In Vivo Uptake of Carbon-14-Colchicine for Identification of Tumor Multidrug Resistance

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A major limitation in the treatment of cancer with natural product chemotherapeutic agents is the development of multidrug resistance (MDR). Multidrug resistance is attributed to enhanced expression of the multidrug resistance gene MDR1. Colchicine (CHC) is known to be one of the MDR drugs. We have previously demonstrated that it is possible to distinguish multidrug-resistant tumors from multidrug-sensitive tumors in vivo on the basis of tritium (^{3}H) uptake following injection of ^{3}H-CHC. **Methods:** The present studies were carried out in xenografted animals using ^{14}C-CHC which may be more indicative of ^{11}C-labeled CHC distribution with regard to circulating metabolites, since metabolic 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). Experiments were carried out at a dose of 2 mg/kg. **Results:** Activity concentration per injected dose was approximately twice as great in sensitive as in resistant tumors ($p < 0.05$) at 60 min following intravenous injection of ^{14}C-CHC. About 75% of total activity was CHC in the sensitive tumors. The findings are further confirmed by the quantitative autoradiographic evaluation of resistant and sensitive tumors. **Conclusions:** These studies confirm our previous observations that it is possible to noninvasively distinguish multidrug-resistant tumors from sensitive tumors in vivo based on uptake of an injected MDR drug using a ^{14}C-labeled CHC at the same position and of comparable specific activity to a ^{11}C-CHC tracer used for PET imaging.

Key Words: multidrug resistance; colchicine; P-glycoprotein

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Resistance to chemotherapeutic agents (intrinsic or acquired) is a major limitation to the clinical treatment of cancer. Tumors 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) which is attributed to enhanced expression of the multidrug resistance gene MDR1 (*2,3*). A 170-kD membrane phosphoglycoprotein (P-glycoprotein), frequently overexpressed as a result of MDR1 gene expression, is believed to act as an ATP-dependent efflux pump for natural product drugs (*1–3*).

Early identification of MDR in vivo could be important in the expeditious design of chemotherapeutic regimens. P-glycoprotein has been quantitated in tumor biopsy specimens by histochemical and electrophoretic methods to identify resistant cell populations (*4,5*). The efficacy of this approach in guiding chemotherapy depends on establishing a correlation among P-glycoprotein concentration, drug accumulation and therapeutic response. A direct, noninvasive method for identifying clinical MDR would be measuring concentrations of radiolabeled chemotherapeutic agents in tumors in vivo by external radionuclide imaging.

Our primary objective is to develop the use of PET in planning chemotherapy. PET imaging of MDR drugs labeled with positron-emitting radionuclides could predict drug uptake in tumors, identify acquired resistance and evaluate potential inhibitors of P-glycoprotein.

Colchicine (CHC) (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, CHC may be relatively easy to label with ^{11}C. The pharmacokinetics of CHC appear favorable for PET studies since metabolism of the drug is limited (*6,7*). It is further suggested that recirculated labeled metabolites of labeled CHC may be negligible (*6,7*).

Few quantitative studies identifying MDR in vivo have been reported. Recently we described our preliminary efforts to detect the MDR phenotype in vivo based on accumulation of ^{3}H-labeled CHC in tumors (*8*). In these studies, we measured the tissue distribution of (ring C, methoxy-^{3}H)-CHC 60 min after intravenous injection in immunosuppressed mice xenografted with CHC-resistant and CHC-sensitive tumor cells. Studies at trace and maximal pharmacologic (LD_{50}) doses of CHC were performed to determine dose dependency of the kinetics of the radiolabel, showed no significant differences. A limited metabolite analysis performed on both tumor and blood samples ascertained the metabolic stability of the labeled site and showed no significant metabolism of the radiolabel. Selec-

