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**POTENTIAL FOR TUMOR THERAPY WITH TRITIATED
TETRACYCLINE. SUMMARY EVALUATION.**

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October 26, 1976

Prepared for U.S. Energy Research & Development
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Foreword

The major portion of this paper was presented verbatim for publication in Cancer Research under the title, "Localization and Therapeutic Potential of Tritiated Tetracycline in Rodent Tumors," by Richard C. Davis, Paula Wood, and Mortimer L. Mendelsohn. This report contains additional material describing the synthesis of 7-tritiotetracycline and the localization of nonradioactive tetracycline in human malignancies.

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POTENTIAL FOR TUMOR THERAPY WITH TRITIATED TETRACYCLINE. SUMMARY EVALUATION.

Abstract

Reports of tetracycline accumulation in human and animal tumors have led a number of investigators to postulate that this drug, if radio-labeled, might have potential as a therapeutic or diagnostic agent. This paper describes four aspects of our attempts to investigate this potential:

- 7-Tritiotetracycline (TTC), 9 Ci/mole, was synthesized, purified, and characterized.
- Tumor therapy experiments were carried out with the high specific activity TTC in two transplanted tumor lines.
- Localization and concentration of TTC was measured in rodents with autochthonous mammary tumors and a large variety of transplanted tumors and correlated with the local rate of cellular proliferation as measured by ^{14}C -thymidine incorporation.
- Tetracycline (TC) concentration was measured by a quantitative extraction procedure in six human malignancies and compared with concentrations found in normal tissues of the same patients after injection of 2 g of non-radioactive tetracycline.

The therapeutic studies demonstrated that while a significant reduction in the growth rates of transplanted tumors could be obtained by the administration of heavy doses of TTC relative to uninjected controls, similar reductions were observed in the growth rates of tumors in animals receiving unlabeled TC. In the localization studies in rodents, the concentrations of TTC in normal tissues and tumors were compared and were correlated with the corresponding concentrations of ^{14}C -thymidine, a measure of proliferative activity. Tumor binding of TTC significantly in excess of normal tissue was found only in those areas of tumor that failed to bind ^{14}C -thymidine. Rapidly proliferating areas of the tumors, as measured by ^{14}C -thymidine incorporation, bound tetracycline in concentrations similar to muscle and significantly lower than kidney. The lack of localization in viable tumor and the absence of a tritium effect in treated tumors argues strongly against the therapeutic value of TTC at least to the extent that the results on this broad spectrum of tumors can be generalized. In human malignancies, viable tumor

tissue demonstrated similarly low relative retention of tetracycline. Necrotic areas of human tumors

retained up to 15 times as much TC as viable areas of tumor or normal tissues.

Introduction

Tetracycline (TC) fluorescence in human malignancy was first noted in 1957 by Rall. Since that time a number of researchers have explored the localization of TC in malignancies in efforts to determine its potential as either an aid to malignancy diagnosis^{1,2,3} or as a vehicle for therapeutic agents.⁴ Therapeutic usefulness would require that tetracycline specifically bind to malignant tumors in high concentrations and for long periods of time compared to normal tissues.

Most efforts to study the localization of tetracycline have used the natural fluorescence of the material. But the fluorescence of tetracycline is sensitive to pH, is dependent on the presence in solution of bivalent metal ions, is second order in tetracycline concentration, and is subject to various organic and inorganic inhibitors, thus making it unsuitable for the quantitation of *in situ* TC concentrations. Studies using nonfluorescence methods to determine TC concentrations in human or animal malignancies are rare and yet a

quantitative estimate of the relative affinity for tetracycline of normal and malignant tissues is essential to the assessment of tetracycline's potential in therapeutic schemes.

Following administration of therapeutic doses of tetracycline, Phillips *et al.*⁵ measured TC concentrations in a variety of human tumors using a *semiquantitative extraction* procedure. Wood *et al.*⁶ compared the tumor concentration of TC found by Phillips with the maximum concentrations that would have been expected in the blood of these patients for the amount of material administered and the route of administration. It was concluded that the TC concentration measured in the malignancies at various times after administration constituted 3 to 2500 times the maximum concentration in the blood at any time after administration. McLeay *et al.*⁷ found levels in three osteogenic sarcomas that were about 1.5 times the calculated peak blood levels.

