Carbon-11-Labeled Daunorubicin and Verapamil for Probing P-Glycoprotein in Tumors with PET

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One of the mechanisms for multidrug resistance (MDR) of tumors is an overexpression of the P-glycoprotein (P-gp). The cytostatic agent daunorubicin and the modulator verapamil were labeled with ^{11}C to probe P-gp with PET. **Methods:** Carbon-11-daunorubicin was prepared from $^{11}CCH_2N_2$ with an aldehyde precursor, followed by hydrolysis. Carbon-11-verapamil was synthesized by ^{11}C-methylation. Both tracers were evaluated by investigating pharmacokinetics in rats and in vitro cell kinetics using human ovarian carcinoma cells. **Results:** Amounts of 111 MBq ^{11}C-daunorubicin were prepared. Biodistribution studies of ^{11}C-daunorubicin in male Wistar rats showed dose-dependent pharmacokinetics, whereas with ^{11}C-verapamil the pharmacokinetics were dose independent. In in vitro experiments with cells, the ratio of accumulation of ^{11}C-daunorubicin in drug sensitive/resistant cell lines was 16. Addition of verapamil resulted in increased accumulation of ^{11}C-daunorubicin in the resistant cell line. The ratios of ^{11}C-verapamil accumulation in drug-sensitive versus the MDR counterpart were 4–5. **Conclusion:** Carbon-11-daunorubicin and ^{11}C-verapamil both have potential for in vivo probing of P-glycoprotein with PET.

Key Words: multidrug resistance; carbon-11-daunorubicin; carbon-11-verapamil; PET

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Multidrug resistance (MDR) is a major obstacle to the clinical treatment of cancer with several anti-cancer agents (*1*). One of the mechanisms resulting in MDR is overexpression of the 170-kD membrane phosphoglycoprotein P-gp (*2*) that is considered to act as an efflux pump for cytostatic agents. The result is a decreased intracellular concentration of anti-cancer drugs to sublethal levels. Until now, P-gp was detected in human tumor with immunohistochemistry or MDR-1 RNA detection. It is unknown whether this expression relates with P-gp function in vivo. Detection of functional P-gp-mediated MDR might be very useful to select the right chemotherapy protocols. To decrease the effect of P-gp-mediated MDR, modulators have been used. The working mechanism is based on competitive blocking of P-gp, resulting in higher intracellular cytostatic drug concentrations and an intracellular shift of the drug from cytoplasm to the nucleus. One of the most applied modulators in vitro is verapamil (VER) (*3,4*). In vivo, the efficacy of the MDR modulators has been low (especially in solid tumors), which may be ascribed to the low affinity of the clinically assessed modulators as compared to newer second generation modulators (*5,6*) or the fact that overexpression of P-gp is only one of the causes that can lead to MDR. It has also been difficult to assess whether the modulator reaches the tumor in an effective concentration.

For imaging purposes, Piwnica-Worms et al. (*7*) used ^{99m}Tc-sestamibi, which allows noninvasive P-gp function measurements in vivo with SPECT. Other groups have reported similar studies using ^{99m}Tc-analogs such as tetrofosmin (*8*). So far, no PET studies on MDR have been reported. In addition, PET may provide a tool for a quantitative functional assay of P-gp.

With the purpose of radiolabeling the natural product ^{11}C-colchicine, Mehta et al. evaluated ^{3}H- and ^{14}C-labeled analogs (*9,10*) as possible probes for P-gp. At 1 hr postinjection, colchicine levels in sensitive tumors were three times higher than the resistant tumors. Another candidate is the cytostatic agent daunorubicin (DNR), since its labeling with ^{11}C was considered to be possible (*11*) (Fig. 1).

Instead of application of a positron-emitting anti-cancer drug, an alternative to monitor P-gp is the use of the radiolabeled P-gp-modulator VER (Fig. 2), since both cytostatic agents and some P-gp-modulators like VER are actively transported as a substrate by P-gp. In this article, the synthesis of ^{11}C-labeled DNR and VER is described as well as in vivo tissue distribution studies in rats and in vitro studies with drug-sensitive (A2780) and drug-resistant (2780^{AD}) human ovarian carcinoma cell lines.