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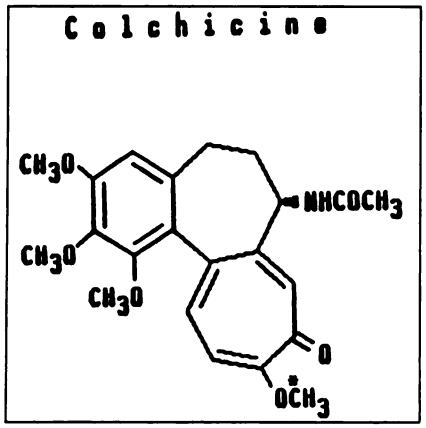

FIGURE 1. Structure of colchicine * Ring C methoxy group, the suggested site of labeling with ^{11}C.

tion of ^{3}H-CHC for these preliminary studies was dictated by the commercial availability of the drug, although ^{14}C-CHC would have been preferable. Hence, in the present paper we evaluate the use of ^{14}C-CHC in identifying MDR in vivo.

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 previously (*9*). 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). (ED_{50} or "effective dose," is that concentration of drug which reduces the cell population by 50%.)

Tumor cells were implanted in female Balb/C nude mice (20–25 g body weight) by subcutaneous flank injection of 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, intraperitonealy) and (ring C, methoxy-^{14}C)-CHC (New England Nuclear, Inc., Boston, MA) was injected intravenously. Animals received 2 mg/kg, containing 4 μCi of 36.0 mCi/mmole [^{14}C]-CHC. In our earlier studies we found that uptake in both resistant and sensitive tumor-bearing animals was very similar regardless of dose (*8*); therefore, we utilized only one dose in the present investigation. Animals were killed by cervical dislocation 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 (no. 86-02-020) approved by the Institutional Animal Care and Use Committee at our institution.

The ^{14}C label was assayed after processing the tissues and blood samples in a Tri-Carb sample oxidizer (Packard Instrument Co., Meriden, CT) (*10*). The oxidized samples were counted in a Tri-Carb 2200 CA Liquid Scintillation Counter (Packard Instrument Co.).

For metabolic analysis the animals were injected intravenously with about 100 μCi (50 μg/kg) of ^{14}C-CHC. Again the animals were killed after 60 min and samples were prepared for HPLC analysis in the following manner. Blood samples were heparinized and from each sample 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 an Centricon 30 ultrafilter (Amicon Division, W.R. Grace and 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 in the liquid scintillation counter to give us the number of total counts associated with tumor. The remainder of the tumor homogenate was divided into two roughly equal portions. One portion was heated in an Eppendorf tube (2 ml) at 100°C for 10 min to release any protein-bound ^{14}C-CHC radioactivity. The heat-denatured sample released the ^{14}C-CHC bound to tissue proteins, thus its ultrafiltrate contained the total ^{14}C-CHC activity. Whereas the nonheated sample following ultrafiltration was representative of only free unbound ^{14}C-CHC activity. A comparison of the two ultrafiltrates is used to show the difference between the protein-bound ^{14}C-CHC activity in sensitive and resistant tumors. The ultrafiltration was carried out using Centricon 30 ultrafilter cartridges at 10,000 rpm for 30 min.

Colchicine injectates, tissue extracts and plasma samples were chromatographed on an analytical (25 × 4.1 mm) reverse-phase C-18 (PRP-1 10 μ, 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. It was soon discovered that the ^{14}C label was not enough to be determined by Spectromonitor so we collected fractions by collecting 0.5-ml fractions using Fraction Collector (Pharmacia LKB Nuclear Inc., Gaithersburg, MD). The samples were then counted in a scintillation counter as mentioned earlier.

The concentrations of total radioactivity and radioactivity associated with a particular molecular species were expressed as percentages of injected activity per gram of tissue or organ. Statistical analysis of differences in tumor percentage of injected dose per gram (%ID/g) between the two groups was done by a planned comparison using the Student's t-test (*11,12*). Nontumor tissue or organ %ID/g in the two tumor groups, as well as tumor and nontumor %ID/g at CHC dose employed in this investigation within a given tumor group, were compared post hoc by the t-test using Bonferroni's criterion to account for Type I errors due to multiple comparisons (*11*). Mean intra-animal differences in ^{14}C-CHC and metabolite concentrations between tumor and plasma within the same tumor group were compared by a 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.