At least three groups have carried out preliminary radiotherapy studies in rodent tumors using the

carrying and binding properties of tetracycline. McLeay *et al.*⁷ used ¹³¹I labeled tetracycline, Wood *et al.*⁶ at this laboratory used tritiated tetracycline, and Gilchrist and Shaw⁸ used boronated tetracycline in combination with neutron capture therapy. McLeay and Gilchrist noted significant extensions of the lifetime of the treated animals over controls. Wood, using spontaneous mammary tumors in Sprague Dawley rats, noted remission in smaller tumors and slowing of growth in some moderate and large tumors.

This paper outlines the further investigation of the therapeutic

potential of tritiated tetracycline. In an effort to obtain better controls and to increase animal numbers, therapy was carried out in two transplanted tumor lines. Also, tetracycline levels in a variety of rodent malignancies and tissues were measured following a single injection of TTC and correlated with the local rate of cellular proliferation as monitored by binding of ¹⁴C-thymidine. Finally, the relevance of these animal data to human malignancy was investigated by the quantitative extraction of TC from the tumors and normal tissues of six human patients.

Materials and Methods

SYNTHESIS OF TRITIATED TETRACYCLINE (TTC).

7-Chlorotetracycline hydrochloride (CTC•HCl, Lederle Lab^{*} primary standard) was dissolved in methyl cellosolve (5 ml/g) with 10% Pd on powdered charcoal (Matheson Coleman and Bell) included as the hydrogenation catalyst. Triethylamine (TEA) was added in the molar ratio 2.1:1

CTC•HCl to remove the HCl and the Cl released during the reaction. The mixture was first slowly frozen under vacuum to degas the solution. A temperature of 35 to 45°C and vigorous agitation was maintained while tritium (T₂) gas was introduced to the reaction flask to total pressures of ≤ 1 atmosphere and the reaction was allowed to progress down to ≤ 0.1 atmosphere pressure. This process was repeated until the reaction was essentially completed. A large excess of deuterium was then added to finish the reaction and the excess reactant gas was then trapped

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on a molecular sieve. The catalyst was removed by filtration and most (~90%) of the solvent evaporated under vacuum.

Labile tritium on the tetracycline molecule (e.g. hydroxyl and amine hydrogens) was removed by dissolving the residue in about 50 volumes of water adjusted to pH 1.7 with HCl and stirring at 30 to 40°C for 12 to 16 h. The solution was then filtered and crude TTC was precipitated as the tetracycline-urea complex⁹ after the solution was brought to pH 4.5 to 5.5.

Purification was carried out by a six-stage fractional crystallization of tetracycline alternately as the hydrochloride and as the tetracycline-urea adduct. Crude TTC-urea was initially dissolved in minimal (3 to 5 cc/g) 2 n-butanol:1 methyl cellosolve. The solution was brought to pH 1.7 with concentrated HCl and maintained there while seeding the liquor with crystalline $\text{TTC} \cdot \text{HCl}$ (Lederle Lab primary standard) and cycling the temperature between 5 and 50°C to promote crystallization. Crystallization, once begun, was allowed to proceed for up to an hour before filtration through a fine frit; the filter cake was washed with drops of butanol-cellosolve. The damp (air suction-dried) filter cake was weighed and dissolved in about 5 cc/g urea-saturated water from which the TTC-

urea adduct could usually be crystallized rapidly. This entire procedure was repeated twice more for a total of six crystallizations and the final filter cake washed with drops of 2-propanol. A 0.01 to 0.05 M aqueous solution of the $\text{TTC} \cdot \text{HCl}$ with a 10-fold molar excess of cysteamine hydrochloride added as a radio-protective agent¹⁰ was brought to a pH of 1.7 with HCl, quickly frozen by spraying obliquely against a liquid nitrogen-cooled metal surface with a syringe, and then stored in liquid nitrogen to minimize auto-radiochemical damage.

Considerable care was taken during the entire procedure to reduce the time TTC was in concentrated solution and crystalline form to minimize autoradiolytic damage and consequent loss of radiochemical purity, particularly in the last recrystallization cycle. Overall yield suffered accordingly and was typically 40%.