MATERIALS AND METHODS

9-Formyltrifluoroacetyl DNR was a gift from Farmitalia Carlo Erba. The μBondapak C18 (300 × 3.9 mm, 5 μm), Si-RAD-Pak (100 × 8 mm, 5 μm) HPLC columns Zorbax-NH_2-column (250 × 4.6 mm, 7 μm) as well as (±)VER and (±)normethyl VER were obtained commercially. All chemicals were analytical grade.

Chemistry

Carbon-11-Daunorubicin. Carbon-11-CH_2N_2 was prepared as described by Crouzel et al. (*12*). One equivalent of $CHCl_3$ with respect to 9-formyl trifluoroacetyl daunorubicin was added to the KOH/hydrazine/ethanol solution. Carbon-11-CH_2N_2 was distilled into the precursor solution (1 mg aldehyde **1** in 1 ml $CHCl_3$/MeOH 9/1 v/v) using a gas flow of N_2 with 2% O_2. After stirring the reaction mixture for 5 min at 60°C in a closed reaction vial, the solvent was evaporated under reduced pressure at 50°C. The residue was dissolved in acetonitrile (0.1 ml). Thereafter, icecold 0.1 *M* NaOH (0.4 ml) was added. After 10 min at 0°C, 0.1 *M* HCl was added to neutralize the reaction mixture. To purify ^{11}C-DNR **3**, the reaction mixture was applied onto a μBondapak C18-HPLC-column with the eluant acetonitrile/water/acetic acid 30/70/1 (v/v/v) at pH = 4 by addition of Na-acetate. Using a flow rate of 2 ml/min, the retention time of ^{11}C-DNR **3** was 10 min (Fig. 3). A second HPLC system was used to characterize the radiotracer. Using the same μBondapak C18-column eluted with a methanol/water mixture (65/35, v/v) containing monobasic ammonium phosphate (1.15 g) and acetic acid (5 ml) at pH = 4, the retention time of **3** was 8 min. The flow rate was 1.5 ml/min. The UV spectra of the end-product and authentic daunorubicin were identical as determined with a photodiode array UV-detector.

To prepare [^{11}C]DNR for pharmacological studies, the organic solvent was removed from the collected HPLC-fraction by evaporation under reduced pressure for 3 min at 50°C.

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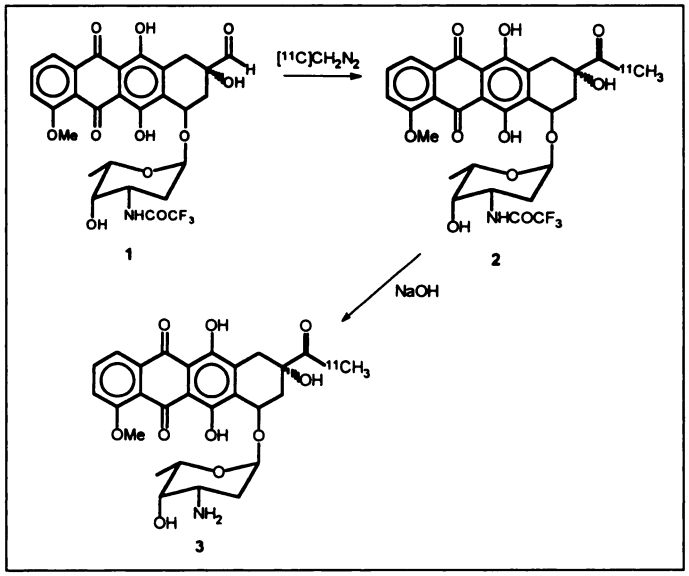

FIGURE 1. Synthesis of ^{11}C-daunorubicin.