Quantitative autoradiographic studies were carried out to determine the association of the radioactivity with both the sensitive and resistant tumors and compared with H and E staining. Tumors were frozen immediately after excision and 20-μm slices were cut on the Cryomicrotome (Hacker Instruments, Fairfield, NJ). Slices adjacent to the ones used for autoradiography were used for H and E staining procedures. Slices for autoradiography were mounted on the microscopic slides with respective ^{14}C stan-

TABLE 1
Tissue Distribution of Radiolabel from (ring C, methoxy-^{14}C) Colchicine at One Hour Postinjection

Tissue	%ID/g Tissue BE(2)-C (n = 12)	BE(2)-C/CHCb (n = 12)
Blood	1.48 ± 0.34	0.62 ± 0.16*
Brain	0.22 ± 0.03	0.42 ± 0.16
Liver	6.45 ± 0.74	6.41 ± 1.92
Kidneys	1.89 ± 0.36	2.21 ± 0.65
Spleen	3.94 ± 1.14	3.63 ± 1.15
Intestines	23.55 ± 4.54	28.55 ± 9.98
Muscle	0.98 ± 0.18	1.39 ± 0.47
Tumor	1.33 ± 0.19	0.55 ± 0.11*

Data stated as mean ± standard error.
*$p < 0.05$ relative to BE(2)-C tumor by t-test (planned comparison).

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

Tissue	Molecular species	Tissue concentration (%ID/g) Tumor type: Sensitive (n = 10)	Resistant (n = 10)
Tumor	CHC		
	Total	2.11 ± 0.62	1.27 ± 0.29*
	Metabolite	0.54 ± 0.12	0.53 ± 0.13*
	Soluble	1.57 ± 0.54	0.72 ± 0.19
Plasma	CHC		
	Total	0.85 ± 0.28	0.53 ± 0.13
	Metabolite	0.66 ± 0.20	0.42 ± 0.18
	Soluble	0.19 ± 0.77†	0.11 ± 0.04†

Data stated as mean ± standard error.
*$p < 0.05$ relative to sensitive tumor by t-test.
†$p < 0.05$ relative to total CHC in tumor by paired t-test.

dards placed in a cassette with SB-5 x-ray film. Films were developed after 10 days and read on a computer using a quantitative autoradiography program package (MCID Software, Imaging Research Inc., St. Catherines, Ontario, Canada). Slide mounted with the adjacent slices, the films were then developed with a standard H and E staining procedure and compared with the autoradiographic films.

RESULTS

Table 1 summarizes the distribution of the ^{14}C label in tissues. Carbon-14 radioactivity concentration was higher in the CHC-sensitive animals than in the resistant tumor-bearing animals ($p < 0.05$) in both blood and tumors. The highest concentration of radioactivity was seen in the intestines. Radioactivity concentration was low in both tumor types; only brain had a lower %ID/g radioactivity. There was no statistically significant difference in ^{14}C activity between the two groups for any other nontumor organ or tissue at this dose level.

The molecular distribution of the radiolabel in blood after 60 min was examined both in vitro and in vivo. HPLC analysis of blood samples incubated with ^{14}C-CHC in vitro revealed that about 55% of total blood ^{14}C concentration was recovered in plasma. Chromatographic analysis following ultrafiltration indicated that none of the plasma radioactivity was bound to protein, and that 95% of the plasma ^{14}C radioactivity was still associated with colchiocine.

In contrast to in vitro observations, 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 21% of the radioactivity in plasma at 60 min postinjection was associated with CHC (Table 2). A labeled metabolite eluting with the solvent front accounted for most of the remaining activity (Fig. 2A). A small amount of another labeled metabolite, presumably an A-ring demethyl derivative of CHC, was also present. The mean CHC %ID/g in plasma was 0.19 and 0.11 for sensitive and resistant groups, respectively (Table 2). We estimated that 0.4%–0.8% of the injected CHC remained in the blood at 60 min (using the mean weight of the animals to be 22 g, a value for mice of 0.08 ml blood/g body weight (*10*), and further assuming that, as in vitro, plasma contained 55% of whole blood CHC).

