In-Vivo Identification of Tumor Multidrug Resistance with Tritium-3-Colchicine

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Multidrug resistance (MDR) is a major obstacle in the clinical treatment of cancer with natural-product anticancer agents. Identification of MDR in vivo could be important in the design of chemotherapeutic regimens. As a first step in developing radiolabeled drugs to detect MDR, we measured the in vivo distribution of radiolabel from [ring C, methoxy-^{3}H]-colchicine ([^{3}H]-CHC) in immunosuppressed mice bearing xenografts of colchicine-resistant and sensitive tumor cell lines. Experiments were done at trace (1 μg/kg) and LD_{50} (4 mg/kg) dose levels. Activity concentration/injected dose was more than twice as great in sensitive as in resistant tumors ($p < 0.01$) at 60 min following retroorbital injection of [^{3}H]-CHC. There was no significant difference in activity distribution between trace- and high-dose injections for any of the tissues sampled. Chromatographic analysis of plasma and tumor extracts demonstrated extensive extravascular metabolic degradation of [^{3}H]-CHC. The ratio of [^{3}H]-CHC concentration of injected dose between sensitive and resistant tumors was 3:1 ($p < 0.05$), due primarily to protein-bound [^{3}H]-CHC. This preliminary study demonstrates that it is possible to distinguish multidrug resistant from sensitive tumors in vivo on the basis of radiolabel uptake from an injected MDR drug. Colchicine, labeled with ^{11}C at the [ring C]-methoxy group, may be useful as a radiopharmaceutical for quantitative identification of MDR in human tumors using PET.

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Drug resistance (intrinsic or acquired) is a major obstacle to the clinical treatment of cancer. Tumor cells that are resistant to natural-product anticancer agents, such as vinca alkaloids or anthracyclines, often show cross-resistance to other natural-product chemotherapeutic agents (*1*). Reduced intracellular drug accumulation is an important factor in this phenomenon of multidrug resistance (MDR). Moreover, a membrane phosphoglycoprotein (P-glycoprotein, P-gp), frequently overexpressed in MDR, is believed to act as an ATP-dependent efflux pump for natural product drugs (*1*).

Identification of MDR in vivo could be an important aid in the design of chemotherapeutic regimens. P-glycoprotein has been quantitated in tumor biopsy specimens by histochemical and electrophoretic methods to identify resistant cell populations (*2,3*). The efficacy of this approach in guiding chemotherapy depends on establishing a correlation among P-gp concentration, drug accumulation and therapeutic response. A more direct method for identifying clinical MDR may be the measurement of concentrations of radiolabeled chemotherapeutic agents in tumors in vivo, which could be done noninvasively by external radionuclide imaging.

Our long-range objective is to develop the use of positron emission tomography (PET) in the planning of chemotherapy. PET imaging of MDR drugs labeled with positron-emitting radionuclides could be used to predict drug uptake in tumors, identify acquired resistance and evaluate potential inhibitors of P-gp.

Colchicine (Fig. 1), a naturally occurring alkaloid and a potent inhibitor of cellular mitosis, is a member of the MDR group of drugs (*1*). Compared with other drugs of that group, colchicine (CHC) may be relatively easy to label with ^{11}C, particularly at the chemoreactive vinyl ether site on the C-ring. We have developed methods for the facile C-ring demethylation of CHC and the subsequent remethylation of the isolated crystalline product (colchicein) to CHC with diazomethane. Colchicine thus synthesized was readily separated from excess unreacted material (Fissekis JD, unpublished results). Any isocolchicine in the mixture can be removed chromatographically. The pharmacokinetics of CHC appear favorable for PET studies since, according to the literature, metabolism of the drug is limited (*4,5*). The literature further suggests that recirculated labeled metabolites of [ring C, methoxy]-labeled CHC may be negligible (*4,5*).

