Scintigraphic Imaging of the Hypoxia Marker $^{99m}$Technetium-labeled 2,2'- (1,4-Diaminobutane)bis(2-methyl-3-butanone) Dioxide ($^{99m}$Tc-labeled HL-91; Prognox): Noninvasive Detection of Tumor Response to the Antivascular Agent 5,6-Dimethylxanthenone-4-acetic Acid

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ABSTRACT

5,6-Dimethylxanthenone-4-acetic acid (DMXAA) and combretastatin A4 phosphate (CA-4-P) markedly inhibit tumor blood flow in mice and are both currently in clinical trial. One of the challenges in clinical evaluation of antivascular agents is the monitoring of tumor blood flow inhibition in individual patients. This study investigates, using mouse models, whether a new marker for tissue hypoxia, $^{99m}$technetium-labeled 2,2'- (1,4-diaminobutane)bis(2-methyl-3-butanone) dioxide ($^{99m}$Tc-labeled HL-91; Prognox) has potential for the scintigraphic monitoring of tumor response to antivascular agents. Determination of radioactivity in dissected tissues 3 h after DMXAA (80 μmol/kg) or CA-4-P (227 μmol/kg) was injected indicated that both drugs inhibited blood flow ($^{86}$RbCl uptake; 84 and 87%, respectively) and increased $^{99m}$Tc-labeled HL-91 levels (350 and 300%, respectively) selectively in murine RIF-1 tumors. Planar imaging of $^{99m}$Tc-labeled HL-91 3 h after DMXAA injection showed a dose-dependent increase in tumor levels above a threshold of 50 μmol/kg; this same threshold was observed for the inhibition of tumor blood flow (determined using Hoechst 33342). DMXAA also inhibited blood flow and increased $^{99m}$Tc-labeled HL-91 uptake—in MDAH-MCA-4 mouse mammary carcinomas and in NZM10 human melanoma xenografts. Whether $^{99m}$Tc-labeled HL-91 might also be useful as a biomarker for tumor cell killing was investigated by clonogenic assay of surviving cells 15 h after injection $^{99m}$Tc-labeled HL-91 in RIF-1 tumors. Log cell kill in individual tumors showed a statistically significant linear correlation ($P < 0.001$) with $^{99m}$Tc-labeled HL-91 uptake after 60 μmol/kg ($r^2 = 0.79$) and 70 μmol/kg ($r^2 = 0.44$) but not at 80 μmol/kg DMXAA. The lack of correlation at high doses presumably reflects the insensitivity of the tumor-averaged $^{99m}$Tc-labeled HL-91 signal to small regions in which tumor blood flow is preserved (which will limit log cell kill). The results indicate the potential of $^{99m}$Tc-labeled HL-91 for the noninvasive imaging of tumor blood flow inhibition by antivascular drugs in humans.

INTRODUCTION

The tumor vasculature is a promising new target in cancer therapy. A large number of agents have been identified that exert antitumor effects through the inhibition of the development of tumor blood vessels (angiogenic agents) or the function of established vessels (antivascular agents). Tumor-selective antivascular agents include TNF1 (1, 2), the Vinca alkaloids vinblastine and vincristine (3, 4), CA-4-P (5, 6), FAA (7–9), and the structural analogue of FAA, DMXAA (10, 11). Both DMXAA (12, 13) and CA-4-P are currently in Phase I clinical trials as tumor-selective antivascular agents.

The mechanisms of antivascular effect of DMXAA and CA-4-P appear to differ. The latter is a tubulin binder, and causes a rapid increase in vascular resistance in tumors (5), possibly as a result of the induction of endothelial cell shape changes (14). Tumor blood flow inhibition by DMXAA may, like that by FAA (15), be attributable to the induction of TNF. Recent studies have shown the selective induction of TNF within tumors in DMXAA-treated mice (16). The decision to evaluate DMXAA clinically, despite the failure of FAA (17), was based in part on the difference in species specificity of TNF induction by the two agents; DMXAA induces TNF in both murine and human cells (18, 19), whereas FAA has less activity in human cells (18, 20). DMXAA also induces a number of other bioactive products (including IP-10, IFNs, IFN regulatory factors, nitric oxide, and serotonin) which could play a role in its antitumor activity (21).

