A Systematic Comparison of ¹⁸F-C-SNAT to Established Radiotracer Imaging Agents for the Detection of Tumor Response to Treatment

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Abstract

Purpose: An early readout of tumor response to therapy through measurement of drug or radiation-induced cell death may provide important prognostic indications and improved patient management. It has been shown that the uptake of ¹⁸F-C-SNAT can be used to detect early response to therapy in tumors by positron emission tomography (PET) via a mechanism of caspase-3–triggered nanoaggregation.

Experimental Design: Here, we compared the preclinical utility of ¹⁸F-C-SNAT for the detection of drug-induced cell death to clinically evaluated radiotracers, ¹⁸F-FDG, ⁹⁹mTc-Annexin V, and ¹⁸F-ML-10 in tumor cells in culture, and in tumor-bearing mice in vivo.

Results: In drug-treated lymphoma cells, ¹⁸F-FDG, ⁹⁹mTc-Annexin V, and ¹⁸F-C-SNAT cell-associated radioactivity correlated well to levels of cell death (R² > 0.8; P < 0.001), with no correlation measured for ¹⁸F-ML-10 (R² = 0.05; P > 0.05). A similar pattern of response was observed in two human NSCLC cell lines following carboplatin treatment. EL-4 tumor uptake of ⁹⁹mTc-Annexin V and ¹⁸F-C-SNAT were increased 1.4- and 2.1-fold, respectively, in drug-treated versus naïve control animals (P < 0.05), although ⁹⁹mTc-Annexin V binding did not correlate to ex vivo TUNEL staining of tissue sections. A differential response was not observed with either ¹⁸F-FDG or ¹⁸F-ML-10.

Conclusions: We have demonstrated here that ¹⁸F-C-SNAT can sensitively detect drug-induced cell death in murine lymphoma and human NSCLC. Despite favorable image contrast obtained with ¹⁸F-C-SNAT, the development of next-generation derivatives, using the same novel and promising uptake mechanism, but displaying improved biodistribution profiles, are warranted for maximum clinical utility. Clin Cancer Res; 21(17) September 1, 2015; 3896–905. © 2015 AACR.

Introduction

The early assessment of drug-induced tumor cell death is of great prognostic value—enabling rapid selection of the most efficacious treatment using a personalized medicine approach. In the clinic, current methods to detect response rely on measuring changes in tumor size under the guidelines of the Response Evaluation Criteria in Solid Tumors (RECIST; ref. 1). This approach, however, lacks sensitivity, and many weeks may elapse before there is evidence on imaging of tumor volume shrinkage.

Cell death, through apoptotic, necrotic or alternate pathways (2), is the surrogate endpoint for most anticancer therapies and treatments (3, 4). Given the inadequacy of current techniques for monitoring drug response, there have been considerable efforts to develop new techniques to detect and image treatment efficacy through measurements of tumor cell death (recently reviewed in ref. 5). As drug-induced cell death manifests itself in many different forms, numerous divergent imaging strategies have been used, targeting a range of both direct and indirect biomarkers. Direct imaging biomarkers include caspase-3, whose activation commits the cell to programmed (apoptotic) cell death (6, 7), and plasma membrane depolarization (8, 9), which occurs downstream of caspase-3 (10) or is exposed as a result of membrane rupture during necrosis (8). Indirect methods include measurements of cell metabolism (11, 12), the release of enzymes from intracellular compartments (13, 14), or the increased diffusion of water that results from loss of cellularity (15).

Radiotracer imaging techniques have shown great promise for the noninvasive detection of cell death. Early alterations in glucose metabolism following therapy, characteristic of both stressed and dying cells, have been detected by ²-¹⁸F-fluoro-2-deoxy-D-glucose (¹⁸F-FDG) positron emission tomography (PET) in a range of tumor types (16–19). Furthermore, a draft framework has been proposed for the routine use of ¹⁸F-FDG-PET for response-assessment in the clinic (20). In addition to ¹⁸F-FDG, the small-molecule radiotracer 2-(5-fluoropentyl)-2-methyl malonic acid (¹⁸F-ML-10) has shown utility for cell death imaging preclinically (21, 22), with first-in-man trials recently reported (23). Out of all the currently available cell death imaging probes, ⁹⁹mTc-HYNIC-labeled Annexin V (⁹⁹mTc-Annexin V) has been the most widely studied, having been used to image drug-induced cell death in human head and neck squamous cell carcinoma (24) and tumors of the breast, lymphoma, and lung (25). ⁹⁹mTc-Annexin V binds to phosphatidylserine (PS), a temporally stable biomarker exposed following either apoptotic or necrotic cell death. ⁹⁹mTc-Annexin V, however, has failed to progress beyond phase II/III clinical trials due to its poor biodistribution profile (26).
Translational Relevance