Few quantitative studies identifying MDR in vivo have been reported, and there have been no reported studies of CHC uptake in tumors. This paper describes our preliminary effort to detect the MDR phenotype in vivo on the basis of accumulation of a radiolabeled drug in tumors. In these studies, we measured the tissue distribution of radiolabel from [ring C, methoxy-^{3}H]-CHC (henceforth denoted [^{3}H]-CHC) 60 min after retroorbital injection in immunosuppressed mice xenografted with CHC-resistant and sensitive tumor cells. Studies at trace and maximal

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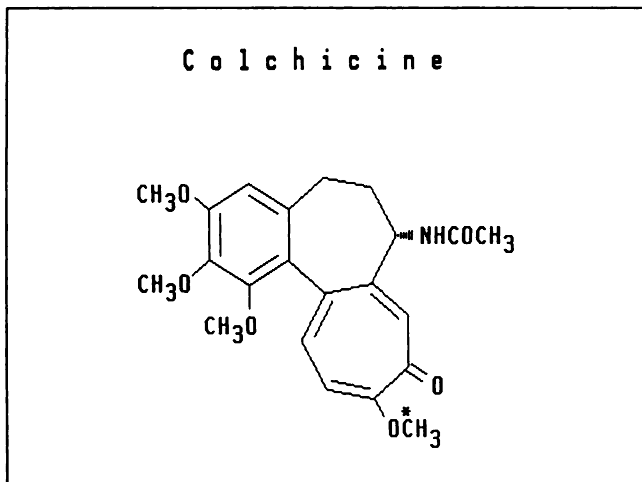

FIGURE 1. Structure of colchicine. * Ring C methoxy group, the suggested site of labeling with ^{11}C.

pharmacologic (LD_{50}) doses of CHC were performed to determine if the kinetics of the radiolabel were dose-dependent. To interpret tumor uptake of the radiolabel, a limited metabolite analysis was performed on tumor and blood samples. The main purpose of this analysis was to check the metabolic stability of the labeled site. Selection of ^{3}H-labeled colchicine for these preliminary studies was dictated by the commercial availability of the drug, although ^{14}C-labeled colchicine would have been preferable.

MATERIALS AND METHODS

The studies were carried out with human neuroblastoma cells selected with 0.2 μg/ml CHC (BE(2)-C/CHCb) and the corresponding, CHC-sensitive, parent line (BE(2)-C). These cell lines have been described in a preliminary report (*6*). When compared with the BE(2)-C cells, the BE(2)-C/CHCb cells were 54-fold resistant to CHC (ED_{50}* 270 ng/ml compared with 5 ng/ml) and were cross-resistant to actinomycin D (65-fold), to Adriamycin (40-fold) and to vincristine (87-fold).

The tumor cells were implanted in female Balb/C nude mice (20–25 g body weight) by subcutaneous flank injection of 1×10^7 cells. When the tumors had grown to 0.5–1.0 g (after 14–21 days), the animals were anesthetized with pentobarbital (50 μg/g body weight, i.p.) and [ring C, methoxy-^{3}H]-CHC (New England Nuclear, Inc. Boston, MA) was injected retroorbitally. Separate groups of animals received either a trace dose of CHC (1 μg/kg, containing 2 μCi of 32.5 μCi/nmol [^{3}H]-CHC) or a high dose of CHC near the LD_{50} for mice (4 mg/kg, again containing 2 μCi of [^{3}H]-CHC). The animals were killed by cervical dislocation at 60 min postinjection. Blood samples were obtained from the heart after dissection of the sternum. Tumors were excised in toto. Samples were also obtained by routine dissection from brain, liver, spleen, kidneys, intestines and skeletal muscle. All procedures conformed to a protocol (Protocol #86-02-020) approved by the Institutional Animal Care and Use Committee at our institution.

The ^{3}H label was assayed after processing the tissues and blood samples in a Tri-Carb sample oxidizer (Packard Instrument Co., Meriden, CT) (*7*). The oxidized samples were counted in a Tri-Carb 2200 CA Liquid Scintillation Counter (Packard Instrument Co., Meriden, CT).