In animal studies, both DMXAA and CA-4-P produce only transient growth inhibition in most tumor models despite profound inhibition of tumor blood flow and extensive hemorrhagic necrosis (6, 11). This modest growth inhibitory activity does not preclude clinical utility because the inhibition of tumor blood flow (especially in poorly perfused regions of tumors) provides an opportunity for enhancing the antitumor activity of other agents (radiation, conventional cytotoxic drugs, bioreductive drugs, and radioimmunotherapy) as illustrated by preclinical studies with DMXAA (11, 22–25) and CA-4-P (6, 26). However, the weak tumor-growth-inhibitory activity of antivascular agents emphasizes the need for the direct measurement of tumor blood flow in clinical studies, rather than reliance on traditional (regression) end points, both for the evaluation of new antivascular agents and as an early assessment of response in individual patients.

The potential of MRI techniques for monitoring tumor-blood-flow inhibition by CA-4-P has been demonstrated in experimental studies (27), and Gd-labeled DTPA-enhanced MRI is being used currently in Phase I studies with DMXAA (13) and CA-4-P. However, this technique is limited to tumor sites distant from the diaphragm, requires access to MRI, and is relatively difficult to interpret. The present study investigates a possible alternative approach for the noninvasive monitoring of tumor blood flow based on scintigraphic imaging.

Inhibition of blood flow in murine tumors by CA-4-P results in the induction of hypoxia as demonstrated by oxygen electrode measurements (28), which suggests the possibility of monitoring changes in tumor hypoxia as a biomarker for blood flow inhibition. Although tumor pO$_2$ has not been measured directly in the case of DMXAA, induction of hypoxia is suggested by the enhanced antitumor activity of hypoxia-selective bioreductive drugs in combination with DMXAA (11, 22, 29) and by the loss of radiation sensitivity of tumors shortly after DMXAA treatment (25). Several methods for detecting tumor hypoxia are under development (30–32), including noninvasive imaging methods (positron emission tomography, MRI, and scintigraphy) based on the selective binding of appropriately labeled $^2$-nitroimidazoles in hypoxic cells (33–35). A number of Tc-labeled agents, 

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3 The abbreviations used are: TNF, tumor necrosis factor; CA-4-P, combretastatin A4 phosphate; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; DTPA, diethylenetriamine pentaacetic acid; FAA, flavone acetic acid; HL-91, 2,2'- (1,4-diaminobutane)bis(2-methyl-3-butanone) dioxime; MRI, magnetic resonance imaging; TC, technetium.
of particular interest because of the ready availability of $^{99m}$Tc in nuclear medicine departments, have also been investigated for imaging tissue hypoxia (36). $^{99m}$Tc-labeled HL-91 (Progonix) is a non-nitroimidazole that is selectively taken up by hypoxic cells in culture by an unknown mechanism (37). It appears to localize in hypoxic regions of murine tumors, as demonstrated by selective binding in poorly perfused regions near necrosis (38), and by the correlation between the uptake of $^{99m}$Tc-labeled HL-91 and the levels of hypoxia as measured with an oxygen electrode (39). Preliminary clinical investigations have shown good tumor-to-normal-tissue background ratios when $^{99m}$Tc-labeled HL-91 is imaged 4 h after administration (40), although its utility as a hypoxia marker in human tumors has not been established.

In the present study, we used planar, scintigraphic imaging to measure the uptake of $^{99m}$Tc-labeled HL-91 in individual tumors in mice. We examined whether $^{99m}$Tc-labeled HL-91 can be used as a marker for the inhibition of tumor blood flow by the antivascular agents DMXAA and CA-4-P and whether the tumor uptake of $^{99m}$Tc-labeled HL-91 after DMXAA treatment is predictive of the antitumor response of individual tumors.

**MATERIALS AND METHODS**

**Drugs.** DMXAA, synthesized in the Auckland Cancer Society Research Centre, was dissolved in PBS, protected from light (41), and stored frozen. CA-4-P, generously supplied by Dr. A. Amiri (OxiGene Europe AB, Lund, Sweden), and Hchoest 33342 (Sigma Chemical Co., St. Louis, MO) were dissolved in saline and stored at $-80^\circ$C. DMXAA and CA-4-P were administered to mice by i.p. injection at 0.01 mg/kg body weight. Hchoest 33342 (0.1 ml/mouse of an 8 mg/ml stock solution) was injected via a tail vein.