Carbon-11-Verapamil. Carbon-11-CH_3I was produced from $^{11}CCO_2$ ($^{14}N(p, \alpha)^{11}C$ nuclear reaction with 17 MeV protons) yielding 30 GBq [^{11}C]CH_3I with a specific activity of 150 TBq/mmol. Carbon-11-CH_3I was trapped in a solution of N-normethyl VER **4** (0.5 mg) in acetonitrile (0.5 ml) in the presence of Al_2O_3/KF according to a procedure described by Schmitz et al. (*13*) (Fig. 2). After heating at 110°C for 10 min, the reaction mixture was filtered over a Millipore FG filter (0.20 μm) to remove Al_2O_3/KF. After evaporation of the solvent under reduced pressure, HPLC-purification was performed, using a Si-RAD-Pak column with dichloromethane/hexane/isopropanol/triethylamine (75/25/4/0.02, v/v/v/v) as eluant. With a flow rate of 1 ml/min, the retention time of [^{11}C]VER **5** was 10 min. To ascertain the chemical identity, the radiotracer was analyzed on a second HPLC system using a Zorbax-NH_2-column with eluant: CH_2Cl_2 with 0.02% Et_3N. Using a flow rate of 1 ml/min, the retention time of **5** was 11 min. Etheral-HCl was added to the collected HPLC-fraction of ^{11}C-VER in order to isolate the HCl-salt. After evaporation of the eluant under reduced pressure, ^{11}C-VER was dissolved in saline.

Biodistribution Studies

All experiments were carried out in compliance with the Law on Animal Experimentation of The Netherlands. Male Wistar rats (250–300 g) were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg body weight). Subsequently, 1 MBq ^{11}C-DNR or ^{11}C-VER (tracer or pharmacological dosage: 10 mg/kg body weight for ^{11}C-DNR and 200 μg/kg body weight for ^{11}C-VER) was injected intravenously in a volume of 0.3 ml saline.

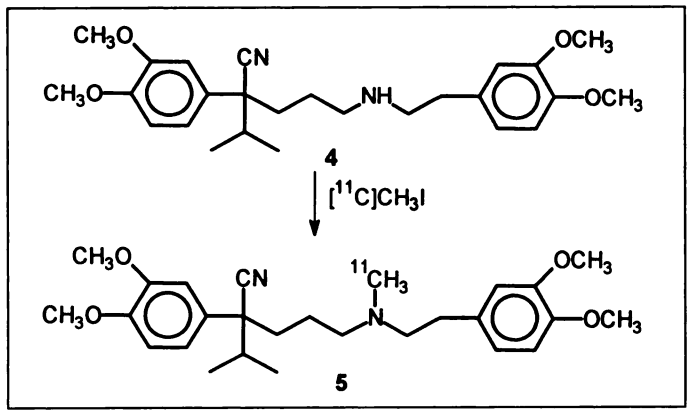

FIGURE 2. Synthesis of ^{11}C-verapamil.

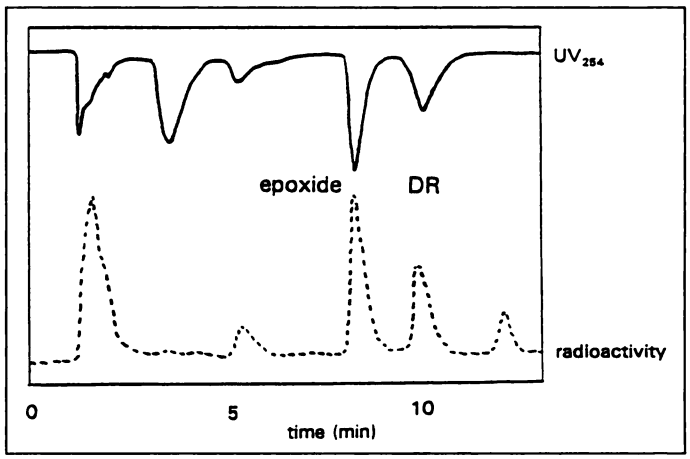

FIGURE 3. HPLC-radiochromatogram obtained for purification of ^{11}C-daunorubicin.