For metabolic analysis, the animals were injected retroorbitally with about 100 μCi (50 μg/kg) of [^{3}H]-CHC. Again, the animals were killed after 1 hr and biopsy samples were prepared for HPLC analysis in the following manner. Blood samples were heparinized and from each a small volume (1–10 μl) was saved for counting. The remainder of each sample was centrifuged at 2,000 rpm for 30 min to separate plasma from cells. A small volume of each plasma sample was put aside for counting, and the corresponding main fraction was centrifuged in a Centricon 30 ultrafilter (Amicon Division, W.R. Grace & Co. Beverly, MA) at 10,000 rpm for 30 min to remove proteins. After counting, each deproteinized plasma sample was analyzed by HPLC. Tumor samples were homogenized with phosphate-buffered saline (pH 6.8) at room temperature. A small aliquot of each crude homogenate was saved and counted, while the remainder was divided into two, roughly equal, portions. One of these was heated in an Eppendorf tube (2 ml) in a water bath at 100°C for 10 min to release any protein-bound radioactivity. The heated and unheated homogenate portions were centrifuged to remove cell debris, the supernatants were recentrifuged in Centricon 30 ultrafilter cartridges at 10,000 rpm for 30 min to remove proteins, and the filtrates were used for HPLC. Small samples taken at each step of the procedure were assayed for radioactivity.

Colchicine injectates, tissue extracts and plasma samples were chromatographed on an analytical (25 × 4.1 mm) reverse-phase C-18 (PRP-1 10μ, Hamilton Co. Reno, NV) column. Samples were eluted with a system of sodium phosphate buffer (0.22 *M*, pH 6.0)-acetonitrile-methanol (70-20-10) flowing at a rate of 2 ml/min. The eluate was assayed with a variable wavelength monitor (Spectromonitor I, Laboratory Data Control, Riviera Beach, FL) at 354 nm and a radiation monitor LB 506B equipped with a Z-200-4 admixer cell (Berthold Analytical Instruments Inc. Nashua, NH). Monoflow-3 (National Diagnostics, Manville, NJ) was used as the scintillator. Each chromatographic run was completed in 20 min.

The concentrations of total radioactivity and radioactivity associated with particular molecular species were expressed as percentages of injected activity per gram of tissue or organ. Statistical analysis of differences in tumor percent dose per gram (%dose/g) between the two groups was done by planned comparison using Student's t-test (*8,9*). Nontumor tissue or organ %dose/g in the two tumor groups, as well as tumor and nontumor %dose/g at high and low CHC doses within a given tumor group, were compared post hoc by t-test using Bonferroni's criterion to account for Type I errors due to multiple comparisons (*8*). Mean intra-animal differences in [^{3}H]-CHC and metabolite concentrations between tumor and plasma within the same tumor group were compared by paired t-test. Differences between the two tumors, and between tumor and plasma, were considered significant if the probability (p) of Type I error was < 0.05. For multiple comparisons, differences were considered significant for $p < 0.05/k$, where k is the number of comparisons.

RESULTS

Table 1 summarizes the observations of the ^{3}H label distribution in tissue. Tritium accumulation was higher in

* ED_{50} or "effective dose" is that concentration of drug which reduces the cell population by 50%.

TABLE 1
Tissue Distribution of Radiolabel from [ring C, methoxy-^{3}H]-Colchicine at One Hour Postinjection

Tumor type	Tissue	%Dose/g tissue	
		High dose	Trace dose
BE(2)-C		(n = 10)	(n = 10)
	Blood	1.15 ± 0.12	1.96 ± 0.43
	Brain	0.14 ± 0.01	0.23 ± 0.04
	Liver	5.93 ± 0.59	10.07 ± 2.12
	Kidneys	7.31 ± 1.77	10.56 ± 3.78
	Spleen	4.67 ± 0.75	4.03 ± 1.31
	Intestines	19.04 ± 3.07	23.93 ± 5.25
	Muscle	2.40 ± 0.28	5.35 ± 0.94
	Tumor	1.66 ± 0.15	1.84 ± 0.31
BE(2)-C/CHCb		(n = 10)	(n = 10)
	Blood	0.97 ± 0.16	1.20 ± 0.27
	Brain	0.13 ± 0.02	0.23 ± 0.10
	Liver	6.27 ± 1.54	4.55 ± 0.68
	Kidneys	2.94 ± 0.54	3.45 ± 0.99
	Spleen	3.71 ± 0.28	3.59 ± 0.60
	Intestines	23.21 ± 2.21	16.03 ± 2.96
	Muscle	2.87 ± 0.51	1.43 ± 0.22*
	Tumor	0.70 ± 0.09†	0.79 ± 0.09‡

Data stated as mean ± standard error.