HL-91 was kindly supplied by Drs. X. Zhang and J. Ballinger (Ontario Cancer Institute, Toronto, Canada). The labeling of HL-91 was carried out by the addition of $^{99m}$Tc-pertechnetate (~200 MBq) to vials containing 0.25 mg/ml HL-91, 95 mM sodium bicarbonate, and 0.26 mM stannous chloride (stored at $-4^\circ$C as 2-ml aliquots under nitrogen). After a 15-min incubation at room temperature, the radiochemical purity of the $^{99m}$Tc-labeled HL-91 was checked by chromatography using Instant Thin Layer Chromatography-Silica Gel (Gelman Systems) with saline to determine pertechnetate impurity, and Whatman paper 31ET with distilled water to determine insoluble impurity. Labeling purity was $>95\%$ for each preparation. $^{99m}$Tc-labeled DTPA was prepared by the addition of $^{99m}$Tc-pertechnetate (~200 MBq) to vials (1-ml containing 10 mg/ml DTPA trisodium salt, 2.1 mM stannous fluoride, and 5.7 mM ascorbic acid. Radiochemical purity was checked as above. For animal studies, $^{99m}$Tc-labeled HL-91 or $^{99m}$Tc-labeled DTPA was diluted with PBS and injected i.p. at 0.2 MBq/kg body weight.

**Animals and Tumors.** Murine RIF-1 fibrosarcoma cells were obtained from Dr. J. M. Brown (Stanford University, Stanford, CA) and maintained using an in vitro/in vivo passaging protocol (42). Murine mammary carcinoma MDAH-MCa-4 tumors (43) were grown from stocks stored in liquid nitrogen and restrained by clips attached to the ankle so that they were positioned over a window in the lucite base containing a layer of the same polyvinyl chloride plastic. The jig was positioned on the face of a GE Starcam 4000 I gamma camera equipped with a high-resolution collimator. Static images were obtained with an imaging time of 16 min. Radioactivity in each tumor was determined by comparing the tumor-bearing and contralateral leg using the following equation, which corrects for differences in injected dose, rates of excretion, and tumor weight:

\[
\text{Fraction of remaining dose per g of tumor} = \frac{R_T - B_T}{R_W \times W_T}
\]

where $R_T$ is the radioactivity (counts/s) in the tumor, $B_T$ is the background in the normal leg corrected to an area similar in size to the tumor, \(i.e., \frac{B_T}{A_T} = \frac{R_T}{A_L} \) with \(A_T\) being the area of tumor (pixels), \(R_T\) the radioactivity in the normal leg, and \(A_L\) the area of the normal leg. \(R_W\) is the radioactivity in the whole body; and \(W_T\) is the tumor weight, estimated from leg + tumor diameter using a calibration curve based on dissected tumors.

**Hoechst 33342 Staining of Tumor Vessels.** Four h after the administration of DMXAA or of vehicle, mice were injected with Hoechst 33342 and killed 2 min later. The tumors were excised and rapidly frozen. Frozen sections (14 mm) were cut and examined at X10 using a Nikon epifluorescopic fluorescence microscope with a UV-1A filter block [excitation filter 365 (bandwidth 10) nm, barrier filter 400 nm, and dichroic mirror 400 nm]. The number of Hoechst-positive vessels in each field (area = 1.59 mm$^2$) was counted. Ten fields were counted for control sections, and the whole area of each section was scored for tumors from treated mice.

**Antitumor Activity.** Tumor response was assessed by the excision of tumors and clonogenic assay 18 h after treatment, or by the measurement of tumor growth delay. For excision assays, $400-500$ mg of minced tumor were dissociated using an enzyme cocktail (0.5 mg/ml Pronase (Sigma), 0.2 mg/ml collagenase (Sigma), and 0.1 mg/ml DNase 1 (Sigma)) using 1 ml/60 mg tumor, with incubation at $37^\circ$C for 30 min, followed by plating of up to $10^3$ cells.

Fig. 1. Planar image of $^{99m}$Tc-labeled HL-91 in mice bearing i.m. RIF-1 tumors in the right leg, 3 h after i.p. administration of $^{99m}$Tc-labeled HL-91 (0.2 MBq/g).
cells for clonogenic survival. Colonies were stained and counted after incubation for 10 days at 37°C. To assess tumor growth delay, tumor-plus-leg diameters were measured 3 days a week after treatment, and the growth delay (GD) determined as the difference in time to reach 13 mm (1.5-g tumor) between treated and control groups. Specific-growth delay was calculated as GDI, where t is the control volume-doubling time between 10- and 13-mm leg diameter (4.2 days for RIF-1 and 5.4 days for MDAH-MCa-4). Regression analysis was performed using Sigmastat version 2.0 for Windows.