CHARACTERIZATION OF THE TTC.

Paper chromatography of the $\text{TTC} \cdot \text{HCl}$ was carried out by the methods of Kelly and Buyske¹¹ with EDTA-impregnated paper. With the upper phase of 4 n-butanol:1 ammonium hydroxide:5 water mixture as solvent, the purified synthesis products were chromatographed in parallel with

tetracycline hydrochloride (TC•HCl). The chromatograms were run for 20 to 24 h, dried, developed by exposure to ammonia vapor, and examined under ultraviolet (Hg) light. The products of the synthesis had the same R_f as the TC•HCl. The strip of the chromatogram containing the radioactive synthesis products (350 mm from origin to solvent front) was cut into 62 equal pieces (1/4-in widths) and the tritium level in each piece determined by oxidation and liquid scintillation counting. A graph of the results is

shown in Fig. 1. The TTC•HCl peak constituted more than 98% of the activity in the products, setting a lower bound on the TTC radiochemical purity, as partial decomposition during the adverse conditions for TC stability¹² during chromatography was not accounted for.

Vacuum dehydration of the stored material and liquid scintillation assay of the released vapors indicated that 0.3% of the activity was volatile (probably almost entirely as HTO). It would be expected that this

Fig. 1. Radiochromatogram of TTC. Note logarithmic scale. Total radioactive impurities constitute $\approx 2\%$ of activity with principal contaminants probably being anhydro- and epianhydrotetracyclins. Detector background levels represent activity detected in blanks processed between samples.

impurity resulted more from sharply accelerated autoradiochemical damage accumulated during the concentrated phases of the purification at room temperature, than product decomposition under storage conditions.

The 7-tritiotetracycline product had a specific activity of 9 Ci/mmole, about 30% of that corresponding to 1 triton per tetracycline molecule. Higher specific activities were observed in procedures that omitted or abbreviated the acid wash step immediately after synthesis, suggesting that some initially bound tritium may be washed off the 7-carbon position by these relatively vigorous conditions.

THERAPY OF TRANSPLANTED TUMOR SYSTEMS.

Therapeutic trials with TTC were carried out in the 13762NF mammary carcinoma implanted subcutaneously in 30-day-old Fisher 344 female rats and in the 512 Morris hepatoma similarly implanted in 3-month-old Buffalo female rats. The drug was chewed immediately before use, neutralized to $\sim$ pH 7.0, mixed with an isotonic phosphate buffer designed to have the same potassium concentration as rat blood ($\sim 4 \times 10^{-3}M$),¹³ and administered via the jugular vein at a dose of 58 mg (1.1 Ci) per kg bodyweight per injection while the animal was held under Metofane anesthesia.

Animals that received large daily doses of either TC or TTC sometimes showed severe respiratory difficulties during the injection, requiring that the injection rate be slowed or stopped until a normal breathing pattern returned. The injections were given either daily or every third day for the duration of the experiment. Some controls were administered 3H_2O so that the animals received the same average whole body radiation dose over the period between injections. This involved injecting an initial dose of 3H_2O sufficient to give the required integrated dose between the first and second injections and then restoring the material excreted between injections at subsequent injections. TC-HCl with a biological half-life in rats of $\sim$ 100 min was assumed to be completely excreted at 24 h after injection, whereas tritiated water has a biological half-life of about 85 h, so that 82% of the material remains 24 h after injection.

The tumor dimensions were monitored by caliper measurements.¹⁴ Tumor diameter was estimated as the cube root of the product of length, width, and height ($\sqrt[3]{lwh}$) and the mean tumor diameter for each group of animals was plotted against time. Such plots were approximately linear, allowing growth rates to be readily

compared. Two-to four-week-old transplanted mammary tumors tended to rupture. Tumors that changed dimensions abruptly because of rupture and loss of fluid were excluded from subsequent averages of tumor dimensions.