Patients with similar tumor types frequently respond differently to the same therapy. An early assessment of treatment response in individual patients would allow rapid selection of the most effective treatment. Therapy response in the clinic currently relies on measurements of tumor size, which is ineffectual as an early response marker. In response, a number of positron emission tomography (PET) and single photon emission computed tomography (SPECT) radiotracers have been developed to noninvasively measure therapy-induced cell death in a range of human cancers. Despite early promise, none of these imaging agents have made it into routine clinical practice. The development of new radiotracers to assess response to therapy is required if the goal of personalized medicine for the treatment of cancer is to be realized. Here, we designed a novel caspase-3–triggered cyclization and nanoaggregation radiotracer with improved sensitivity for the detection of cell death in comparison with current gold standard in molecular imaging.

Despite the promise of noninvasive imaging techniques for the assessment of tumor cell death, no imaging agent has yet been accepted into routine clinical practice. This has prompted the development of a new generation of agents designed to monitor cell death. One such radiotracer, 18F-C-SNAT, has been shown in preliminary studies to detect early response to therapy in tumors by PET via a novel mechanism of caspase-3–triggered cyclization and nanoaggregation (Supplementary Fig. S1; ref. 7). Here, we directly compared the performance of 18F-C-SNAT to established radiotracers, 18F-FDG, 99mTc-Annexin V, and 18F-ML-10 (Fig. 1), for the noninvasive detection of cell death. We found that all radiotracers except 18F-ML-10 were able to sensitively measure drug-induced cell death in cell culture. However, only 18F-C-SNAT was able to measure treatment response in vivo. Together, our analysis suggests that 18F-C-SNAT may have utility for the detection of drug-induced cell death in the clinic.

Materials and Methods

Cell culture

EL-4 murine lymphoma cells and PC-9 human NSCLC (ATCC) were grown in complete RPMI medium (Life Technologies; 11.1 mmol/L glucose), supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. A549 cells (ATCC) were grown in DMEM medium (Life Technologies; 25 mmol/L glucose), supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were propagated at 37°C in a humidified atmosphere containing 5% CO2. EL-4 cells could not be authenticated as they were of non-human origin, with A549 and PC-9 cells purchased from an authenticated cell biobank just prior to publication.

Radiotracers

Radiosynthesis of 18F-FDG, 99mTc-Annexin V, 18F-ML-10, and 18F-C-SNAT was based on previously described methodology, as detailed in the Supplementary Data.

Cell uptake studies

Eighteen to 20 hours after either etoposide (Sigma-Aldrich; 15 μmol/L) or vehicle treatment (DMSO), EL-4 cells were split into two fractions for analysis: (i) assessment of radiotracer cell uptake and binding, (ii) Cell death determination by flow cytometry. For cell uptake studies, 104 EL-4 cells were incubated for 10, 30, or 60 minutes in fresh RPMI (1 mL) at 37°C with respective radiotracer to determine uptake kinetics. PC-9 and A549 cells were seeded at 105 cells per well of a 6-well plate 24 hours prior to carboplatin treatment (Sagent; 200 μmol/L; 72 hours), as previously described (27). For all cell uptake experiments, the radiotracers were added at the following concentrations and specific activities: 18F-FDG, 0.185 MBq/mL, <0.38 ± 0.08 μmol/L, >2.6 GBq/μmol; 99mTc-Annexin V, 10 nmol/L, >0.185 MBq/mL, 21.1 ± 2.2 GBq/μmol; 18F-ML-10, 1.48 MBq/mL, <0.18 μmol/L, >7.4 GBq/μmol; 18F-C-SNAT, 1.48 MBq/mL, 0.15 ± 0.007 μmol/L, 123.2 ± 61.4 GBq/μmol. Following incubation for the allotted time, adherent cells were trypsinized (0.25% trypsin; 1 mmol/L EDTA) and harvested by centrifugation (1,200 g; 3 minutes; 4°C). Detached cells present in the media before trypsinization were retained and pooled with the trypsinized cells. Suspension cells were collected by centrifugation (1,200 × g; 3 minutes; 4°C), and all cells were washed three times with ice-cold Hank’s Buffered Salt Solution (HBSS; 1 mL, 1,200 × g; 3 minutes; 4°C), with the final cell pellet lysed in 200 μL RIPA buffer (Thermo Fisher Scientific Inc.). One hundred microliters of cell lysate was transferred to counting tubes and decay-corrected radioactivity was determined on a gamma counter (Cobra II Auto-Gamma counter, Packard Biosciences Co.). The remaining lysate was frozen and used for protein determination following radioactive decay using