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. Both sensitive and resistant tumors had elevated CHC concentrations ($p < 0.05$) relative to blood plasma. The tumor-to-plasma ratio for total CHC was significantly higher in the sensitive tumor group. Figure 3 shows the comparison of tumor-to-plasma concentration ratios at 60 min in sensitive and resistant tumor-bearing animals following both ^{3}H-CHC and ^{14}C-CHC administration. Total CHC uptake was about two times greater in sensitive than in resistant tumors ($p < 0.05$). Most of the CHC in the tumors was bound to protein. The predominant metabolite was equilibrated between plasma and tumor (Table 2). The mean bound-to-total CHC ratios, viz., 0.70 ± 0.05 (S.E.) for sensitive tumors and 0.57 ± 0.06 (S.E.) for resistant tumors, were not significantly different. Ratios of bound-to-total CHC obtained with [^{3}H]-CHC administration (*8*) were 0.88 ± 0.03 and 0.82 ± 1.0 for sensitive and resistant tumors, respectively, were also not significantly different.

Autoradiographic analysis showed that the radioactivity was associated with tumor sites as seen with the H and E staining procedure (Figs. 4A–D). The difference in radioactivity concentrations between sensitive and resistant tumors was significant. Comparison of autoradiographic and H and E images clearly revealed that there was less radioactivity associated with resistant tumors (Figs. 4A and C) compared to sensitive (Figs. 4B and D) tumors. Figure 5 shows the mean nCi/g of ^{14}C-CHC obtained from autoradiographic images in sensitive and resistant tumor slices.

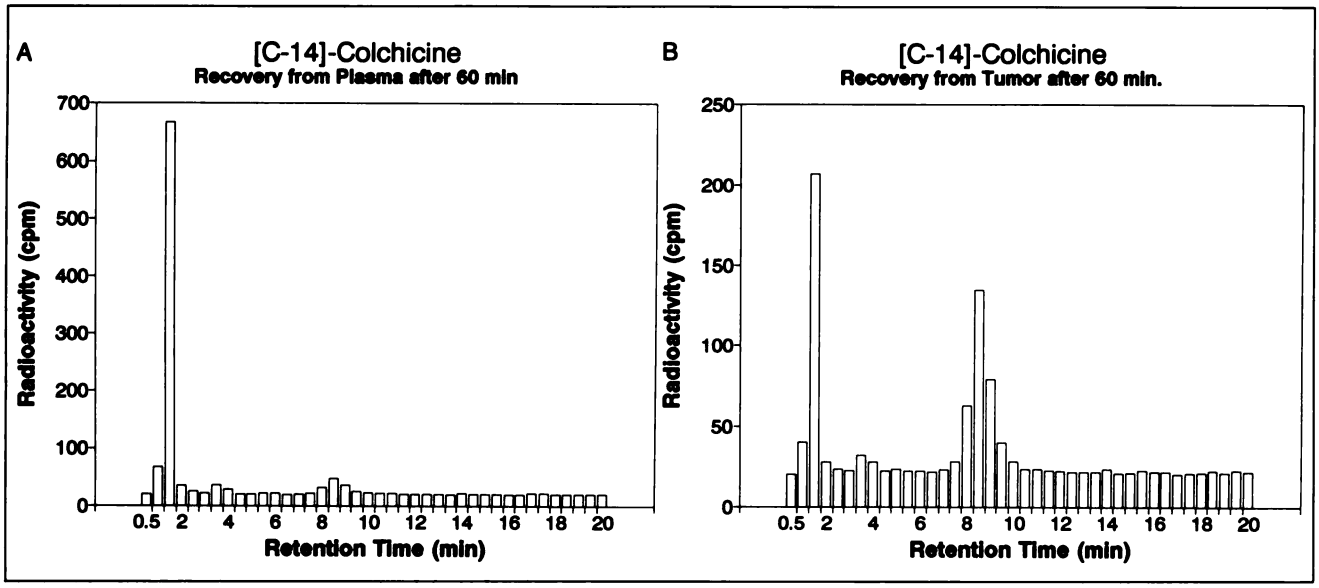

FIGURE 2. Chromatographic profiles of deproteinized plasma (A) and of tumor extract (B) obtained 60 min after the injection of [ring C, methoxy-^{14}C]-colchicine in nude mice.