After 60 min, the rats were killed by extirpation of the heart and several tissues were dissected. Plasma was obtained from blood by centrifugation (2 min, 3000 g). Uptake values were expressed as differential absorption ratios

$$\text{DAR} = \frac{\text{(counts per min recovered/g tissue)}}{\text{(counts per min injected/g body weight)}}.$$

Plasma clearance of ^{11}C-VER was determined. The carotid artery was cannulated and arterial blood samples (100–200 μl) were drawn at various time points from 0 to 60 min after injection of 1 MBq ^{11}C-VER. Plasma and red blood cells (RBCs) were separated by short centrifugation (2 min, 3000 g). Plasma (25 μl) and pellet samples were then counted in a calibrated gamma counter.

In Vitro Experiments with Human Ovarian Carcinoma Cell Lines

Accumulation of ^{11}C-DNR or ^{11}C-VER was investigated in a human ovarian carcinoma cell line (A2780) and its 90-fold adriamycin resistant counterpart 2780^{AD} (*15,16*). P-gp was detected by immunohistochemical staining with the monoclonal antibody C219, directed against P-gp. In the resistant ovarian carcinoma cell line 2780AD, P-gp expression was high, but not in the parental cell line A2780. The cells [2×10^6 in cell medium containing RPMI medium and 10% heat-inactivated fetal calf serum (FCS)] in polystyrene tubes were loaded with either a tracer or a pharmacological concentration of ^{11}C-DNR (2 μM) or ^{11}C-VER (5 μM) (NCA or CA) (100 μl) in a final volume of 5 ml. For the ^{11}C-DNR experiments, the accumulation experiments as described above were repeated in the presence of the MDR-modulator verapamil (50 μM). The cells were incubated for 30 min at 37°C. Subsequently the cells were washed by addition of ice-cold PBS (5 ml), followed by centrifugation (5 min, 180 g, 4°C). The amount of ^{11}C in the pellet was determined. In order to correct for extracellular binding of ^{11}C-DNR or ^{11}C-VER, incubation experiments were repeated at 4°C. The intracellular accumulation was expressed as pmole/10^6 cells.

RESULTS AND DISCUSSION

Chemistry

[14-^{11}C]DNR **3** was prepared by the reaction of $^{11}CCH_2N_2$ with the aldehyde **1**, followed by the alkaline hydrolysis of the trifluoro-acetic group (Fig. 1). This synthetic route is analogous to the synthesis of the [^{14}C]DNR as described by Penco et al. (*16*). Since the reported reaction times, as described for the

synthesis of ^{14}C-DNR, were too long for ^{11}C chemistry, the synthetic procedure had to be adapted.

Sufficient amounts in the range of 0.6–3 mCi (27–111 MBq) could be prepared to perform animal studies. The overall radiochemical yield was 3% ± 1% with a total synthesis time of 53 min. The specific activity was 20–30 Ci/mmol. The radiochemical purity was >99%.

The synthesis of ^{11}C-DNR via $^{11}C\text{-}CH_2N_2$ proceeds with several drawbacks, resulting in a low overall radiochemical yield. From the literature, it is known that this type of reaction is slow and usually takes several hours at room temperature before completion (*17*). The incorporation of ^{11}C-diazomethane after 5 min reaction time at 50°C was 10%–15%. To reduce the amount of unidentified side products, carrier $CHCl_3$ was added to the solution containing KOH, ethanol and hydrazine-H_2O. Possibly small amounts of 9-formyltrifluoroacetyl DNR **1** were oxidized to the corresponding carboxylic acid, which is much more reactive to diazomethane. A possible result is the formation of undesirable ^{11}C-methyl ester.