* $p < 0.02$ relative to skeletal muscle in BE(2)-C tumor group by t-test (post hoc comparison).

† $p < 0.001$ relative to BE(2)-C tumor by t-test (planned comparison).

‡ $p < 0.01$ relative to BE(2)-C tumor by t-test (planned comparison).

the CHC-sensitive than in the resistant tumors at both "trace" ($p < 0.01$) and "high" ($p < 0.001$) colchicine doses. The highest activity accumulations were seen in the intestine. Activity accumulation was low in both tumor types; only the brain had a lower %dose/g. There was no statistically significant difference in activity distribution between high- and trace-dose injections of CHC. Skeletal muscle had a higher %dose/g at trace dose in the BE(2)-C tumor group than in the BE(2)-C/CHCb tumor group ($p < 0.02$); there was no statistically significant difference in activity concentration between the two groups for any other non-tumor organ or tissue at either dose level.

The molecular distribution of the radiolabel after 60 min was examined in blood both in vitro and in vivo. Blood samples were incubated with [^{3}H]-CHC at 37°C for 60 min and then separated into red cell and plasma fractions as described in Materials and Methods. About 55% of total blood activity was recovered in plasma. Ultrafiltration indicated that none of the plasma activity was bound to protein. Colchicine is lipophilic (octanol:water partition coeff. >4) (*10*). Since the fraction of total blood activity present in plasma approximated the ratio of plasma volume-to-whole blood volume, it is reasonable to assume that the compound equilibrated between plasma and red cells with little or no intracellular binding. Chromatographic analysis showed that over 95% of the plasma activity was still associated with colchicine.

In contrast to the observations in vitro, analysis of plasma isolated from blood samples taken from normal and tumor-bearing mice revealed substantial metabolism of CHC in vivo. Observations in the two groups were essentially the same. Only about 15% of the radioactivity in plasma at 60 min postinjection was associated with CHC (Table 2). A labeled metabolite (most likely ^3H-H_2O) eluting with the solvent front accounted for most of the remaining 85% of the activity (Fig. 2A). A small amount of another labeled metabolite, presumably an A-ring demethyl derivative of CHC, was also present. The mean CHC %dose/g in plasma was 0.06 for both animal groups (Table 2). By using the mean weight of the animals (22 g) as well as a value for mice of 0.08 ml blood/g body weight (*11*) and assuming that, as in vitro, plasma contained 55% of whole blood CHC, we estimate that only about 0.2% of the injected CHC dose remained in the blood at 60 min.

A representative chromatographic profile from a tumor sample extract is shown in Figure 2B; the secondary metabolite (peak 2) seen in plasma was also present in the tumor samples. Numerical results are summarized in Table 2. Both sensitive and resistant tumors had elevated CHC concentrations ($p < 0.01$) relative to blood plasma; tumor-to-plasma concentration ratio for total CHC was significantly higher in the sensitive tumor group (Fig. 3). The predominant metabolite on the other hand equilibrated between plasma and tumor (Table 2). Total CHC accumulation was about three times greater in the sensitive than in the resistant tumors ($p < 0.05$). Most of the CHC in the tumors was bound to protein. The mean bound-to-total ratios (± standard error), 0.88 ± 0.03 for sensitive tumors and 0.82 ± 0.10 for resistant tumors, were not significantly different.

TABLE 2
Comparison of Colchicine (CHC) and Labeled Metabolite Concentrations in Tumor and Plasma

Tissue	Molecular species	Tissue concentration (%dose/g) — Tumor type: Sensitive (n = 5)	Resistant (n = 4)
Tumor	CHC		
	Total	0.81 ± 0.20	0.26 ± 0.03*
	Bound	0.71 ± 0.17	0.21 ± 0.03*
	Soluble	0.10 ± 0.04	0.05 ± 0.03
	"Water"	0.36 ± 0.05	0.40 ± 0.04
Plasma	CHC (soluble)	0.06 ± 0.02†	0.06 ± 0.01‡
	"Water"	0.31 ± 0.07	0.42 ± 0.06

Data stated as mean ± standard error. "Water" represents predominant labeled metabolite interpreted to be ^3H-H_2O.

