wave length of 370 mμ and the fluorescence wave length of 410 mμ. While, total amounts of TC-17 in each 1.0 ml of the incubated mixtures without gel filtration were assayed spectrophotofluorometrically by the above mentioned method, and the binding capacity of the drug towards serum proteins was calculated.

Electrophoresis :

One milliliter of rat serum was incubated with 1.0 ml of 0.6% TC-17 physiological saline solution at the temperature of 37°C for 30 min. *in vitro* and then gel filtration of 1.0 ml of the incubated mixture on Sephadex G-25 (column size, 10 mm×130 mm) was made with physiological saline pH 7.0 at flow rates of 30 ml/hr. for the purpose of removing intact TC-17 in the mixture. Effluent was separated each 2 ml by a fraction collector. Main protein fraction was eluted at 6～10 ml (fraction No. 3～5) and the effluent solution with strong ultraviolet-absorption (fraction No. 4) was selected for electrophoresis.

Electrophoresis was carried out according to KOHN's cellulose acetate method[3] at 25°C for 45 min. until the different protein components have migrated far enough to constitute separate zones. In this experiment, veronal buffer solution (0.06 M, pH 8.6) was used and binding of TC-17 with different serum protein components was determined by ultra-violet light after staining the zones with solutions of Ponceau 3R.

RESULTS

Distribution in Tissues and Serum

Mice : The distribution at various time intervals was studied in mice following 7 successive intraperitoneal doses of 75 mg/kg/day of TC-17, with the results shown in Table 1.

The concentration of TC-17 in various tissues increased with increasing number of injections until 24 hrs. after the last injection and were lower than serum concentration at this period. But at 15 th and 22 nd day (8 and 15 days after the last injection), the tissue levels were higher than that of serum. At 8 th day, the levels in kidney, liver and testis were higher than those in spleen, lung, tumor and skin except that the serum levels were extremely high.

The levels in spleen increased gradually throughout the experimental periods. Testis and lung levels decreased gradually as compared with those in other tissues.

Rats : The distribution in tissues, serum and lymphatic fluid in a single injection on rats is shown in Table 2.

Lymph levels in rats receiving TC-17 intraperito-

Table 1. Levels of TC-17 in mouse tissues following 7 successive intraperitoneal injections of 75 mg/kg/day

Tissues	Concentration (μg/g or ml)			
	4 th day	8 th day	15 th day	22 nd day
Liver	127.9± 22.6	276.4± 69.3	165.7± 51.0	104.6± 22.8
Testis	90.7± 38.1	256.8± 75.2	261.4±116.0	183.6± 84.1
Kidney	154.3± 28.2	315.0±169.4	175.3± 72.3	78.4± 17.1
Spleen	105.2± 36.0	157.8± 45.2	195.0±100.5	213.6±147.5
Lung	90.7± 29.6	135.0± 17.8	133.3± 51.1	115.8± 39.5
Skin	169.6± 57.2	144.5± 38.0	168.8± 75.1	65.5± 23.5
Tumor	70.6± 36.1	176.0± 34.5	44.0± 6.3	60.5± 5.5
Serum	783.1±214.0	800.0±399.0	87.5± 20.6	35.4± 25.4

Five mice were used at each time interval, and the average value ± S.E. was given.

All figures in this table were corrected to 100% recovery value.

Table 2. Levels of TC-17 in rat and rabbit tissues at various time intervals

Tissues	Concentration (μg/g or ml)											
	Rabbit*						Rat**					
	24	72	120	240	480	720(hrs.)	24	48	120	240	480	720(hrs.)
Brain	1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	.00	0.0	0.0	0.0
Liver	162.8	167.8	203.7	235.4	132.5	31.7	148.5	170.5	164.1	132.8	66.1	33.8
Testis	61.2	28.5	28.0	24.2	18.8	9.5	88.2	98.3	148.0	92.5	56.3	32.5
Kidney	295.3	260.4	303.0	293.7	164.4	77.3	202.8	286.2	1758.8	874.5	216.2	188.4
Spleen	141.4	88.2	98.2	79.5	38.2	15.0	179.5	188.0	200.3	216.2	77.3	85.7
Lung	120.6	94.3	96.0	54.6	24.8	10.4	245.3	270.2	300.5	248.6	90.8	87.3
Skin							27.7	31.7	33.3	27.5	15.3	13.1
Adrenals	28.6	12.5	11.3	8.8	12.3	8.0						
Tumor	207.1	139.2	58.0	94.8	30.0	—						
Skeletal muscle	5.0	4.5	1.0	2.0	0.0	0.0						
Bile	50.0	15.5	12.0	10.3	2.5	6.5						
Serum	100.0	28.3	27.8	13.6	7.5	4.0	1350.0	1317.0	858.3	787.5	38.3	58.3
Lymph							881.3	480.0	312.5	75.0	50.0	0.0

* Three rabbits transplanted BROWN-PEARCE carcinoma were used at each time interval. Fifty mg/kg of TC-17 were administered intravenously.

** Three rats were used at each time interval. Hundred mg/kg of TC-17 were administered intraperitoneally. All figures in this table were corrected to 100% recovery value.

neally were lower than that in serum and the rate of disappearance was relatively rapid. Half-life in serum and lymphatic fluid was about 14 and 3 days respectively.