DOUBLE LABEL EXPERIMENTS

To determine how areas of high TTC uptake related to cellular proliferation, double-label experiments employing ^{14}C -thymidine in conjunction with TTC were carried out. Tumor-bearing animals were administered TTC (11 mCi/kg body wt) and ^{14}C -thymidine (New England Nuclear, methyl- ^{14}C , 40 to 60 mCi/mmol, 0.1 mCi/kg body wt) in a volume of 10 ml/kg body wt. Rats were injected via the jugular vein and mice via the tail vein while under Metofane anesthesia. Animals were sacrificed 24 h after injection and samples of abdominal muscle, kidney, and liver were removed together with the complete tumor. The tumor was dissected and photographed, and samples of 5 to 50 mg from various regions were removed and weighed. Triplicate 25 mg samples of the normal tissues were prepared. The samples were oxidized in a ^3H - ^{14}C sample oxidizer (Packard Model 305) and counted. The $^3\text{H}_2\text{O}$ was counted in 10 ml Instagel (Packard Industries), while the $^{14}\text{CO}_2$ was

reacted with 3 ml ethanolamine and mixed with 9 ml methanol and 7 ml scintillant (15 g of 91% PPO, 9% bis-MSB mixture per litre of scintillation grade toluene). Sample quenching was monitored and counts were corrected for background counting efficiency and recovery of the isotopes from the oxidizer. A high concentration of ^{14}C was taken to indicate a high fraction of cells synthesizing DNA as would occur in areas of rapid cellular proliferation.

ANALYSIS OF TC CONCENTRATION IN HUMAN TISSUES:

Human subjects were given a total of 2 g of nonradioactive $\text{TC}\cdot\text{HCl}$ (~ 30 mg/kg) in 4 injections over a period of 48 h ending 72 h prior to surgical removal of malignancy. Surgical specimens of malignant and normal tissues were analyzed for TC content with a modification of the method of Kohn.¹⁵ Samples were finely minced and 500 mg aliquots were placed in test tubes with 9.5 ml 0.1 N HCl at 5°C . This mixture was sonicated 6 times for 5 s with 15-s intervals between, and then centrifuged for 1 h at 4800 g and 5°C . One ml of a solution of 1.5 N trichloroacetic acid and 0.004 M KH_2PO_4 was added to 4 ml of supernatant and the mixture briefly centrifuged. To 4 ml of the resulting supernatant was added 2 ml

of a solution of 0.06 M $\text{Pb}(\text{NO}_3)_2$ and 2.0 M sodium acetate and the resulting mixture allowed to sit for 30 min. After brief centrifugation 1 ml 0.32M KIO_3 was mixed with 4 ml of supernatant and allowed to stand for 1 h before centrifuging again. Four ml of supernatant were then added to a tube containing 3 ml 0.9M Sodium Barbitol, 3ml 0.1M CaCl_2 , and 4 ml redistilled ethyl acetate. The tube was capped and shaken vigorously for several minutes. The fluorescence of the organic phase was measured in a Farrand Spectrofluorometer (Cat # 104244) with the excitation set at 395 nm and emission measured at 520 nm. Kohn calibrated this procedure against microbiological assay and found it to be quite linear in the region of 3 to 19 μM concentrations. In our hands, rats given a single injection (~ 50 mg/kg) of $\text{TC}\cdot\text{HCl}$ with a tracer level of $\text{TTC}\cdot\text{HCl}$ were found by the extraction procedure to have TC concentrations that correlated very well with the radioactivity levels as

measured by oxidation of sample tissues and liquid scintillation counting (Fig. 2).

Fig. 2. Correlation between [TC] as measured by extraction of fluorescent complexes and as determined by tracer technique with TTC and oxidation - scintillation counting. Concentrations vary according to tissue type and time between injection and sample collection.

Results

THERAPY EXPERIMENTS.

Four animals carrying the Morris hepatoma were given TTC injections every third day beginning thirteen days after implantation when the mean tumor diameter was 12.4 mm (tumor

mass ~ 1 g). Four uninjected controls were also followed. The mean tumor diameter of the treated animals increased at the rate of 0.62 mm/day compared with 0.775 mm/day in the uninjected controls, representing about a 20% reduction in growth rate.