* $p < 0.05$ relative to sensitive tumor by t-test.

† $p < 0.01$ relative to total CHC in sensitive tumor by paired t-test.

‡ $p < 0.01$ relative to total CHC in resistant tumor by paired t-test.

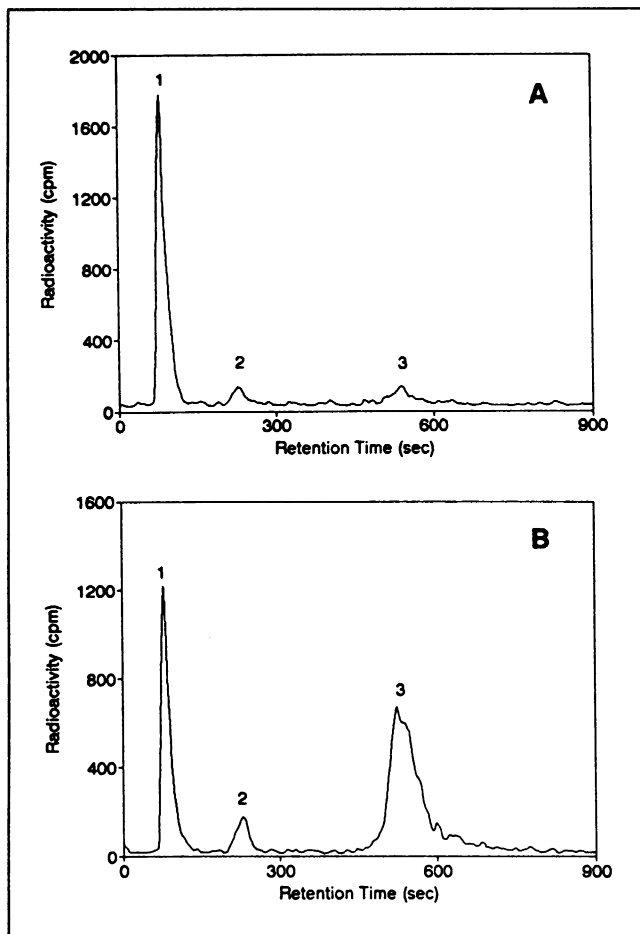

FIGURE 2. Chromatographic profiles of deproteinized plasma (A) and of tumor extract (B) obtained 1 hr after the injection of [ring C, methoxy-^{3}H]-colchicine in mice. 1, $^3H\text{-}H_2O$; 2, unknown metabolite structurally related to colchicine; 3, colchicine.