RESULTS

Effects of Antivascular Agents on 99mTc-labeled HL-91 and 86RbCl Uptake by Tumors and Normal Tissues. The biodistribution of 99mTc in RIF-1 tumor-bearing mice was determined 3 h after administration of 99mTc-labeled HL-91 either alone or simultaneously with DMXAA (80 μmol/kg) or CA-4-P (227 μmol/kg); Table 1). In untreated control mice the liver and kidneys retained the highest concentrations of 99mTc-labeled HL-91 (3.23 ± 0.30 and 1.58 ± 0.18% injected dose/g tissue, respectively), with the lowest concentration in the brain (0.10 ± 0.01% injected dose/g tissue) and intermediate levels in tumor, skeletal, and cardiac muscle, spleen, and lung. The administration of DMXAA or CA-4-P resulted in a selective and statistically significant increase (3.5-fold and 3.0-fold, respectively) in tumor concentrations of 99mTc-labeled HL-91. There was no statistically significant change in retention of 99mTc-labeled HL-91 in any of the normal tissues after either antivascular agent, with the exception of a 35% decrease in 99mTc-labeled HL-91 in the liver after DMXAA treatment (Table 1). Tumor:muscle ratios of 99mTc-labeled HL-91 were greatly increased after treatment with DMXAA (12.2 ± 1.7) or CA-4-P (8.0 ± 1.5) compared with the ratios for control mice (2.3 ± 0.6).

Fraction of cardiac output to tumor and normal tissues was assessed in the same animals, from the biodistribution of 86RbCl. The kidneys had the highest concentrations, and the brain the lowest (Table 1). Treatment with the antivascular agents did not significantly alter blood flow to any of the normal tissues, except for a 33% decrease to the spleen after treatment with CA-4-P. Tumor blood flow was substantially inhibited by both DMXAA and CA-4-P, with a reduction in blood flow of 84 or 87%, respectively, compared with that for controls (Table 1).

Imaging Studies. Initial imaging studies were performed on RIF-1-tumor-bearing mice at various times after the administration of 99mTc-labeled HL-91. Fig. 1 shows a planar image of six mice 3 h after administration of 99mTc-labeled HL-91, showing high levels of radioactivity in the viscera and stools relative to the chest and head. 99mTc levels were clearly increased in the tumor-bearing right leg of all the mice. 99mTc-labeled HL-91 uptake in individual tumors, calculated as the fraction of 99mTc-labeled HL-91 remaining per g of tumor, varied little between 2 and 6 h after administration (Fig. 2). Subsequent experiments were performed by imaging 3 h after the injection of 99mTc-labeled HL-91.

Imaging of 99mTc-labeled HL-91 after coadministration with DMXAA demonstrated increased retention of the hypoxia marker in RIF-1 tumors above a threshold DMXAA dose of 50 μmol/kg (Fig. 3). In separate experiments, RIF-1 tumor blood flow was assessed 4 h after DMXAA; the density of vessels supporting blood flow (stained with Hoechst 33342) showed an inverse relationship with 99mTc-labeled HL-91 uptake. Blood flow was inhibited progressively above a threshold dose of ~50 μmol/kg DMXAA, reaching 97% inhibition at 80 μmol/kg (Fig. 3). DMXAA (70 μmol/kg) also produced a severe (80%) inhibition of blood flow in MDAH-MCa-4 murine mammary carcinomas and NZM10 human melanoma xenografts (92% inhibition), determined 4 h after drug administration (Fig. 4a). A DMXAA-induced inhibition of tumor blood flow was associated with a 2.0-fold or 3.1-fold increase in tumor uptake of 99mTc-labeled HL-91 for MDAH-MCa-4 or NZM10 tumors, respectively (Fig. 4b).