![Chemical structure and molecular weight for 18F-FDG, 99mTc-Annexin V, 18F-ML-10, and 18F-C-SNAT. The radioisotope is shown in bold type.](Image)

Figure 1.
a bicinchoninic acid (BCA) 96-well plate assay (Thermo Fisher Scientific Inc.). Ten microliters of standards from the original nonwashed cell/media suspension was counted to quantitate the percentage of radiotracer uptake/binding in cells.

To correlate cell-associated radioactivity with levels of drug-induced cell death, mixtures of vehicle and etoposide-treated EL-4 cells were prepared 18 to 20 hours after either vehicle or drug addition. Mixtures contained 0%, 25%, 50%, 75%, and 100% v/v etoposide-treated cells in 6-ml total volume, with the remaining volume made from vehicle-treated cell suspension. One million cells were subsequently collected for cell death analysis by flow cytometry, with the remaining cells used to assess cell-associated radioactivity 60 minutes after radiotracer addition, as described above.

Detection of cell death in vitro

Apoptosis and necrosis in cell mixtures containing 0% to 100% drug-treated cells were visualized by flow cytometry using a method adapted from ref. 12, in parallel to cell uptake studies. Fluorescein isothiocyanate (FITC)–Annexin V (BioLegend) in combination with 7-amino-antimycin D (7-AAD; BioLegend) were used for cell death determination. Early apoptotic cells were defined as cells positively stained for FITC–Annexin V but not 7-AAD, with both late apoptotic and necrotic classes as cells positive for both stains. FlowJo (v.7.6.5; Tree Star, Inc.) was used for analysis.

In vivo tumor models

All experimental procedures involving animals were approved by Stanford University Institutional Animal Care and Use Committee. EL-4 tumor cells (5 × 10⁶ in 100 μl PBS) were injected subcutaneously on the back of female C57BL/6 mice (aged 6–8 weeks; Charles River Laboratories) and grown to approximately 150 mm³, over 6 days. Tumor dimensions were measured daily using a caliper, with tumor volumes calculated by

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\text{volume} = \frac{a \times b \times c}{6} \quad (a, b, \text{ and } c \text{ represent three orthogonal axes of the tumor (27). At day 6, animals were size-matched and either treated with clinically formulated etoposide (Topsar, Novaplus; 70 mg/kg) or left untreated. Treatment–response analysis was performed 24 hours after drug treatment, 7 days after tumor cell implantation. In a separate cohort of mice, the effect of drug-treatment on tumor volume was followed up to 96 hours after therapy. For all other experiments, animals were culled 25.5 hours after therapy and tissues excised for analysis.}

Imaging studies

PET imaging scans were carried out on a docked Siemens Inveon PET/CT scanner (matrix size, 128 × 128 × 159; CT attenuation-corrected; nonscatter corrected), following a bolus i.v. injection of approximately 10 MBq of the radiotracer into tumor-bearing mice (n = 3–4 per group). Dynamic scans were acquired in list mode format over 90 minutes, as described in detail in the Supplementary Data.

Biodistribution studies

Approximately 10 MBq of radiotracer was injected via the tail vein of anesthetized EL4 tumor-bearing C57BL/6 mice (n = 4–8 per group). These mice were maintained under anesthesia and warmed to 37°C to replicate imaging conditions. At 90 minutes after radiotracer injection, animals were sacrificed by exsanguination via cardiac puncture. For all animals, tumors were excised immediately upon death, weighed, and rapidly placed in 10% formalin (Fisher Scientific) for fixation. Tumor and tissue radioactivity was subsequently determined on a gamma counter (decay-corrected; Cobra II Auto-Gamma counter, Packard Biosciences Co.). Predefined 3.7 MBq standards (250 μl) were also counted for data normalization to injected dose. Data were expressed as percent injected dose per gram of tissue (%ID/g).