Uptake of ^{14}C-CHC activity in sensitive tumors was approximately twice that of resistant tumors ($p < 0.05$). Autoradiographic analysis thus corresponds to the HPLC analysis of ^{14}C-CHC in the tumors.

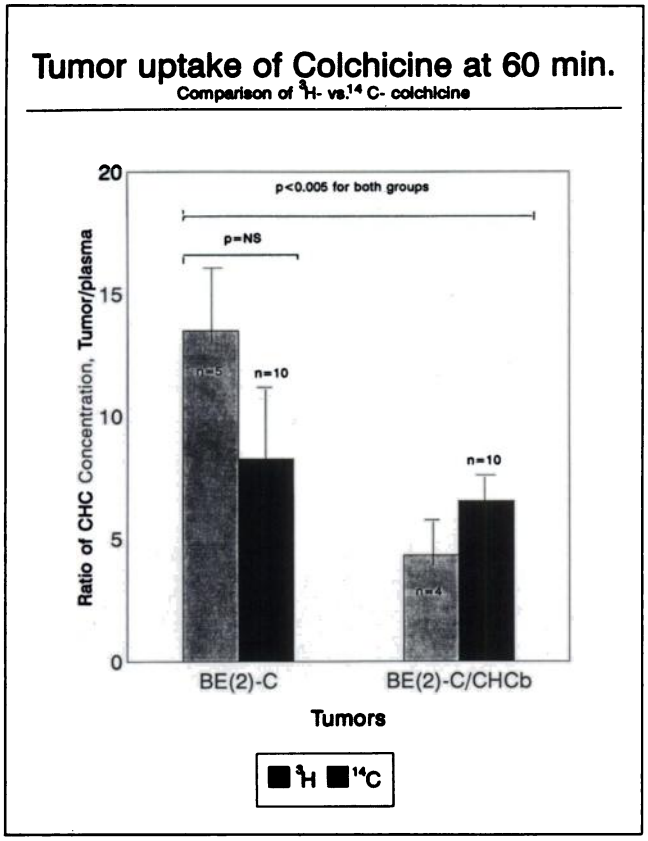

FIGURE 3. Comparison of tumor-to-plasma ratios of total CHC concentration 60 min after injection of [^{3}H] and [^{14}C]-CHC in nude mice. Data are averages among individual mice; error bars represent standard errors.

DISCUSSION

Since the BE(2)-C/CHCb tumor cells are multidrug resistant, the retention of radiolabeled-CHC in vivo was expected to be less in BE(2)-C/CHCb than in BE(2)-C tumors. This was discovered in our earlier studies (*8*) and further confirmed with the present data which show ^{14}C label concentrations 2–2.5 times greater ($p < 0.01$) and CHC concentrations about 2 times greater ($p < 0.05$) in sensitive than in resistant tumors.

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 bowel as well as its contents. We observed earlier (*8*), as have Bennett et al. (*13*), a relative exclusion of radiolabel from the brain of ^{14}C-CHC. This phenomenon may be explained by the presence of elevated P-glycoprotein expression in cerebral capillary endothelial cells (*14*). As reported by Trnavska et al. (*15*), we observed that the drug did not bind to plasma proteins. Our chromatographic analysis indicated essentially total removal of CHC from blood within 60 min of injection.

Earlier, we showed that biodistribution of CHC was independent of dose (*8*). 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 2 mg/kg of CHC, are probably representative of the metabolic fate of the ^{14}C label in the tissue distribution studies as well. The small differentiation 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.