A second explanation for the low radiochemical yield is the formation of a substantial amount of side products. HPLC-analysis of the trifluoroacetyl DNR **2** revealed that besides the homologization rearrangement resulting in the desired ^{11}C-methyl ketone **2**, formation of the ^{11}C-epoxide had taken place. The ratio of methyl ketone to epoxide under these conditions was 1:2. In contrast, Penco et al. (*16*) reported a ratio of methyl ketone to epoxide of 3:2 for the synthesis of the ^{14}C-analog. Some additional unidentified side products are formed during the first reaction step using ^{11}C-diazomethane. These products might be formed due to the sensitivity to light or air (*18,19*). When the composition of the obtained reaction mixture is studied by HPLC (Fig. 3), about 20% of the total radioactivity was the desired ^{11}C-DNR.

A third drawback is the hydrolysis of the trifluoroacetyl group. The hydrolysis was performed for 10 min with 0.1 *M* NaOH to form ^{11}C-DNR. The radiochemical yield of the hydrolysis step was about 80%.

To prepare ^{11}C-VER, several bases and solvents were tested for the ^{11}C-methylation reaction (Fig. 2), e.g., KOtBu/THF, NaH/MeCN, $Bu_4N^+OH^-$/MeOH and Al_2O_3/KF in acetonitrile or THF or DMF. The use of bases did not improve the radiochemical yield as compared to the blank reaction without base. Al_2O_3/KF proved to be superior to the other bases or to the reaction without base with respect to chemical purity of the end product. Some impurities were present when using the other bases in the ^{11}C-VER-HPLC-fraction. Given that chemically pure ^{11}C-VER could be obtained only by using the solid support, we conclude that other impurities might absorb to the support. The overall radiochemical yield was 16% (corrected for decay) with a total synthesis time of 45 min. The radiochemical purity was >99%. The specific activity was >300 Ci/mmol, when $^{11}CCO_2$ was produced with an integrated beam of 10 μAh. Carbon-11-VER was converted to its HCl-salt by addition of etheral-HCl.

Biodistribution Studies

Biodistribution studies with ^{11}C-DNR in male Wistar rats were performed to determine the pharmacokinetics. It is important that the influx of ^{11}C-DNR to tumor tissue and its efflux take place within the time scale appropriate for ^{11}C. In Table 1, DAR values at 60 min postinjection are presented. Two dosages were used: non-carrier-added and a pharmacological dose of 10 mg/kg body weight. Differences in uptake are observed in the radioactivity level in plasma, liver and urine. Since DNR binds to plasma proteins, the observed dose-dependent kinetics may be the result of altered drug or metabolite clearance. For humans, dose-dependent pharmacokinetics of the related adriamycin were also reported (*20,21*). For a tracer dose, the proportion of unbound radiolabeled DNR is lower. Therefore, in a tracer dose, DNR may be transported more slowly from plasma to tissue and renal excretion may also be decreased.

TABLE 1
Biodistribution Studies of Carbon-11-DNR in Male Wistar Rats (n = 3 for Each Group) 60 Minutes Postinjection

Tissue	Non-carrier-added	Body weight (10 mg/kg)
Heart	0.62 ± 0.07	0.78 ± 0.19
Kidney	3.20 ± 0.73	1.83 ± 0.34
Liver	2.50 ± 0.76	0.64 ± 0.18
Lung	1.11 ± 0.21	1.51 ± 0.13
Muscle	0.45 ± 0.13	0.20 ± 0.08
Pancreas	0.89 ± 0.28	0.68 ± 0.10
Stomach	0.66 ± 0.12	0.59 ± 0.10
Bone	0.75 ± 0.24	0.39 ± 0.05
Spleen	1.54 ± 0.33	2.57 ± 0.22
Bladder	0.91 ± 0.18	0.90 ± 0.44
Urine	4.55 ± 1.80	9.29 ± 0.94
Brain	0.48 ± 0.06	0.12 ± 0.02
RBC	0.52 ± 0.08	0.10 ± 0.02
Plasma	0.91 ± 0.13	0.17 ± 0.04

Uptakes are expressed as DAR. Errors are s.d. RBC = red blood cells.