Immunohistochemistry

Formalin-fixed tumors were embedded in paraffin, sectioned into 5-μm thick slices and placed on microscope slides according to standard procedures (Histo-Tec Laboratory). Sections were taken at regular intervals across the entire tumor volume in order to capture previously described heterogeneity of EL-4 tumors (28). Sections were stained with either hematoxylin and eosin (H&E; Histo-Tec) or with a colorimetric terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay using a kit by Promega (DeadEnd), according to the manufacturer’s instructions, with each section digitized using a NanoZoomer 2.0RS whole-slide scanner (Hamamatsu). The percentage of TUNEL-positive cells was measured across the entire tumor section using ImageJ (v.1.47; NIH, Bethesda, Maryland). Six sections were analyzed per tumor, with three tumors evaluated per treatment group (naïve or drug-treated) for each radiotracer studied. TUNEL positivity was compared with the corresponding tumor uptake values previously determined by biodistribution.

Statistical analysis

Data were expressed as mean ± SD, with the exception of tumor volume measurements, which were presented as mean ± SEM. Statistical significance was determined using the Student t test (Microsoft Excel). For correlation analysis, linear regression, statistical significance, and 95% confidence levels were determined using Prism software for Mac OS X (v.6.0e; GraphPad Software). Differences between groups were considered significant if P < 0.05.

Results

Measurements in cells

Time course of radiotracer accumulation. EL-4 murine lymphoma cells rapidly undergo caspase-3–mediated cell death following etoposide treatment (12). By 18 hours after therapy, cell death had reached 48.9% ± 6.7% in comparison with 2.6% ± 1.4% in vehicle-treated cells, as determined by flow cytometry (Fig. 2A; P < 0.001; n = 4). Temporal 18F-FDG, 99mTc-Annexin V, 18F-ML-10, and 18F-C-SNAT uptake/cell binding was measured at 18 to 20 hours after drug treatment to permit the comparison of their uptake kinetics (Fig. 2B). 18F-FDG cell uptake increased linearly with time in vehicle-treated cells, rising from 3.1% ± 1.2% radioactivity/mg protein at 10 minutes after radiotracer addition, to 10.7% ± 2.2% radioactivity/mg protein by 60 minutes. In comparison, 18F-FDG uptake in drug-treated cells at 60 minutes was 2.0% ± 0.7% radioactivity/mg protein, 5.3-fold lower than vehicle controls (n = 4; P = 0.0008). Conversely, high 99mTc-Annexin V binding to drug-treated cells was evident by just 10 minutes after radiotracer addition, at 110.4% ± 4.0% radioactivity/mg protein, with significantly lower binding measured in vehicle controls (6.0% ± 1.9% radioactivity/mg protein; P < 0.001; n = 3). By 60 minutes after radiotracer addition, 99mTc-Annexin V binding was 24.3-fold lower than vehicle controls.

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higher in drug-treated versus vehicle-control cells, at 173.2% ± 15.7% radioactivity/mg and 7.1% ± 2.4% radioactivity/mg, respectively (P < 0.001; n = 3). No difference in EL-4 cell uptake in treated versus control cells was detectable with 18F-ML-10 over the 60 minutes time course (P > 0.05; n = 3). Cell-associated radioactivity at 60 minutes in etoposide-treated cells was 3 orders of magnitude lower than 99mTc-Annexin V, at 0.10% ± 0.04% radioactivity/mg. 18F-C-SNAT retention in drug-treated cells followed an initial phase of slow uptake for the initial 30 minutes, followed by a rapid increase in the rate of retention over the proceeding 30 minutes. There was no change in 18F-C-SNAT cell uptake in vehicle control cells over the 60 minutes time course. At 60 minutes, 18F-C-SNAT cell uptake was 4.5-fold higher in drug-treated cell in comparison with vehicle controls, at 1.5% ± 0.2% radioactivity/mg and 0.3% ± 0.1% radioactivity/mg, respectively (P < 0.001; n = 3).