Various tissue levels showed peak values at 5 days except liver and spleen, and disappearance of the drug from these tissues were not observed at a long period for 30 days after TC-17 injection.

In rats, TC-17 was concentrated in kidney and lung, and skin levels were lower than those of all other tissues. The lung levels increased promptly after the drug injection and maintained high values for a long period.

Rabbits : The distribution in tissues and serum of rabbits bearing BROWN-PEARCE carcinoma which received single intraveous injection of TC-17 is shown in Table 2.

The serum half-life was about 4 days. The tissue levels increased rapidly in lung, spleen and tumor, and accumulated gradually in liver and kidney showing peak values at 5 or 10 days after injection. Testis or adrenal levels were lower than those in tumor and spleen, and brain and skeletal muscle levels were negligible. Disappearance of these tissue levels were not observed throughout this experimental period.

Excretion

Urinary excretion both in rats and rabbits was studied at various time intervals. The spectrophotofluorometrical determination of TC-17 in urine was difficult because the interferences caused by some other biological fluorescent materials might give variable blanks from animal to animal. But it was suggested that urinary excretion of unmetabolized TC-17 was negligible, because the mean fluorescence intensity in the urine of the drug treated animals was approximately the same as that of the drug untreated ones. Small amounts of the drug were excreted in the bile of rabbit.

Binding of TC 17 to Serum Protein

The extent to which TC-17 in rabbit serum was bound to the serum proteins was determined by the application of gel filtration method. At serum levels of TC-17 in the range of 100～500 μg/ml, about 50% at pH 7.0 and 20% at pH 8.6 were bound to the serum proteins. And 68～71% at pH 7.0 and 35% at pH 8.6 were bound at serum levels of 1,000 μg/ml.

Electrophoresis of Rat Serum incubated with TC-17

Electrophoresis using KOHN's cellulose acetate method was carried out to prove binding of TC-17 to serum proteins.

Specific electrophoretic pattern of TC-17 bound serum proteins could not be recognized and only the normal pattern of protein components in rat

serum was obtained. TC-17 migrated to the cathode.

DISCUSSION

The physiological disposition studies have shown that TC-17 administered parenterally in a single injection was distributed rapidly in various tissues and was retained in high level for 10 days or more. Repeated injections on tumor-bearing mice showed accumulation of the drug in lung, liver, kidney, testis and tumor. In this case, the retention in testis lung and spleen for a long time and the distribution in skin were noted. In a single injection into rats, strong affinity of the drug was observed in lung and kidney. Peak tissue level in rats received TC-17 intraperitoneally appeared at 5 or 10 days after the injection, and serum half-life was about 14 days.

Urinary excretion of unmetabolized TC-17 was almost none in rats and rabbits, and small amounts were excreted in the bile of rabbit. Although the metabolism of TD-17 is not yet made clear, these findings suggest that significant absorption is still occuring for at least several days after intraperitoneal injection and metabolism or excretion of the drug is very slow.

The results presented in the previous papers[1,2] have suggested that the tumor-inhibiting mechanism of TC-17 might be related to an inhibition of the lodgment of tumor cells which were implanted into host animals. It is therefore postulated that high tissue levels following the pretreatment with TC-17 may play a role in the inhibiting process of tumor-cell lodgment. If TC-17 is related to inhibit the lodgment of tumor cells on various tissues, it would be expected that the drug has an inhibitory activity on metastasis formation in lung and other organs which showed a persistent retention at hight levels, and the study in connection to metastasis inhibition is now under way.

Although it was noted that TC-17 was retained in body for long periods because of its very slow elimination and its high affinity with various tissues, the finding was similar to results presented for stilbamidine[4] which was a diamidine related compound having stilbene structure. Stilbene group on the structural similarity between TC-17 and stilbamidine may be associated with the affinity for tissues.

It has been found that the binding capacity of TC-17 towards rabbit serum proteins is relatively high (about 50% or more at pH 7.0) and the complex formation may be proportional to total amounts of TC-17 in serum. TC-17 bound serum proteins did not show a specific electrophoretic pattern. This phenomenon has originated in the dissociation of the TC-17-serum protein complex caused in electrophoretic process, probably because of high negative charge of TC-17. Therefore, it is concluded that the binding between TC-17 and serum proteins is reversible. A further study on the metabolic transformation of TC-17 is of interest in an attempt to gain some informations on its mechanism of action.

SUMMARY

The physiological disposition of TC-17 in tumor-bearing mice, rabbits and normal rats was investigated.

When TC-17 was injected parenterally, various tissue levels increased rapidly and were retained in high levels in the body for 10 days or more. Peak values in various tissue levels in rats receiving TC-17 intraperitoneally in a single does of 100 mg/kg were found at 5 or 10 days after the injection and the drug was concentrated temporarily in lung and kidney. Serum half-life was about 14 days.

Serum half-life in tumor-bearing rabbits receiving 50 mg/kg of the drug intravenously was about 4 days.

Urinary excretion of the drug was almost none in rats and rabbits, and its biliary excretion was a little.

Repeated intraperitoneal injections on tumor-bearing mice showed accumulation of the drug in liver, kidney and testis.

At rabbit serum levels of 100 to 500 μg/ml, about 50% of TC-17 bound with the serum proteins, and TC-17 serum protein complex occurred the ionic dissociation in electrophoreetic process.

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