Five uninjected animals, bearing mammary tumor 13762NF, showed a growth rate of ~ 1.8 mm/day in mean tumor diameter. Five animals given injections once every third day beginning 8 days after implantation when the mean tumor mass was about 1 g showed a 22% reduction in growth rate. Another four animals injected daily with TTC showed a reduction in growth rate of $\sim 60\%$ compared to uninjected controls. However, in a control study with 6 animals/group, animals given the same daily dosage of nonradioactive tetracycline in combination with $^3\text{H}_2\text{O}$ showed a nearly identical reduction in the growth rate of mean tumor diameter ($\sim 60\%$) as those injected with TTC. See Figs. 3 and 4. Further experiments involving the injection of tetracycline only, or tritiated water only, indicated that the TC rather than the $^3\text{H}_2\text{O}$ led to the tumor response.

CONCENTRATION RATIOS OF TTC IN NORMAL AND MALIGNANT TISSUES.

To measure the levels of tetracycline uptake in tumor samples and simultaneously monitor the rate of cell proliferation in these samples, a double label experiment was carried out.

The results of the comparative uptake of TTC and ^{14}C -thymidine for the R-1 rhabdomyosarcoma, the 13762NF

Fig. 3. Therapeutic response of 13762NF mammary tumors. Animals injected every day and every third day with 7-tritiotetracycline were compared with uninjected controls in separate experiments. Mean tumor diameter = $^3\text{H}/\text{Iwh}$. Growth rates were compared by the slopes of straight line approximations to the data. There were four animals per group. Tumors whose dimensions changed abruptly because of rupture and loss of fluid were dropped from subsequent measurements.

mammary adenocarcinoma and a set of autochthonous mammary tumors in Simonsen albino rats are shown in Figs. 5, 6, and 7, plotting the logarithm of the ^3H concentration ($\mu\text{Ci/g}$) vs the logarithm of the

Fig. 4. Therapeutic response of 13762NF mammary tumors. Animals injected each day with 7-tritiotetracycline were compared with controls either uninjected or injected each day with tetracycline + $^3\text{H}_2\text{O}$. There were six animals per group. Mean tumor diameter = $\sqrt[3]{1wh}$.

Fig. 5. Concentration of ^{14}C and ^3H in samples of 5-wk-old rhabdomyosarcomas from animals injected 24 h previously with ^{14}C -thymidine and ^3H -tetracycline. The concentration of ^{14}C is taken to indicate relative local cellular proliferation rate while the concentration of ^3H indicates the relative binding of tetracycline. Identification of necrotic and viable areas was made by direct observation at time of dissection.

Fig. 6. Concentration of ^{14}C -thymidine and ^3H -tetracycline in samples of 1-, 2-, and 4-week-old mammary tumors 13762NF from animals injected 24 h previously. Samples from 1-week-old tumors have uniformly high ^{14}C content and low ^3H concentration, indicating high average cellular proliferation rate and low affinity for tetracycline respectively. Samples from older tumors have increased tetracycline uptake and decreased average proliferation rate.

Fig. 7. Concentration of ^{14}C -thymidine and ^3H -tetracycline in samples of spontaneous mammary tumors from 6 Simonren albino rats injected 24 h previously. Uptake of tetracycline indicated by ^3H -concentration is uniformly lower than that of kidney from the same animal.

^{14}C concentration ($\mu\text{Ci/g}$). The rhabdomyosarcoma (Fig. 5) at 5 weeks after implantation developed sharply defined regions of necrosis that had a relatively low ^{14}C content in combination with a high ^3H concentration. The necrotic regions of the mammary adenocarcinoma 13762NF (Fig. 6) were not as strongly delineated, but 1-week-old tumors had uniformly high ^{14}C and low ^3H concentrations. The autochthonous tumors (Fig. 7) show a great degree of heterogeneity in local rates of cellular proliferation, but, in general, ^{14}C -thymidine uptake was lower than that by transplanted tumors and zones of necrosis were scarce. Taking the level of ^{14}C -thymidine as a measure of cellular proliferation rate in the sample and the ^3H level as an indicator of TC uptake, the following points can be made:

- Rapidly proliferating, non-necrotic tumor samples retain tetracycline in concentrations similar to those in muscle and liver, but lower than those in kidney.
- The maximum degree of TC retention in tumors occurs in necrotic material and in areas of low proliferation, and is roughly 100 times the retention of rapidly proliferating tumor areas or normal tissues with low

retention such as muscle or blood.