To clarify whether the mechanism by which DMXAA increases uptake of 99mTc-labeled HL-91 in tumors involves (a) trapping of the hypoxia marker as the result of decreased clearance or (b) induction of tumor hypoxia, similar imaging experiments were performed using 99mTc-labeled DTPA. There is no evidence of selective accumulation of 99mTc-labeled DTPA in hypoxic cells. Groups of six mice received 99mTc-labeled DTPA (0.2 MBq/g) alone, at the same time as DMXAA (80 μmol/kg), or 1 h after DMXAA; tumors were imaged

Table 1 Biodistribution data in RIF-1-tumor-bearing mice for 99mTc-labeled HL-91 (0.2 MBq/g) coadministered with DMXAA (80 μmol/kg) or CA-4-P (227 μmol/kg), and for 86RbCl (0.05 MBq/g) administered 3 h after 99mTc-labeled HL-91

<table>
<thead>
<tr>
<th>Tissue</th>
<th>99mTc-CL-HL-91a</th>
<th>86RbClb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>0.41 ± 0.04</td>
<td>1.58 ± 0.07</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.24 ± 0.07</td>
<td>1.85 ± 0.19</td>
</tr>
<tr>
<td>Liver</td>
<td>3.23 ± 0.30</td>
<td>2.20 ± 0.13</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.57 ± 0.02</td>
<td>4.36 ± 0.23</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.58 ± 0.18</td>
<td>25.67 ± 3.25</td>
</tr>
<tr>
<td>Lung</td>
<td>0.53 ± 0.06</td>
<td>8.64 ± 0.81</td>
</tr>
<tr>
<td>Brain</td>
<td>0.10 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

Values represent the % injected dose per g tissue and are expressed as mean ± SE.
Statistically different from control (P < 0.001).
Statistically different from control (P < 0.01).

Fig. 2. Time dependence of 99mTc-labeled HL-91 retention in individual i.m. RIF-1 tumors. Different symbols refer to individual mice.
3 h after the injection of $^{99m}$Tc-labeled DTPA. There was a small increase in tumor uptake from $3.2 \pm 0.5$ in mice treated with $^{99m}$Tc-labeled DTPA alone, to $4.6 \pm 0.9$ for mice receiving $^{99m}$Tc-labeled DTPA and DMXAA simultaneously, and to $5.0 \pm 0.7$ for mice injected with $^{99m}$Tc-labeled DTPA 1 h after DMXAA, but these effects were not statistically significant.

Relationship between $^{99m}$Tc-labeled HL-91 Uptake and Antitumor Activity in DMXAA-treated Mice. The relationship between $^{99m}$Tc-labeled HL-91 uptake and the antitumor activity of DMXAA in the same tumors was assessed by tumor growth delay after scintigraphic imaging in the same animals. Plotting the mean tumor growth delay against mean tumor $^{99m}$Tc-labeled HL-91 levels at 3 h gave a consistent trend for both RIF-1 and MDAH-MCa-4 tumors (Fig. 5). This was statistically significant by linear regression only for RIF-1 ($P = 0.034$), although it could not be concluded that the relationship was different for the two tumors.

Subsequent investigation of the $^{99m}$Tc-labeled HL-91 uptake and antitumor activity in the same animals was based on the measurement of clonogenic cells in RIF-1 tumors 18 h after treatment with DMXAA (Fig. 6), which showed a much larger response than the growth delay assay. Mean values for groups treated at different DMXAA doses (Fig. 6a) showed no enhancement of $^{99m}$Tc-labeled HL-91 uptake or cell killing by DMXAA at $\leq 50 \mu$mol/kg but showed a highly significant linear correlation at higher doses. Examination of the individual tumors within each dose level indicated that at 60 and 70 $\mu$mol/kg, there was a highly significant correlation between tumor cell killing and $^{99m}$Tc-labeled HL-91 uptake, with explained variance ($r^2$ values) of 0.79 and 0.44, respectively (Fig. 6b and 6c). At the highest DMXAA dose of 80 $\mu$mol/kg, the correlation between these two parameters was not significant (Fig. 6d).