To assess the predictive value of PET-measurements with ^{11}C-DNR for the clinic, it will be necessary to determine the correlation between pharmacokinetics for tracer and pharmacological dose. In practice, patients who will be investigated are those with tumors that developed MDR during adriamycin chemotherapy. In this case, a constant and relatively high level of adriamycin is present in the tumor. The ultimate experiment to prove P-gp-related uptake is measuring uptake of ^{11}C-DNR before and after treatment with a MDR-modulator during chemotherapy.

Biodistribution studies with ^{11}C-VER in male Wistar rats were performed with both a tracer dose of ^{11}C-VER and a pharmacological dose (200 μg/kg body weight) (Table 2). High DAR values were obtained for lung, spleen and kidney. No

TABLE 2
Biodistribution Studies of Carbon-11-Verapamil in Male Wistar Rats 60 Minutes Postinjection (n = 3)

Tissue	Non-carrier-added	Body weight (200 μg/kg)
Heart	0.80 ± 0.15	0.73 ± 0.08
Kidney	3.84 ± 0.81	3.49 ± 0.60
Liver	1.35 ± 0.36	1.30 ± 0.18
Lung	9.45 ± 1.31	8.22 ± 4.03
Muscle	0.57 ± 0.25	0.35 ± 0.11
Pancreas	2.11 ± 0.93	2.64 ± 0.24
Stomach	1.15 ± 0.29	0.97 ± 0.33
Bone	1.03 ± 0.73	1.08 ± 0.29
Spleen	4.54 ± 0.94	4.41 ± 0.52
Bladder	0.67 ± 0.26	0.84 ± 0.02
Urine	0.16 ± 0.08	1.29 ± 1.36
Brain	0.14 ± 0.09	0.18 ± 0.06
RBC	0.19 ± 0.11	0.16 ± 0.02
Plasma	0.29 ± 0.15	0.27 ± 0.02

Uptakes are expressed as DAR. Errors are s.d.

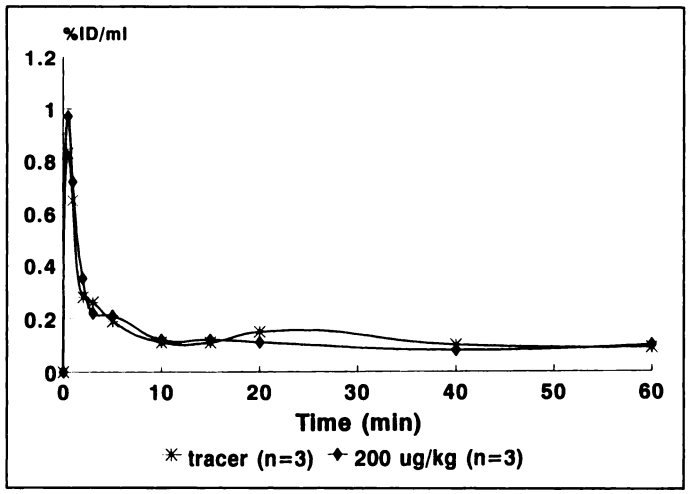

FIGURE 4. Plasma clearance of ^{11}C-verapamil in Wistar rats.

significant differences were observed between application of the tracer dose and pharmacological dose of ^{11}C-VER.

Another favorable property of ^{11}C-VER is its rapid clearance from plasma within 5 min postinjection (Fig. 4). Plasma clearance of ^{11}C was similar after injection of a tracer dose ^{11}C-VER and a pharmacological dose. The first phase (distribution) has a half-life of 0.9 min, whereas the second phase has a half-life of 90 min.

