RELATIVE TISSUE DISTRIBUTION OF RADIOACTIVITY IN RATS WITH ENDOCRINE "AUTONOMOUS" BREAST CARCINOMAS AFTER ^{3}H-, ^{99m}Tc-, AND ^{64}Cu-BLEOMYCIN

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The localization of ^{64}Cu-, ^{3}H-, and ^{99m}Tc-labeled bleomycin was studied in tissues of Fisher rats bearing an endocrine autonomous breast tumor. The ^{64}Cu-bleomycin showed a greater uptake than ^{3}H- and ^{99m}Tc-bleomycin in the breast tumor at all time intervals studied, from 2 to 72 hr after the tracer. This same differential uptake was shared by the liver and kidney.

Since bleomycin was reported as an effective antitumor agent by Umezawa (*1*), clinical trials using this agent on various neoplasms have been carried out by several institutions (*2–4*). However, the chemical structure and the mechanism of antitumor activity of bleomycin have not been clarified (*5*).

Evidence of binding of bleomycin to DNA of growing bacteria and mammalian cells (*6*) and relatively high concentration of bleomycin in tumor cells (*7*) strongly suggested that the compound might be an effective tumor-scanning agent when labeled with a gamma-emitting nuclide. The use of radioactive cobalt-labeled bleomycin and ^{111}In-labeled bleomycin for tumor scanning with promising results have been reported (*8,9*). Bleomycin, labeled with ^{99m}Tc, has been used as a tumor-scanning agent in Japan (*10*).

Renault, et al (*11*) have presented data showing that the antibiotic and antitumor agent bleomycin is a chelating agent with a binding capacity of 26 μg of Cu^{2+}/mg of bleomycin, 25 μg of Zn^{2+}/mg, 17 μg of Co^{2+}/mg, 15 μg of Ni^{2+}/mg, 9.5 μg of Hg^{2+}/mg, and all other cations <1.0 μg/mg.

Since bleomycin is complexed with copper in nature (*12*), the objective of this study was to label bleomycin with ^{64}Cu and to compare ^{64}Cu-bleomycin uptake in breast tumors with bleomycin labeled with ^{3}H or ^{99m}Tc.

We wish to report that ^{64}Cu-bleomycin shows a greater uptake in the Fisher rat endocrine "autonomous" breast tumor than ^{3}H- and ^{99m}Tc-labeled bleomycin at all intervals studied from 2 to 72 hr after the tracer was administered. This same differential uptake was shared by the liver and kidney.

METHOD

Radiopharmaceuticals. Bleomycin was obtained from Bristol Laboratories. Bleomycin was labeled with ^{3}H by New England Nuclear. Production of the ^{64}Cu [$Cu(No_3)_2$] and labeling of bleomycin with ^{64}Cu was done by the Nuclear Pharmacy and the Phoenix Memorial Laboratory, University of Michigan, as described. The specific activities of the labeled bleomycins used were: ^{3}H, 1.88 mCi/mg; ^{64}Cu, 2–7 mCi/mg; and ^{99m}Tc, 1–2 mCi/mg.

Rats. A DMBA-induced (*13*) endocrine "autonomous" breast tumor, 13762 T.P. (Mason Research Institute, Worcester, Mass.) was transplanted subcutaneously into each flank of 46 Fisher rats weighing 150 gm each (*14*). Three to four weeks after the transplantation, radionuclide-labeled bleomycin or free ^{64}Cu was administered to the rats intravenously through the tail vein at a dose of 20–50 μCi of ^{3}H-bleomycin, 2 mCi of ^{99m}Tc-bleomycin, or 1 mCi of ^{64}Cu-bleomycin per rat. As shown in Table 1, three rats were sacrificed at various time intervals from 2 to 72 hr after the dose was administered. The rats were deeply anesthetized with chloroform. While the rats were still alive, the chest was opened and blood was removed from the cardiac chamber into a 3-ml heparinized syringe. The animals were then sacrificed by cutting out the heart with scissors. Samples of tissue were dissected free, cleared of fat and connective tissues, and weighed. Representative 30–50-mg samples of tissue were obtained.