**DISCUSSION**

The results confirm previous reports (11, 22) that DMXAA profoundly inhibits blood flow in MDAH-MCa-4 tumors, as demonstrated here by decreased perfusion measured by in vivo staining of microvessels with i.v.-administered Hoechst 33342 (Fig. 4). We extend this observation to the RIF-1 fibrosarcoma and NZMN10 human melanoma xenografts and show that this inhibition is accompanied in all three of the tumors by an increased uptake of $^{99m}$Tc-labeled HL-91. The present study also confirms that the antivascular effect of DMXAA (80 $\mu$mol/kg) and of CA-4-P (227 $\mu$mol/kg) is tumor-specific in mice, with 84 and 87% reduction, respectively, in fraction of cardiac output to RIF-1 tumors at 3 h but with no significant change in most normal tissues (Table 1). Similar data have been reported for mice bearing CaNT tumors treated with CA-4-P (6). The only normal tissue to be affected in the present study was the spleen with a small (35%) reduction in blood flow after CA-4-P; Tozer et al. (44) also found the spleen to be the most affected normal tissue in a study of blood flow inhibition by CA-4-P in BD9 rats.

The inhibition of blood flow by DMXAA was associated with significantly increased uptake of the hypoxia marker $^{99m}$Tc-labeled HL-91 in all of the three tumors investigated, as determined by noninvasive imaging of mice with tumors in the gastrocnemius muscle 3 h after coadministration of the antivascular agent and hypoxia marker (Fig. 4). Two lines of evidence suggest that the increased $^{99m}$Tc-labeled HL-91 uptake is a direct consequence of blood flow inhibition: (a) a comparison of $^{86}$RbCl and $^{99m}$Tc-labeled HL-91 uptake in the same animals (by dissection of tissues and off-line gamma counting) shows that increased $^{99m}$Tc-labeled HL-91 uptake, after treatment with DMXAA or CA-4-P, is restricted to the tissue showing blood flow inhibition (i.e., tumor), with the exception of minor changes in blood flow to the spleen and $^{99m}$Tc-labeled HL-91 uptake by the liver, after treatment with CA-4-P and DMXAA, respectively; (b) the dose threshold of 50 $\mu$mol/kg for the inhibition of blood flow is the same as that for enhanced $^{99m}$Tc-labeled HL-91 uptake in RIF-1 tumors (Fig. 3).

These observations support the original hypothesis that increases in
NONINVASIVE MONITORING OF $^{99m}$Tc-LABELED HL-91 UPTAKE

Fig. 6. Relationship between cell killing in i.m. RIF-1 tumors and $^{99m}$Tc-labeled HL-91 uptake. $\mu$g, averaged data for groups of at least 6 mice, shown as mean $\pm$ SE. DMXAA doses (mg/kg) are indicated beside data points. Relationship for individual tumors treated with (b) 60 mg/kg, (c) 70 mg/kg, and (d) 80 mg/kg DMXAA.

Fig. 5. Relationship between growth delay of i.m. MDAH-MCa-4 tumors (open symbols) or RIF-1 tumors (filled symbols) and $^{99m}$Tc-labeled HL-91 uptake after treatment with DMXAA doses (mg/kg) as indicated. Values are mean $\pm$ SE for 5–6 mice for controls and 9–12 mice for DMXAA-treated groups.

The present results argue that $^{99m}$Tc-labeled HL-91 has considerable potential for the noninvasive monitoring of tumor blood flow in clinical studies of antivascular agents. In off-line gamma counting studies (Table 1), large increases in tumor levels of $^{99m}$Tc-labeled HL-91 were achieved 3 h after DMXAA or CA-4-P treatment (3.5- and 3.0-fold increases, respectively), providing high tumor:muscle ratios (12.2 $\pm$ 1.7 and 8.0 $\pm$ 1.5, respectively). Increased tumor uptake of $^{99m}$Tc-labeled HL-91 after coadministration with DMXAA could be readily detected using planar scintigraphic imaging of mice with ~0.6-g tumors in the leg. In the imaging studies, DMXAA, at 80 mg/kg, increased tumor levels of $^{99m}$Tc-labeled HL-91 by 2.9-fold at 3 h, which was broadly consistent with the 3.5-fold increase determined by off-line counting. This large change in $^{99m}$Tc-labeled HL-91 uptake in tumor relative to non-DMXAA-treated animals was confirmed in the other two tumors investigated, using 70 mg/kg DMXAA (2.8-, 2.0-, and 3.1-fold increases for RIF-1, MDAH-MCa-4, and NZMN10, respectively; Fig. 4b), and demonstrated the possibility of detecting drug-induced changes in tumor blood flow/hypoxia in individual patients by imaging with $^{99m}$Tc-labeled HL-91 before and after DMXAA treatment. This approach would have a number of advantages over Gd-labeled DTPA-enhanced MRI, including the ready availability of $^{99m}$Tc-based planar and single-photon emission computed tomography imaging and the applicability to most tumor sites. $^{99m}$Tc-labeled HL-91 was developed as a hypoxia marker for clinical application by Nycomed Amersham, although the company is no longer pursuing its development. The proposed application is based on the detection of acute changes in hypoxia, by comparing $^{99m}$Tc-labeled HL-91 scans before and after treatment with antivascular agents, which is likely to be more straightforward than its use to determine absolute levels of hypoxia in tumors.