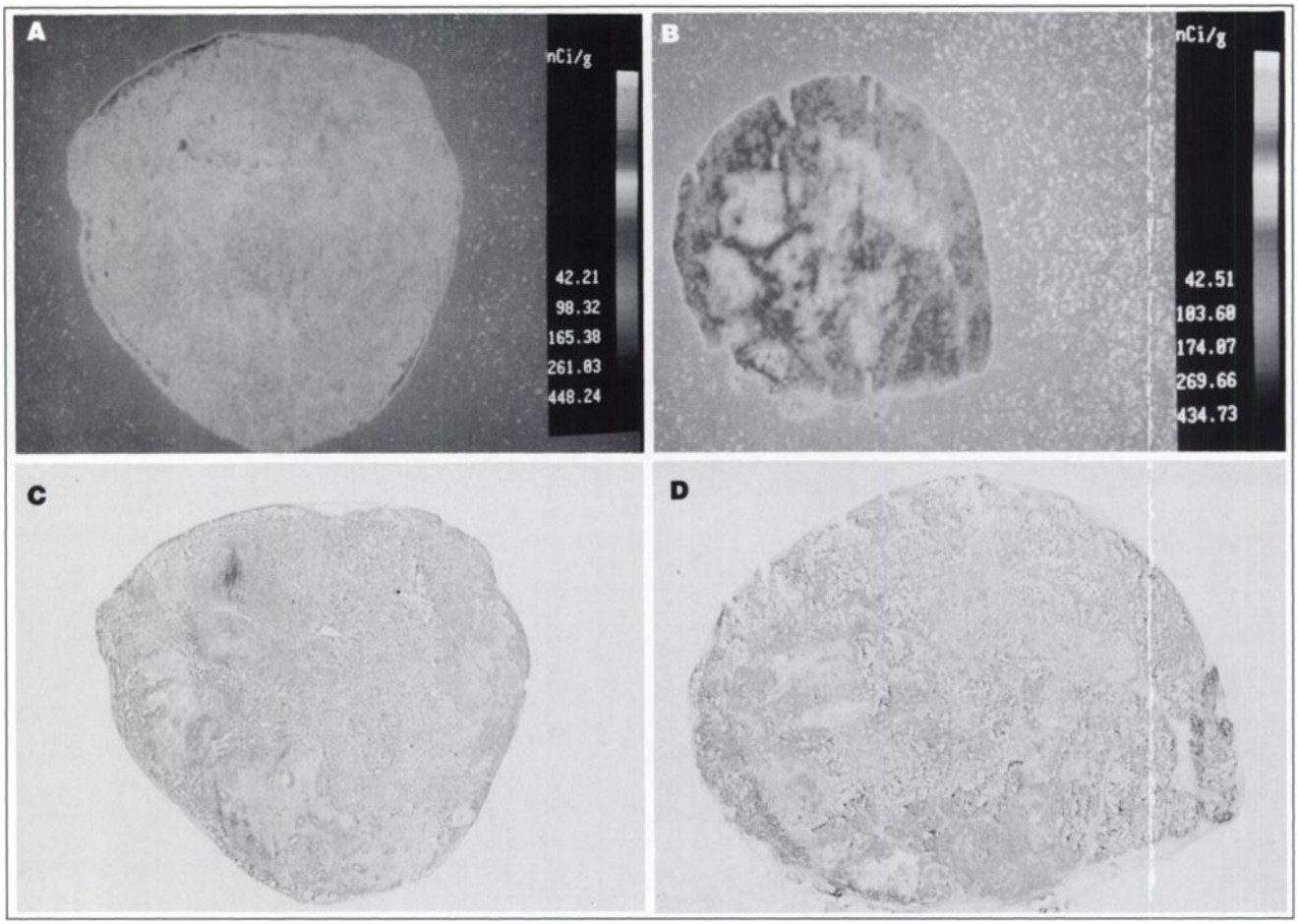

FIGURE 4. Comparison of BE(2)-C (sensitive) and BE(2)-C/CHCb (resistant) tumor slices of both the H and E staining and the autoradiography. (A and B) the radioactivity concentrations of the BE(2)-C (sensitive) and BE(2)-C/CHCb (resistant) tumors in comparison with the H and E staining for the respective adjacent slices in C and D.