- TC uptake by spontaneous mammary tumors in Simonsen albino rats is rarely higher than that by the kidney in the same animal.

The same experimental format was used to study the labeling patterns in about 20 other transplanted tumor strains and about 15 other autochthonous mammary tumors. The results for these tumors are shown with the same format in Appendix A. The results of the double label experiments for the tumor types investigated are summarized in Table 1. The maximum tetracycline content of the tumor samples relative to kidney were used to list the tumors in descending order of the maximum concentration ratio. The number of animals tested is given, and when animals bore multiple tumors, the total number of tumors tested is given in parenthesis. In an effort to reduce tumor necrosis as a factor in tetracycline accumulation, most other transplanted tumors were tested soon after they became palpable, as indicated by the small number of tumor samples/animal.

The correlation coefficient between the $\log ^{14}\text{C}$ concentration and $\log ^3\text{H}$ concentration was calculated. Tumor samples that had a lower concentration of ^{14}C than kidney were excluded from this correlation on the

Table 1. Summary of tetracycline localization in rodent tumors.^a

Tumor Designation	Tumor Type	Type of Animal Strain or Mouse No.	Comments	# of Animals (Tumors)	Total of Tumor Samples	Mean [Tumor TC] [Kidney TC]	Max [Tumor TC] [Kidney TC]	#Samples in Corr.	Corr. Coeff.	Sig.
W75	Mammary Squamous Cell Carcinoma	Fischer 344 (R)		3	45	1.03	11.26	31	+0.56*	+
1376/29 (old)	Mammary Adenocarcinoma	Fischer 344 (R)	28 and 35 days	1	14	1.33	9.527	26	+0.81*	+
5-1-1020	Hard-Mastoma	Rag/131 (R)	5 weeks post transplant	2	13	1.94*	6.892	32	+0.922	+
LAW	Mammary Adenocarcinoma	Fischer 344 (R)	Metastasis from 1376/29F	2(3)	38	1.51	5.157	53	+0.913	+
W71/14	Anterior Pituitary Tumor	Fischer 344 (R)		4	10	1.73*	4.735	10	+0.43	
W77/85	Anterior Pituitary Tumor	Wistar-Kurth (R)		2	13	1.12	3.91*	13	0.191	0
58 T.B. 1376/111 Median 5*	Spontaneous Mammary Melanoma	Swiss-Mehler(M) SCL (R)	Autochthonous	1 5(8)	24 29	1.149 0.31	3.473 3.168	17 29	0.531 +0.555	+ 0
W77/10	Anterior Pituitary Tumor	Wistar-Kurth (R)		1	19	0.6673	3.024	36	+0.572	+
15 Costello 1-1,2*	Mammary Carcinoma Spontaneous Mammary Tat	A/? Unknown Hooded Rat (R)	Autochthonous	4 1	18 19	0.695 0.311*	1.731 1.557	17 9	+0.537* +0.44*	+ 0
Dunn	Spontaneous Pituitary Carcinoma	FA (R)	Transplant of kidney Metastasis	2	20	0.9219	1.488	18	+0.143	0
4-1 (new)	Melanocarcinoma	Rag/131 (R)	5 weeks post transplant	2	11	0.3523	1.267	11	0.464	0
2100 1316 1317 1318 1319 1320 1321 1322 1323 1324 1325 1326 1327 1328 1329 1330 1331 1332 1333 1334 1335 1336 1337 1338 1339 1340 1341 1342 1343 1344 1345 1346 1347 1348 1349 1350 1351 1352 1353 1354 1355 1356 1357 1358 1359 1360 1361 1362 1363 1364 1365 1366 1367 1368 1369 1370 1371 1372 1373 1374 1375 1376 1377 1378 1379 1380 1381 1382 1383 1384 1385 1386 1387 1388 1389 1390 1391 1392 1393 1394 1395 1396 1397 1398 1399 1400 1401 1402 1403 1404 1405 1406 1407 1408 1409 1410 1411 1412 1413 1414 1415 1416 1417 1418 1419 1420 1421 1422 1423 1424 1425 1426 1427 1428 1429 1430 1431 1432 1433 1434 1435 1436 1437 1438 1439 1440 1441 1442 1443 1444 1445 1446 1447 1448 1449 1450 1451 1452 1453 1454 1455 1456 1457 1458 1459 1460 1461 1462 1463 1464 1465 1466 1467 1468 1469 1470 1471 1472 1473 1474 1475 1476 1477 1478 1479 1480 1481 1482 1483 1484 1485 1486 1487 1488 1489 1490 1491 1492 1493 1494 1495 1496 1497 1498 1499 1500 1501 1502 1503 1504 1505 1506 1507 1508 1509 1510 1511 1512 1513 1514 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basis that they did not represent proliferative areas of the tumor. The total number of tumor samples as well as the number included in the correlation are listed. The significance of the correlation was evaluated by the critical values for correlation coefficients supplied by Rohlf and Sokal.¹⁶ A correlation with a probability of greater than 5% of having been produced by randomly distributed samples was given a zero significance, a positive correlation with a probability of less than 1% of occurring with randomly distributed samples was given a ++ significance, a probability between 1 and 5% was rated as +, and negative correlations were correspondingly rated - - and -.