If changes in $^{99m}$Tc-labeled HL-91 levels provide a noninvasive method for monitoring the inhibition of tumor blood flow, the question then arises as to whether this might also have utility as a biomarker for the antitumor activity of antivascular agents. The relationship between $^{99m}$Tc-labeled HL-91 uptake 3 h after DMXAA treatment and subsequent antitumor response in the same tumors was, therefore, assessed, with tumor response initially determined using tumor regrowth delay as the end point. As observed in previous studies (11, 22), there was rapid regrowth of both RIF-1 and MDAH-MCa-4 tumors (Fig. 5) despite substantial inhibition of tumor blood flow (Fig. 4). Histological studies have shown that there are islands of residual viable tissue after DMXAA treatment, especially at the tumor periphery, as also noted with CA-4-P (5), and that tumors regrow rapidly from this surviving tissue (11). This small antitumor effect made it difficult to discern the relationship between $^{99m}$Tc-labeled HL-91 uptake and tumor growth delay for individual tumors, although the group means did show a trend toward higher growth delays with increasing uptake. Linear regression analysis showed this trend to be statistically significant for the RIF-1 tumor but not for the MDAH-MCa-4 tumor, although the slopes of the regression lines were not significantly different for the two tumors, and the explained variance...
The role of hypoxia markers such as 99mTc-labeled HL-91 in assessing tumor response to antivascular drugs needs to be determined in clinical studies. A direct comparison, between Gd-labeled DTPA-enhanced MRI and 99mTc-labeled HL-91 uptake, for detecting inhibition of tumor blood flow by antivascular agents should be made during these studies. If increases in marker uptake in tumors can be demonstrated after drug treatment, the challenge will be to establish that the enhanced uptake reflects blood flow inhibition and that it provides a useful biomarker of antitumor response. The above tumor growth-delay studies suggest that the latter question may be difficult to answer using tumor regression end points after treatment with antivascular drugs as single agents. However, if the large responses seen in preclinical studies when DMXAA is combined with other classes of drugs (11, 23, 29, 45) can be translated into the clinic, then noninvasive monitoring of hypoxia marker uptake may provide a useful early-response marker.

ACKNOWLEDGMENTS

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REFERENCES


8. Zwi, L. J., Baguley, B. C., Gavin, J. B., and Wilson, W. R. Blood flow failure as a marker of tumor response to DMXAA was more readily defined by determining clonogenic survival of RIF-1 cells 18 h after treatment, which provided a much more sensitive end point (Fig. 6). As expected from the blood flow inhibition data, there was no detectable tumor cell kill at DMXAA doses less than 50 μmol/kg, but there was clear cell killing at higher doses. There was a highly significant correlation between tumor cell killing, measured by clonogenic assay after the excision of tumors, and tumor uptake of 99mTc-labeled HL-91 as illustrated in Fig. 6a, which shows the group means. Analysis of these data for individual tumors at each DMXAA dose level revealed that at intermediate doses of 60 and 70 μmol/kg, there was an excellent correlation between tumor cell killing and tumor uptake of 99mTc-labeled HL-91 (Fig. 6, b and c). The lack of a significant correlation at a dose of 80 μmol/kg DMXAA (Fig. 6d) may result from saturation of 99mTc-labeled HL-91 uptake at high doses. Because 99mTc-labeled HL-91 uptake is an arithmetic mean for the whole tumor, it will be relatively insensitive to small “cold” regions in which blood flow is not inhibited, and is not expected to discriminate the magnitude of effect in tumors with a marked inhibition of blood flow (as illustrated by the data of Fig. 3, which shows no significant further increase in 99mTc-labeled HL-91 uptake at DMXAA doses giving >75% inhibition of Hoechst perfusion). Also, if inhibition of blood flow were too severe, it might interfere with the supply of the hypoxia marker to the hypoxic tissue, which could also contribute to the apparent saturation of effect at high DMXAA dosage. In contrast, tumor cell kill is readily measured on a logarithmic scale; under these conditions, differences in residual tumor perfusion will have a marked effect on tumor cell killing that is attributable to ischemia. To illustrate this, one would not expect to detect a difference in 99mTc-labeled HL-91 uptake between two tumors with 90 versus 99% loss of perfusion, but if antivascular effect translates directly into the magnitude of cell kill, then the expected 10-fold difference in surviving fraction should be readily detectable. This interpretation (and the results of Fig. 6) argue that, as a potential biomarker for DMXAA response in the clinic, 99mTc-labeled HL-91 is more likely to be useful in discriminating nonresponders from responders rather than ranking the extent of response in responding patients.