In Vitro Experiments with Human Ovarian Cancinoma Cell Lines

To investigate the suitability of ^{11}C-DNR and ^{11}C-VER for PET, in vitro studies were performed with human ovarian carcinoma cell lines. In case of DNR, the experiments were carried out with tracer and pharmacological concentrations (2 μM). The steady-state drug concentrations are presented in Table 3. In case of the tracer concentration of DNR, the intracellular concentration of ^{11}C-DNR was 16-fold higher in the MDR-sensitive cells as compared to the resistant P-gp overexpressing cells. These results are in good agreement with data published by Broxterman et al. (*22*). Addition of a pharmacological dose of P-gp-modulator VER (50 μM) resulted in an increased accumulation of ^{11}C-DNR in the resistant cell line to a level comparable to the sensitive A2780 cell line. Addition of VER did not influence the accumulation of ^{11}C-DNR in the sensitive A2780 cell line. The absolute values for the pharmacological dosages were decreased to about 50% as compared to the values for the tracer dosages. Addition of VER (50 μM) caused an increase of accumulation of ^{11}C-DNR in the resistant 2780^{AD} cell line to a level comparable to the sensitive cell line. These results unambiguously demonstrate P-gp mediated pharmacokinetics of ^{11}C-DNR.

The accumulation of ^{11}C-VER in the A2780 sensitive cell line was 4–5 times higher as compared to the resistant 2780^{AD} cell line (Table 3). The overexpression of P-gp results in an increased efflux of the P-gp-modulator ^{11}C-VER. This ratio of 4–5 is less than ^{11}C-DNR, where a ratio of 16 was obtained. The results with ^{11}C-VER are in good agreement with experiments described by Broxterman et al. (*22*). Since the cell membranes reveal a high passive permeability for VER, the obtained ratio of 4–5 might be underestimated (*23*), since the cells are centrifuged and washed with buffer to determine the radioactivity uptake.

TABLE 3
In Vitro Accumulation Studies with Carbon-11-Daunorubicin and Carbon-11-Verapamil in Human Ovary Cancer Cell Lines (n = 3)

Concentration Radiotracer	A2780 pmole/ 10^6 cells	2780^{AD} pmole/ 10^6 cells
[^{11}C]DNR (tracer)	7.68 ± 0.47	0.47 ± 0.47
+ verapamil	7.89 ± 1.32	5.63 ± 0.50
[^{11}C]DNR (2 μM)	129 ± 29	7 ± 5
+ verapamil	319 ± 18	151 ± 4
[^{11}C]VER (tracer)	0.0863 ± 0.0388	0.0188 ± 0.0100
[^{11}C]VER (5 μM)	345 ± 102	77 ± 15

^{11}C-VER might be a promising tracer to investigate P-gp mediated efflux in MDR-resistant tumors with PET. This modulator approach could be an attractive alternative for PET-measurements of radiolabeled cytostatic agents. One drawback might be that the cytostatic efflux function per se of P-gp was not investigated, which is relevant in evaluating treatment protocols with modulators. For this purpose, a radiolabeled cytostatic agent will be required, since the uptake can be manipulated by modulators, but not vice versa.

CONCLUSION

The results obtained from the biodistribution studies and the in vitro experiments with cell lines suggest that P-gp-mediated accumulation in cancer cells can be measured with tracer dosages of a positron-emitting anti-cancer drug or modulator within 1 hr postinjection. Since membrane properties might be different between drug-sensitive and resistant tumors, it could be important to perform blood flow corrections to obtain reliable P-gp efflux measurements in vivo. Work is in progress to develop an animal model with MDR-resistant and sensitive tumors.

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Technetium-99m-Sulfur Colloid for Lymphoscintigraphy: Effects of Preparation Parameters