Several features are apparent. The highest concentration ratios are ~ 10 and occur in tumors with strongly negative correlations between tetracycline uptake and cellular proliferation. The highest average TC concentration ratio was about two. The R-1 and 13762NF tumors are shown twice, once with small young tumors (~ 0.5 g) and once with older, larger tumors (2 to 20 g). The only tumors that have high maximum concentrations of TTC relative to kidney are older necrotic tumors that have a negative correlation between TTC and ¹⁴C thymidine uptake. Many of the tumors, while showing low concentrations of

TC relative to kidney, do have a positive correlation between tetracycline uptake and ¹⁴C-thymidine uptake. This could either be a reflection of the binding capacity of the younger tumor cells or of the blood supply in these tumors.

It is known that tetracycline complexes divalent cations such as Ca⁺⁺, and that growing bone strongly binds the drug. For this reason a bone-forming tumor, the Dunn osteosarcoma, was included in our survey. Though the average tumor tetracycline concentration ratio was relatively high, the maximum tetracycline uptake was still only about 1.5 times that of kidney, and no significant correlation between tetracycline uptake and cellular proliferation was noted.

Our results indicate that the concentration ratios remain of the same order in the 13762NF mammary adenocarcinoma from 12 to 192 h after a single injection into young tumors (Fig. 8), though at very long times after injection the concentration ratios may change. One Sprague Dawley female bearing spontaneous mammary tumors was given eight TTC injections and was sacrificed for analysis 1.3 y after the last injection. The maximum levels of tetracycline found in these tumors was about 200 times that in kidney

Fig. 8. Concentration of ^3H -tetracycline in 13762NF mammary tumors compared with kidney and muscle from the same animals at 12, 24, 48, 96, and 192 h after injection. The tumors were injected one week after implantation. Their average weight at 12 h after injection was 0.3 g and at 192 h was 12 g. Tissue concentrations shown are the average found in 3 animals except at 192 h (4 animals). Biological half life for unbound tetracycline is > 100 min in rats so that residual activity after 24 h consists almost entirely of bound tetracycline and labeled impurities with longer biological half lives. The rate at which ^3H elutes from the muscle and tumor after 48 h is consistent with the biological half life of tritiated water.

and the average tumor tetracycline concentration was about 7 times that in kidney. These are the only spontaneous tumors in which more than 50% of the samples had higher TTC concentrations than kidney. The proliferation rate in these samples is unknown.

EXTRACTIONS FROM HUMAN TUMORS

Figure 9 shows the levels of TC found in six human malignancies and their associated normal tissues. Only two of the tumors contained

areas with TC levels significantly higher than those found in the normal tissues and in both cases these were either grossly necrotic or hemorrhagic areas of the tumors. The maximum concentration of TC found was about 15 times that of the corresponding normal tissues. The infiltrating ductal carcinomas however are rather diffuse in nature and, since large samples (0.5 g) were required for analysis, some high local concentrations may have been lost in an average over surrounding normal tissue.

Fig. 9. Tetracycline concentrations in human tumors and normal tissues as determined by extraction of fluorescent complexes. Patients had received a total of 2 g of nonradioactive tetracycline in four I.V. injections ending 3 da before surgical removal of the malignancy.