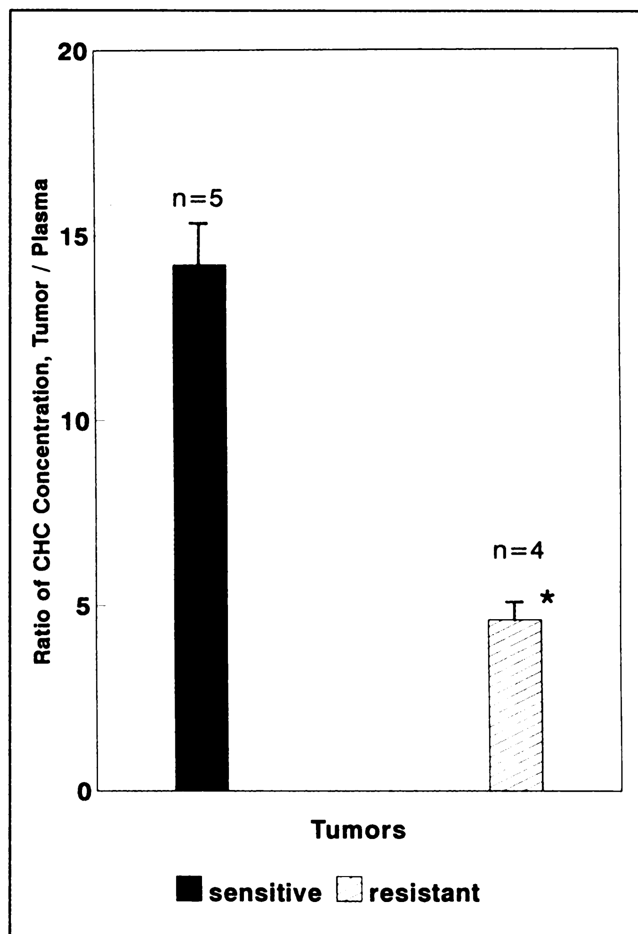

FIGURE 3. Tumor-to-plasma ratios of total colchicine concentration 1 hr after injection of [ring C, methoxy-^{3}H]-colchicine in mice. Data are averages among individual mice; error bars represent standard errors. * $p < 0.001$ relative to sensitive tumor by t-test.

DISCUSSION

Since the BE(2)-C/CHCb tumor cells are multidrug resistant, the retention of [^{3}H]-CHC in vivo was expected to be less in BE(2)-C/CHCb than in BE(2)-C tumors. This was borne out by our data, which show ^{3}H label concentrations two to two and a half times greater ($p < 0.01$) and CHC concentrations three times greater ($p < 0.05$) in sensitive than in resistant tumors.

The observed systemic distribution of the ^{3}H label is consistent with previous reports on colchicine behavior in vivo. The high concentrations of activity in liver and intestine are in accord with the accepted detoxification of CHC via the bile (*4*). (We were unable to satisfactorily express intestinal contents; our data, therefore, represent activity in both bowel and its contents.) We observed, as have others (*12*), a relative exclusion from brain of radiolabel from [^{3}H]-CHC. This phenomenon may be explained by the presence of elevated P-gp expression in cerebral capillary endothelial cells (*13*). As previously reported by others (*14*), we observed that the drug did not bind to plasma proteins. Our chromatographic analysis indicated essentially total removal of CHC from blood within 1 hr of injection.

Systemic distribution of the ^{3}H label was found to be approximately independent of CHC administered dose between trace (1 μg/kg) and LD_{50} (4 mg/kg) levels. This implies that the mechanisms of CHC transport, binding and metabolism in the mouse are linear (i.e., nonsaturable) within physiologic limits of CHC concentration. Thus, the chromatographic studies, which employed injections of 50 μg/kg of CHC, are probably representative of the metabolic fate of the ^{3}H label in the trace- and high-dose tissue distribution studies as well. That there was little difference in normal tissue concentrations of the radiolabel between mice injected with either cell line reinforces interpretation of the observed differences in sensitive and resistant tumor uptake as being due to events within the tumors themselves.

Most of the radiolabel circulating at 60 min postinjection was on a single molecular species other than CHC. Typically, the metabolic endpoint of tritium in vivo is ^{3}H-

H_2O. The chromatographic profiles (Fig. 2A–B) and plasma-to-tumor distribution ratios (Table 2) are consistent with that identification of the predominant labeled, non-CHC species in these experiments. Colchicine was shown to retain the label in aqueous buffer solution for several days and in blood at 37° C for 1 hr, indicating that the ^3H-H_2O was formed via an extravascular metabolic process or processes, and not by passive hydrogen exchange.

Colchicine binds to intracellular tubulin, the protein basis of microtubules; this is thought to be the primary mechanism of the drug's cytotoxic action (*15*). Tumors contained the same two labeled metabolites present in blood plasma (Fig. 2B). Concentrations of ^3H-H_2O in tumor and plasma were similar (Table 2); insufficient secondary metabolite was present to permit meaningful, quantitative comparison between tumor and plasma (Fig. 2A–B).

The data suggest that P-gp mediated outward transport is the predominant mechanism underlying the observed reduction of CHC retention in the BE(2)-C/CHCb in comparison to the BE(2)-C tumors. The ratio of bound-to-total CHC was the same in both tumors, indicating that the difference in uptake was not due to impaired binding in the resistant tumor. Our experiments do not rule out differences in blood flow, blood-to-cell transport or density of viable cells as causes of reduced uptake by the BE(2)-C/CHCb tumors. However, we observed that the probabilities of successful implant as well as the growth rates of the two tumor lines were very similar. This plus the fact that the resistant line was derived from the sensitive line by selection for colchicine resistance and is cross-resistant to other natural-product drugs in vitro strongly suggest that MDR is the most likely cause of the observed difference in tumor uptake of CHC.