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There has been a resurgence in the use of lymphoscintigraphy for the external detection of lymph nodes for metastatic melanoma and breast tumors. Technetium-99m-antimony trisulfide colloid was the radiopharmaceutical developed for this procedure and was found to have a narrow distribution of small particles, 0.003–0.03 μm, but it was never approved by the FDA. Technetium-99m-sulfur colloid also forms particles and this article reports on the effects different preparation parameters have on its particle size distribution and stability. **Methods:** Four groups of kits were evaluated, kits which utilized: (a) a reduced heating protocol with a new ^{99m}Tc-elution, (b) a reduced heating protocol with an old ^{99m}Tc-elution, (c) a prolonged heating protocol with a new ^{99m}Tc-elution and (d) a prolonged heating protocol with an old ^{99m}Tc-elution. The particle size distribution and the stability of the different ^{99m}Tc-sulfur colloid kit preparations were evaluated over 6 hr utilizing polycarbonate filters ranging from 0.03 to 10 μm. **Results:** In vitro studies demonstrated no significant change in the particle size distribution over a 6-hr period and all ^{99m}Tc-sulfur colloid preparations had a bimodal particle size distribution pattern. Importantly, heating the kit for shorter periods of times utilizing [^{99m}Tc]pertechnetate, which had a longer ingrowth of [^{99m}Tc]pertechnetate, produced a formulation which had the largest percentage of particles smaller than 0.03 μm. **Conclusion:** In our clinical setting, ^{99m}Tc-sulfur colloid prepared with the reduced heating protocol and utilizing [^{99m}Tc]pertechnetate, which has the highest ingrowth of [^{99m}Tc]pertechnetate has proved to be an excellent agent for lymphoscintigraphy studies. This preparation has demonstrated rapid movement of the particles from the primary site to the lymph nodes in over 97% (106/109) of the patients we have studied.

Key Words: technetium-99m-sulfur colloid; lymphoscintigraphy; particle size distribution

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Increasing data suggest that the absence of tumor in the first node (sentinel node) in a nodal bed receiving lymph drainage from the primary malignant melanoma site predicts the absence of tumor in the other nodes of that bed. Biopsy of a single sentinel node is less costly and produces less morbidity than a total lymph node dissection (*1–4*). Lymphoscintigraphy is an important diagnostic tool for the external detection of the sentinel node. To identify this node, tracer materials are injected around the primary melanoma site, the material is subsequently absorbed into the lymphatic vessels, where it flows through the lymphatic system into the lymph node bed where it becomes trapped. Morton et al. (*4*) demonstrated the usefulness of this procedure by making multiple intradermal injections of a blue dye to localize the lymph nodes. A limitation to the blue dye technique is that the dye is not visible through intervening tissue and therefore is not optimal for external detection of the labeled sentinel node.

Bergqvist et al. (*5*) reported that the particle size of a radiopharmaceutical utilized for lymphoscintigraphy greatly effects its biokinetics and that a particle size of less than 0.1 μm is necessary for migration from the injection site and uptake into the lymph nodes. The radiopharmaceutical initially developed for lymphoscintigraphy was ^{99m}Tc-antimony trisulfide colloid; however, this drug was never approved by the FDA for routine use in the United States and is no longer available even as an investigational agent. Technetium-99m-antimony sulfide colloid had a relatively narrow particle size distribution range, 0.003–0.03 μm (*6*). A second agent which has been utilized overseas is ^{99m}Tc-Nanocoll, which is a ^{99m}Tc-based agent which typically contains 95% of the colloidal particles smaller than 0.08 μm in size (*7*). No agent is available in the United States for lymphoscintigraphy which has the optimal particle size range, but other agents have been utilized for this study, including ^{99m}Tc-human serum albumin (^{99m}Tc-HSA) and ^{99m}Tc-sulfur colloid. Technetium-99m-HSA has been utilized to successfully image flow, but it is not particulate in nature and there is less retention within the lymph nodes (*5*) and delayed images may miss the sentinel node.

In 1969, Hauser et al. (*8*) utilized a gelatin-stabilized ^{99m}Tc-sulfur colloid preparation and to image lymph nodes in rabbits. In addition, a recent report has suggested filtering a standard sulfur colloid preparation through a 0.1-μm membrane filter (*9*). The unfiltered ^{99m}Tc-sulfur colloid was reported to have an average particle size of 305–340 nm and, after filtration, was shown to have an average particle size range of 10 nm with a small (<0.1%) secondary population averaging 89–173 nm (*9*).

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