Discussion

The data presented by Phillips *et al.*⁵ and the analysis of these data by Wood⁶ showed tetracycline concentrations in human malignancies were much greater than would be expected in normal tissues. Phillips *et al.*⁵ also noted that the highest TC concentrations occurred in the rapidly proliferating areas of the

tumors studied and that zones of necrosis had relatively little affinity for tetracycline. A number of authors,^{17,18,19} using tetracycline fluorescence to examine transplanted rodent tumors, concluded in direct contradiction to the results of Phillips that tetracycline localization was solely in necrotic tissue.

As noted earlier, however, TC fluorescence is dependent on pH and the presence of bivalent metal cations, is second order in TC concentration, and is readily quenched by proteins, phosphates, and fixatives. Therefore tetracycline fluorescence cannot be regarded as a reliable indicator of relative TC concentrations. Machado *et al.*¹⁹ did some work with tritiated tetracycline that tended to confirm their fluorescence studies in sarcoma 37 tumors, and recently Chauncy *et al.*²⁰ using ¹³¹I-tetracycline observed that clearance of the labeled drug was slower from the nonviable portions of a rat hepatoma than from viable areas, and that normal tissues including kidney cleared more rapidly than viable tumor. In general, however, there has been no comprehensive quantitation of TC concentration in malignancies that could be compared with the data of Phillips.

The results of our work with an array of transplanted and spontaneous tumors in rats and mice using TTC to measure tetracycline concentrations in normal and malignant tissues indicates that higher TC concentrations appear only in older necrosing tumors, and that high TC concentrations found in tumors are negatively correlated with the local rate of cellular proliferation as measured by ¹⁴C-thymidine uptake. These

results are also consistent with previous fluorescence studies. These results are also consistent with the findings of Sontzeff *et al.*²¹ that tumor necrosis is very rich in calcium, which would provide a binding site for TC.

The question remains as to whether the rodent tumor systems that we examined or those that were studied by others with fluorescence techniques constitute accurate models for human malignancies. The studies of nonradioactive tetracycline concentrations determined chemically in several sets of malignant and normal human tissues showed that areas of heavy necrosis retained as much as 15 times the concentration of TC found in normal tissues, while the remaining malignant tissues had about the same concentration as normal tissue. These results are consistent with the animal data, but the diffuse nature of the human tumors studied and the necessarily large samples required for chemical analysis (0.5 g) could have obscured high local concentrations in viable tumor tissue.

Further studies of the localization of TC in tumor and tissues such as those of Breslow *et al.*²² should clarify its potential in tumor diagnosis. Our animal data suggest

that TTC is of relatively little utility in inflicting selective damage in rapidly proliferating areas of malignancies. Thus we conclude that it is not of therapeutic value, with the reservation that the localization

of TTC in nonproliferating tumor could conceivably be useful as an adjunct to chemotherapy and radiotherapy by selectively irradiating the chemically resistant out-of-cycle and radioresistant hypoxic cells.

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One of the authors, Paula Wood, was killed in an airplane accident prior to the completion of the work.

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Appendix A: Concentrations of ^3H and ^{14}C in Tumors after TTC-HCl and ^{14}C -Thymidine Injections.

Concentrations of ^3H and ^{14}C were determined in each tumor type 24 h after a single injection of TTC-HCl (11 $\mu\text{Ci/g}$ body weight) and ^{14}C -thymidine (0.1 $\mu\text{Ci/g}$ body weight). Samples that were included in the correlation statistics of Table I are shown as +. Samples having lower ^{14}C concentrations than average kidney tissues were considered nonproliferative areas. These were excluded from the correlation calculation and are represented with $\#$. K represents the mean ^3H and ^{14}C found in the kidneys of the tested animals. Weight loss by drying in samples less than 5 mg in some cases significantly altered the measured concentrations of ^{14}C and ^3H . Data from low weight samples are therefore not presented except in the case of SMT-2A where four of the six samples were under 5 mg. Tumor types are shown in same order as in Table I except for R-1 (old), 13762NF, and spontaneous mammary tumors in Simonsen albino rats, that data presented in Figs. 5, 6, and 7, respectively.