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TABLE 1. TISSUE DISTRIBUTION OF ^{3}H-, ^{99m}Tc-, OR ^{64}Cu-BLEOMYCIN AT VARIOUS TIME INTERVALS IN 13762 T.P. BREAST TUMOR RATS (% DOSE/GM TISSUE ± S.E. OF THREE RATS)

Time (hr)	2			5			24			48		72	
Tissue	^{3}H-	^{99m}Tc-	^{64}Cu-	^{3}H-	^{99m}Tc-	^{64}Cu-	^{3}H-	^{99m}Tc-	^{64}Cu-	^{3}H-	^{64}Cu-	^{3}H-	^{64}Cu-
Tumor	0.146	—	0.372	0.136	—	0.256	0.082	0.033	0.796	0.060	0.473	0.047	0.508
	±0.013	—	±0.052	±0.007	—	±0.030	±0.001	±0.0035	±0.048	±0.004	±0.042	±0.001	±0.076
Blood	0.125	0.062	0.323	0.126	0.056	0.614	0.100	0.046	0.525	0.077	0.336	0.088	0.341
	±0.036	±0.019	±0.073	±0.012	±0.010	±0.017	±0.002	±0.000	±0.063	±0.012	±0.034	±0.036	±0.053
Liver	0.312	0.516	1.500	0.330	0.624	1.560	0.186	0.076	2.120	0.084	1.340	0.064	0.479
	±0.038	±0.095	±0.268	±0.018	±0.088	±0.150	±0.026	±0.011	±0.365	±0.006	±0.090	±0.009	±0.069
Kidney	0.462	1.160	3.740	0.841	1.200	4.410	0.663	0.874	2.930	0.377	1.920	0.347	1.320
Cortex	±0.190	±0.039	±0.220	±0.172	±0.340	±0.866	±0.023	±0.235	±0.702	±0.038	±0.214	±0.023	±0.185
Kidney	0.602	0.274	0.450	0.319	0.259	0.270	0.223	0.874	0.674	0.089	0.326	0.069	0.311
Medulla	±0.164	±0.065	±0.090	±0.014	±0.194	±0.088	±0.071	±0.235	±0.133	±0.011	±0.057	±0.004	±0.058
Lung	0.214	1.240	0.314	0.176	0.935	0.330	0.088	0.031	0.336	0.048	0.285	0.036	0.303
	±0.065	±0.191	±0.064	±0.022	±0.500	±0.033	±0.003	±0.004	±0.019	±0.005	±0.017	±0.002	±0.037
Intestine	0.202	0.107	0.485	0.149	0.027	0.511	0.092	0.022	0.518	0.039	0.449	0.023	0.329
	±0.068	±0.073	±0.121	±0.014	±0.002	±0.036	±0.006	±0.005	±0.037	±0.003	±0.023	±0.002	±0.061
Fat	0.105	0.017	0.364	0.101	0.008	0.439	0.009	0.007	0.394	0.042	0.288	0.036	0.284
	±0.012	±0.002	±0.087	±0.008	±0.001	±0.077	±0.001	±0.002	±0.029	±0.003	±0.015	±0.008	±0.069
Skeletal	0.038	0.013	0.065	0.080	0.072	0.063	0.053	0.005	0.151	0.016	0.100	0.014	0.097
Muscle	±0.004	±0.002	±0.011	±0.005	±0.032	±0.005	±0.004	±0.001	±0.023	±0.001	±0.004	±0.001	±0.012
Heart	0.140	0.041	0.164	0.135	0.021	0.251	0.069	0.013	0.374	0.056	0.358	0.035	0.352
	±0.016	±0.006	±0.036	±0.026	±0.004	±0.013	±0.003	±0.001	±0.024	±0.002	±0.023	±0.003	±0.023
Adrenal	0.090	0.032	0.255	0.135	0.026	0.361	0.096	0.022	0.362	0.065	0.355	0.046	0.372
	±0.018	±0.005	±0.056	±0.021	±0.005	±0.012	±0.007	±0.004	±0.014	±0.003	±0.025	±0.006	±0.033
Pancreas	0.146	0.040	0.270	0.135	0.014	0.382	0.089	0.013	0.352	0.044	0.278	0.029	0.269
	±0.015	±0.019	±0.064	±0.020	±0.002	±0.049	±0.006	±0.001	±0.082	±0.007	±0.022	±0.002	±0.033
Ovary	0.121	—	0.300	0.146	—	0.409	0.080	0.024	0.365	0.052	0.374	0.028	0.266
	±0.019	—	±0.053	±0.006	—	±0.046	±0.005	±0.000	±0.010	±0.002	±0.033	±0.005	±0.142
Thyroid	0.115	0.024	0.159	0.149	0.061	0.214	0.078	0.057	0.251	0.042	0.193	0.072	—
	±0.018	±0.006	±0.029	±0.035	±0.017	±0.002	±0.001	±0.016	±0.016	±0.001	±0.026	±0.009	—
Skin	0.044	0.011	0.045	—	—	—	0.055	0.020	0.055	—	—	—	—
	±0.004	±0.000	±0.007	—	—	—	±0.009	±0.006	±0.011	—	—	—	—
Spleen	0.143	0.910	0.302	0.166	0.682	0.547	0.204	0.067	0.423	0.074	0.414	0.048	0.372
	±0.013	±0.057	±0.084	±0.003	±0.229	±0.026	±0.043	±0.012	±0.034	±0.011	±0.027	±0.008	±0.054

Six rats were imaged 24 or 48 hr or both after administration of ^{99m}Tc- and ^{64}Cu-labeled bleomycin with a Pho/Gamma HP camera (Searle Radiographics) using a pinhole collimator.