Most of the radiolabel circulating at 60 min postinjection was on a single molecular species (presumably a polar metabolite) other than CHC. The chromatographic profiles (Figs. 2A and 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 binds to intracellular tubulin, the protein basis of microtubules; this is thought to be the primary mechanism of the drug's cytotoxic action (*16*). Tumors contained the same two labeled metabolites present in blood plasma (Fig. 2B). An insufficient secondary metabolite was present to permit meaningful, quantitative comparison between tumor and plasma (Figs. 2A and B).

Quantitative autoradiographic analysis of both the sensitive and resistant tumors in Figures 4A–D show a clearer picture of the binding of the radioactivity. Although radioactivity in the representative sensitive tumor slice is greater than that in the resistant tumor slice (Figs. 4A and B, respectively), both slices show comparable amounts of tumor cells (Figs. 4C and D). The quantitation is further confirmed in Figure 5, which shows the difference in tumor uptake expressed as nCi/g of both tumors.

Our experiments do not rule out differences in blood flow, blood-to-cell transport or density of viable cell population, as causes of reduced uptake by the BE(2)-C/CHCb tumors. However, we observed that the probabilities of a successful implant and the growth rates of the two tumor lines were very similar. 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 tumors. Finally, the resistant line was derived from the sensitive line by selection for CHC resistance and is cross-resistant to other natural product drugs in vitro. The data taken together strongly suggest that MDR is the most likely cause of the observed differences in tumor uptake of CHC.

The present study further demonstrates that it is possible to distinguish multidrug-resistant tumors from sensitive tumors in vivo using a radiolabeled MDR drug. This, in principle, suggests the possibility of monitoring MDR in patients by PET of drugs labeled with beta-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 polar metabolites. Fur-

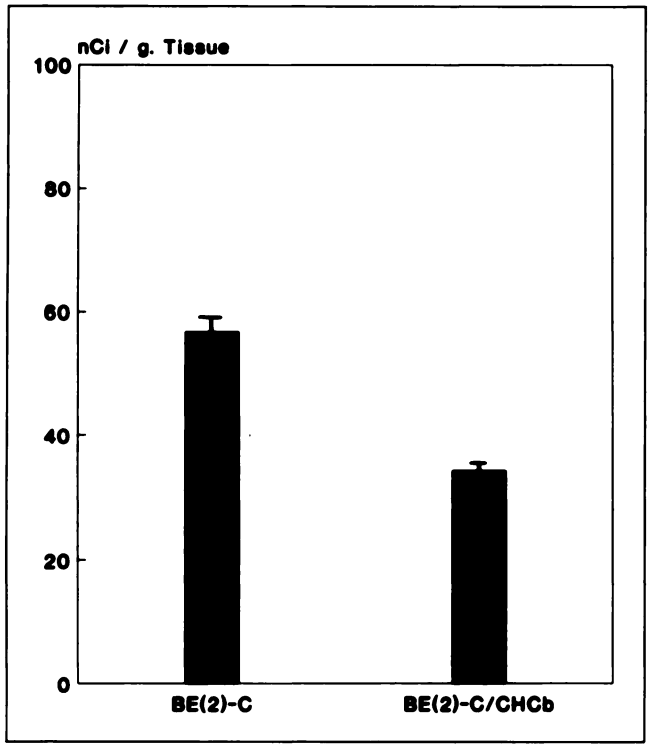

FIGURE 5. Comparison of quantitative autoradiographic images of the BE(2)-C (sensitive) and BE(2)-C/CHCb (resistant) tumor slices.

thermore, the uptake of CHC was low in both tumor types relative to that in most nontumor tissues and organs. Nonetheless, radiolabel uptake did distinguish between the two tumor types. Carbon-14-CHC may 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 60 min 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 ^{14}C-CHC.

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