The relationship between 99mTc-labeled HL-91 uptake and antitumor response to DMXAA was more readily defined by determining clonogenic survival of RIF-1 cells 18 h after treatment, which provided a much more sensitive end point (Fig. 6). As expected from the blood flow inhibition data, there was no detectable tumor cell kill at DMXAA doses less than 50 μmol/kg, but there was clear cell killing at higher doses. There was a highly significant correlation between tumor cell killing, measured by clonogenic assay after the excision of tumors, and tumor uptake of 99mTc-labeled HL-91 as illustrated in Fig. 6a, which shows the group means. Analysis of these data for individual tumors at each DMXAA dose level revealed that at intermediate doses of 60 and 70 μmol/kg, there was an excellent correlation between tumor cell killing and tumor uptake of 99mTc-labeled HL-91 (Fig. 6, b and c). The lack of a significant correlation at a dose of 80 μmol/kg DMXAA (Fig. 6d) may result from saturation of 99mTc-labeled HL-91 uptake at high doses. Because 99mTc-labeled HL-91 uptake is an arithmetic mean for the whole tumor, it will be relatively insensitive to small “cold” regions in which blood flow is not inhibited, and is not expected to discriminate the magnitude of effect in tumors with a marked inhibition of blood flow (as illustrated by the data of Fig. 3, which shows no significant further increase in 99mTc-labeled HL-91 uptake at DMXAA doses giving >75% inhibition of Hoechst perfusion). Also, if inhibition of blood flow were too severe, it might interfere with the supply of the hypoxia marker to the hypoxic tissue, which could also contribute to the apparent saturation of effect at high DMXAA dosage. In contrast, tumor cell kill is readily measured on a logarithmic scale; under these conditions, differences in residual tumor perfusion will have a marked effect on tumor cell killing that is attributable to ischemia. To illustrate this, one would not expect to detect a difference in 99mTc-labeled HL-91 uptake between two tumors with 90 versus 99% loss of perfusion, but if antivascular effect translates directly into the magnitude of cell kill, then the expected 10-fold difference in surviving fraction should be readily detectable. This interpretation (and the results of Fig. 6) argue that, as a potential biomarker for DMXAA response in the clinic, 99mTc-labeled HL-91 is more likely to be useful in discriminating nonresponders from responders rather than ranking the extent of response in responding patients.

The role of hypoxia markers such as 99mTc-labeled HL-91 in assessing tumor response to antivascular drugs needs to be determined in clinical studies. A direct comparison, between Gd-labeled DTPA-enhanced MRI and 99mTc-labeled HL-91 uptake, for detecting inhibition of tumor blood flow by antivascular agents should be made during these studies. If increases in marker uptake in tumors can be demonstrated after drug treatment, the challenge will be to establish that the enhanced uptake reflects blood flow inhibition and that it provides a useful biomarker of antitumor response. The above tumor growth-delay studies suggest that the latter question may be difficult to answer using tumor regression end points after treatment with antivascular drugs as single agents. However, if the large responses seen in preclinical studies when DMXAA is combined with other classes of drugs (11, 23, 29, 45) can be translated into the clinic, then noninvasive monitoring of hypoxia marker uptake may provide a useful early-response marker.

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Scintigraphic Imaging of the Hypoxia Marker 99mTc-labeled 2,2′-(1,4-Diaminobutane)bis(2-methyl-3-butanone) Dioxime (99mTc-labeled HL-91; Prognox): Noninvasive Detection of Tumor Response to the Antivascular Agent 5,6-Dimethylxanthenone-4-acetic Acid

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