Radioactivity measurement. For the measurement of ^{3}H activity, all specimens were placed in liquid scintillation counting vials, digested overnight in 0.3 ml of 10% NaOH, dissolved by warming to 70°C for 20–30 sec, and after cooling, three drops of 30% H_2O_2 were added. Then 10 ml of PCS solubilizer (liquid scintillation mixture, Amersham/Searle, Chicago, Ill.) was added. After overnight dark adaption and cooling, samples were counted for 10 min each in a liquid scintillation counter (Searle Radiographics Unilux IIA). Quenching was corrected using the channels ratio method. Radioactivity for ^{99m}Tc or ^{64}Cu was measured with an autogamma well counter (Searle Radiographics). The data were expressed as percent administered dose per gram of fresh tissue.

Bleomycin labeling procedure I. Copper $(NO_3)_2 \cdot {}^{3}H_2O$ was irradiated in a fused silica tube for 20 hr at a flux of 2.3×10^{13} n/cm²-sec. The radioactivity produced was approximately 3.3 mCi/mg of Cu $(NO_3)_2 \cdot {}^{3}H_2O$ or 11 mCi/mg Cu^{2+}.

The sample vial was placed in a sterile, clean polyethylene breaking tube and broken. The contents of the vial were dissolved in 2 ml of 0.8% NH_4Cl solution prepared with distilled pyrogen-free sterile water. Gentle heating was used to aid in dissolution.

A quantity of bleomycin, such that the bleomycin/$Cu(NO_3)_2 \cdot {}^{3}H_2O$ ratio was greater than 3:1, was measured into 2 ml of copper solution, transferred to a 10-ml vial, and heated for 1 hr at 70°C in a water bath.

Additional 0.8% NH_4Cl was added to make a total volume of 10 ml. The final solution was filtered through a 0.22-micron filter. Specific activity obtained through this procedure ranged from 2 to 5 mCi ^{64}Cu/mg bleomycin.

Bleomycin labeling procedure II. Copper Phthalocyanine (Eastman) was purified by recrystallization from solution in concentrated sulfuric acid and carefully washed free of residual sulfuric acid before drying. Purified copper phthalocyanine (1,200 mg) was sealed in a fused silica tube and irradiated for 2 hr at a flux of 2.3×10^{13} n/cm²-sec.

Two hours after the sample was removed from the reactor, the vial was broken in a polyethylene breaking tube as in Procedure I. The contents of the vial were dissolved in 10 ml of concentrated H_2SO_4 and the solution mixed using a magnetic stirrer.

Distilled water was added dropwise until a total volume of 120 ml was obtained. This solution was chilled and stirred for 1 hr at 10°C using a "cold plate." The sample solution was filtered through a 0.22-micron filter and diluted to 500 ml with distilled water.

The diluted filtrate was then passed through a Dowex-50 ion-exchange column to collect the ionic

copper on the column followed by a rinse of 20 ml with distilled water. The ionic ^{64}Cu was eluted from the column using 20 ml of 7 *N* HNO_3. The eluant was evaporated to dryness three times, each time adding a few milliliters of distilled water and redrying to remove HNO_3.

Two milliliters of 0.8% ammonium chloride was added to the dry residue and Procedure I followed from Step 3 with the exception that the ratio of bleomycin to ^{64}Cu was decreased because of the increased specific activity of ^{64}Cu (8 mCi ^{64}Cu/mg bleomycin).

Technetium-labeling procedure. Under a nitrogen atmosphere, 1 ml of 0.02 *N* HCl containing 0.1 mg of freshly prepared $SnCl_2$ was added to 5 mg of bleomycin. The solution was agitated for 4 min and then 1 ml sodium ^{99m}Tc-pertechnetate (specific activity 40 mCi/ml) was added. This final solution was filtered through a 0.22-micron filter.