The present study demonstrates that it is possible to distinguish multidrug resistant from sensitive tumors in vivo using a radiolabeled MDR drug. This, in principle, suggests the possibility of monitoring MDR in patients by positron emission tomography of drugs labeled with β^+-emitting radionuclides. The evidence provided here regarding the suitability of colchicine as a PET imaging agent is, however, mixed. Contrary to previous reports in the literature, we found that [ring C, methoxy-labeled]-CHC produces large amounts of recirculating, labeled metabolites. Furthermore, the uptake of CHC was low in both tumor types relative to that in most nontumor tissues and organs. Nonetheless, radiolabel accumulation did distinguish between the two tumor types. Tritium-labeled CHC may not be indicative of ^{11}C-labeled CHC with regard to circulating metabolites. Specifically, our observations suggest that a metabolic process or processes following injection of [ring C, methoxy-^{11}C]-CHC may produce significant amounts of circulating 1-Carbon fragments (i.e., methanol and/or formaldehyde). Low blood flow or other physiologic peculiarities of the BE(2)-C and BE(2)-C/CHCb xenografts may account for their low uptake. The near absence of CHC from blood at 60 min suggests that tumor uptake and CHC-to-metabolite ratios may be optimal at times earlier than 1 hr postinjection. These, as well as other questions bearing on the suitability of [ring C, methoxy-labeled]-CHC as an agent for PET, will be explored in further experiments with ^3H- and ^{14}C-labeled CHC.

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REFERENCES

1. Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* 1990;42:155–199.
2. Merkel DE, Fuqua SAW, Tandon AK, Hill SM, Buzdar AU, McGuire WL. Electrophoretic analysis of 248 clinical breast cancer specimens for P-glycoprotein overexpression or gene amplification. *J Clin Oncol* 1989;7: 1129–1136.
3. Verelle P, Meissonmier F, Fonck Y, et al. Clinical relevance of immunohistochemical detection of multidrug-resistance in breast carcinoma. *J Natl Cancer Inst* 1991;83:111–116.
4. Hunter AL, Klaassen CD. Biliary excretion of colchicine. *J Pharmacol Exp Ther* 1975;192:605–617.
5. Wallace SL, Omokoku B, Ertel NH. Colchicine plasma levels: implications as to pharmacology and mechanism of action. *Am J Med* 1970;48: 443–448.
6. Biedler JL, Casals D, Chang T-D, Meyers MB, Spengler BA, Ross RA. Multidrug-resistant human neuroblastoma cells are more differentiated than controls and retinoic acid further induces lineage-specific differentiation. *Adv Neuroblast Res* 1991;3:181–191.
7. Larson SM, Weiden PL, Grunbaum Z, et al. Positron imaging feasibility studies. II: characteristics of 2-deoxyglucose uptake in rodent and canine neoplasms: concise communication. *J Nucl Med* 1981;22:875–879.
8. Hays WL. *Statistics.* New York: Holt, Rinehart and Winston; 1981:413–443.
9. Walker HM, Lev J. *Statistical inference.* New York: Holt, Rinehart and Winston; 1953:157–158.
10. Ling V. Drug resistance and membrane alteration in mutants of mammalian cells. *Can J Genet Cytol* 1975;17:503–515.
11. Wish L, Furth J, Storey RH. Direct determinations of plasma, cell and organ-blood volumes in normal and hypervolemic mice. *Proc Soc Exp Biol Med* 1950;74:644–648.
12. Bennett EL, Alberti MH, Flood JF. Uptake of [^3H]colchicine into brain and liver of mouse, rat, and chick. *Pharm Biochem Behav* 1981;14: 863–869.
13. Cordon-Cardo C, O'Brien JP, Casals D, et al. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA* 1989;86:695–698.
14. Trnavska Z, Kuchar M, Rejholec V, Trnavsky K. The binding of colchicine and its derivatives to bovine and human serum albumin and human plasma. *Pharmacology* 1979;18:123–127.
15. Owellen RJ, Owens AH, Donigian DW. The binding of vincristine, vinblastine and colchicine to tubulin. *Biochem Biophys Res Comm* 1972;47: 685–691.