RESULTS

Copper-bleomycin labeling. The yield of copper-bleomycin labeling assayed by means of thin-layer chromatography was 100% in every trial. In Procedure I of copper labeling, copper nitrate was chosen so that no other radioactive nuclides should appear in the final product such as ^{35}S, ^{32}P, ^{38}Cl, etc. Pure copper metal was not used to avoid difficulty in target dissolution.

Copper labeling Procedure II makes use of the Szilard–Chalmers method of increasing specific activity of the copper to provide a higher specific-activity-labeled bleomycin. Longer irradiation time tended to break down the phthalocyanine reducing the efficiency of producing high specific-activity copper. The shorter irradiation and larger quantity of phytalocyanine improved yield.

Scanning. The tumor masses were well visualized in scintiphoto scans in all breast-tumor-bearing rats. Examples of the tumor scan in the rats are shown in Figs. 1 and 2.

A growing tumor that was not necrotic showed more prominent uptake of the radionuclide (Fig. 1). An area of necrosis in one tumor was clearly visible in the scintiphoto (Fig. 2). No significant difference in the quality of tumor scans was noticed between pictures taken at 24 hr or 48 hr after the dose was administered.

Tissue distribution. Table 1 presents the relative tissue distribution of radioactivity from ^{64}Cu-, ^{99m}Tc-, and ^{3}H-bleomycin. The uptake in percent dose per gram in all tissues at all time intervals was greater (except for kidney medulla at 5 hr) after ^{64}Cu-bleomycin than after ^{3}H- or ^{99m}Tc-bleomycin. The amount of radioactivity in the tumor tissues from

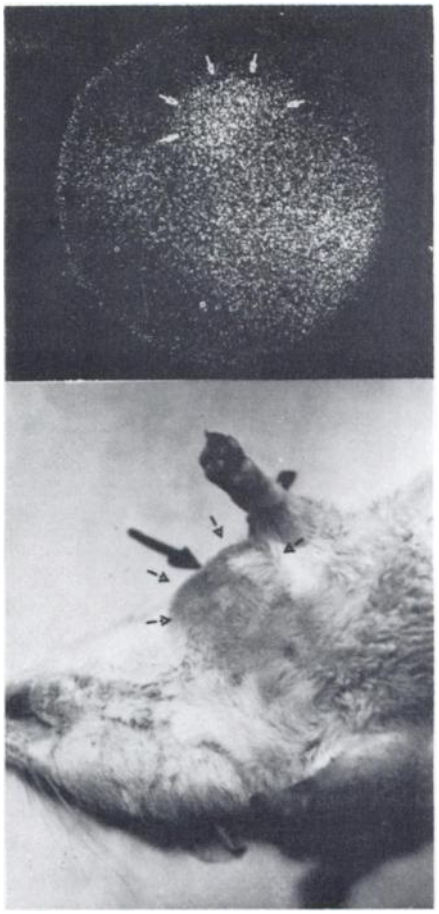

FIG. 1. Scintiphoto obtained 48 hr after i.v. injection of 1 mCi ^{64}Cu-bleomycin in small, growing breast tumor in rat.

^{64}Cu-bleomycin in percent dose per gram was well retained in the tissues through 72 hr whereas the concentrations of ^{3}H-bleomycin decreased with time. Thus, the uptake of ^{64}Cu-bleomycin in breast tumor was ten times that of ^{3}H-bleomycin at 72 hr. At 24 hr ^{64}Cu-bleomycin has a tumor tissue concentration 24 times that of ^{99m}Tc-bleomycin.

Copper-64 radioactivity in breast tumor tissue exceeded that in all tissue except liver and kidney at all time intervals. Tritium tumor radioactivity was less than all tissues except blood, fat, and muscle at 2, 5, and 24 hr but exceeded that of the lungs, intestine, fat, and muscle at 48 and 72 hr. Technetium-99m tissue radioactivity showed the highest concentration in the kidney cortex at all time intervals except in the lungs at 2 hr. The breast tumor content of ^{99m}Tc radioactivity at 24 hr exceeded that of adrenal, pancreas, muscle, fat, intestines, and lungs.

DISCUSSION

Umezawa (*15*) has emphasized that the bleomycins are a group of antibiotics produced by Strep-

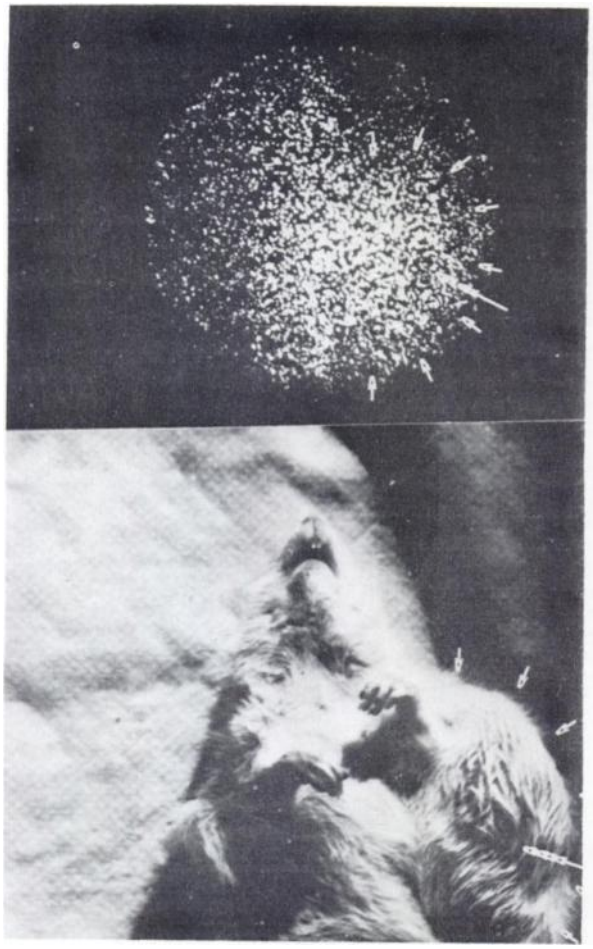

FIG. 2. Scintiphoto obtained 48 hr after i.v. injection of 1 mCi ^{64}Cu-bleomycin in medium-sized breast tumor with smaller central area showing necrosis and lack of uptake in area of necrosis.

tomyces verticillus and their therapeutic effect against the squamous cell carcinoma has been proved by clinical studies. He has isolated in the pure state eight products, viz, bleomycin A_1, A_2, demethyl-A_2, A_2-a, A_2-b, B_2, A_5, and B_4. Structures of the various bleomycins have been elucidated and it has been observed that they differ from each other in their amine moiety. Forty-two artificial bleomycins were synthesized and their biologic activities studied. Both the copper-chelated and the copper-free bleomycins are equally active. Bristol Laboratories removes copper routinely to produce the "copper-free" bleomycin.

Umezawa found that bleomycin A_2 was taken up by a 20-methyl-cholanthrene-induced carcinoma four times more than in a similarly produced sarcoma. Sixty percent of the bleomycin A_2 remained in the active form in the carcinoma 1 hr after the injection but none was in the active form at 1 hr in the sarcoma. He attributed this difference to a lack of an enzyme in the carcinoma that inactivated the bleomycin. He found this enzyme to be active in liver, kidney, and spleen but weak in skin and lungs. These studies, however, were based on antibacterial activity.

When Umezawa, et al (*16*) studied the tissue distribution of ^{3}H-bleomycin in mouse tissue, they used a bleomycin mixture containing A_2 as the main component and B_2 as the second component. The results indicated a high concentration in skin and peritoneum. The concentrations determined by radioactivity were higher than those determined by the antibacterial activity suggesting that tissue contains the same substance that reduces the bacterial activity against B subtilis or inactivates the bleomycin.

The nonspecific distribution of ^{3}H-bleomycin in breast-tumor-bearing rat, the unsuccessful breast-tumor scanning with ^{99m}Tc-bleomycin, and the prominent visualization of the breast tumor with ^{64}Cu-bleomycin we observed in the present study indicate that bleomycin labeled with a different radionuclide is different in its chemical property and/or metabolized in a different way in the tissues. The ^{64}Cu, which we used in the present study to label the bleomycin, is not an optimum nuclide for a scanning agent. Because of the short half-life of ^{64}Cu, a longer half-life radionuclide of copper would be better suited to perform the 3–5 day delayed tumor imaging that results in a better target-to-nontarget ratio. Current work on ^{67}Cu-bleomycin and also on the feasibility of the use of bleomycin labeled with a suitable nuclide to achieve both chemotherapy and radiation therapy effects in tumor treatment are in progress in our laboratory.

The chemical nature of our labeled bleomycins was not determined. We therefore cannot speculate on the reasons for the difference in behavior of the radioactivity uptake from ^{64}Cu, ^{3}H, and ^{99m}Tc in breast tumors and in other tissues of the Fisher rat. The data suggest, however, that either the ^{3}H and ^{99m}Tc labels are removed in vivo more readily than the ^{64}Cu label and excreted, or that the ^{64}Cu is deposited in breast, liver, and kidney selectively.

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