**Correlation to cell death.** Radioactive uptake and EL-4 cell binding were further compared with levels of drug-induced cell death in culture. Here, different ratios of drug-to-vehicle–treated cells were combined to create a large dynamic range of dying cells, from approximately 3% to 80% of the total cell population (Fig. 3). As both early and late apoptotic/necrotic cells were present in the drug-treated samples used to prepare the sample series (Supplementary Fig. S2), it was impossible to determine the contribution of each mechanism of death to cell uptake using this method. Instead, cell-associated radioactivity was compared with cells identified as positively stained by FITC–Annexin V, defined here as “dying cells.” Both 99mTc-Annexin V and 18F-C-SNAT cell uptake/binding significantly correlated with levels of cell death (R² = 0.985 and 0.924, respectively; P < 0.001), with 18F-FDG uptake negatively correlated to death (R² = 0.842; P < 0.001). There was no statistically significant correlation found for 18F-ML-10 (R² = 0.053; P > 0.05). It is important to note that 99mTc-Annexin V was correlated to a fluorescent Annexin V derivative used for the assessment of cell death, a potential limitation of this comparison.

**Monitoring therapy response in NSCLC.** To explore whether the radioactive uptake results in EL-4 lymphoma cells were generalizable to other tumor cell lines taken from solid tumors, we next assessed radioactive uptake in either vehicle or carboplatin-treated human PC-9 and A549 NSCLC cells; a tumor type well known for its relative resistance to common chemotherapeutics (29). Seventy-two hours of carboplatin treatment induced extensive cell death in both lines (Supplementary Fig. S3A). In PC-9 and A549 cells, drug treatment resulted in a significant 69.8% (P < 0.001; n = 3) and 43.9% (P = 0.01; n = 3) reduction in 18F-FDG uptake 60 minutes after radiotracer addition (Supplementary Fig. S3B). For 99mTc-Annexin V, radioactivity was increased 5- and 4-fold in PC-9 and A549 cells, respectively in comparison with vehicle control cells (P < 0.001; n = 3). Similarly, 18F-C-SNAT was increased 3.8-fold (P = 0.03) and 2.9-fold (P < 0.001) in PC-9 and A549, respectively (n = 3). For 18F-ML-10, no significant increase observed in drug-treated cells versus vehicle for either PC-9 or A549 cells, in agreement with data obtained for EL-4 cells.
Monitoring treatment response in tumors

We next evaluated the specificity of each radiotracer to noninvasively measure tumor cell death in vivo. Subcutaneous EL-4 tumors grew rapidly in C57BL/6 mice, detectably by day 4 and reaching approximately 150 mm³ 6 days after implantation (Fig. 4A). On day 6, mice were size matched and either treated with drug or left untreated. Drug treatment resulted in a significant 40% reduction in tumor volume after 24 hours, reduced from 159.8 ± 26.3 mm³ to 95.3 ± 16.0 mm³, whereas the tumor volume increased from 145.3 ± 32.2 mm³ to 256.4 ± 36.3 mm³ in naïve animals (80% increase; P < 0.001, n = 7/group). At this time point, drug-treated tumors were characterized by loss of tumor cellularity, immune cell infiltration (Fig. 4B), and extensive DNA damage throughout multiple slices of the tumor, determined by histochromatic staining (Fig. 4C). In naïve tumor-bearing animals, DNA damage was limited to the peripheral margins of the tumor. By 96 hours after therapy, tumors had shrunk to 42.8 ± 7.2 mm³, growing to 393.4 ± 23.2 mm³ in naïve animals (Fig. 4A).

Tumor uptake and radiotracer biodistribution. 18F-FDG, 99mTc-Annexin V, 18F-ML-10, and 18F-C-SNAT tumor uptake, illustrated in Fig. 4D, and ex vivo biodistribution profiles, summarized in Supplementary Table S1, were measured in EL-4 tumor-bearing animals 90 minutes after radiotracer injection in either drug-treated or naïve animals. 18F-FDG, 99mTc-Annexin V, and 18F-ML-10 pharmacokinetics were dominated by renal excretion. In addition, high heart and kidney retention were measured with 18F-FDG and 99mTc-Annexin V, respectively. 18F-C-SNAT elimination was through both the renal and hepatobiliary routes. 18F-ML-10 radioactivity had almost completely cleared from all organs by 90 minutes. For drug-treated mice, 18F-FDG was significantly higher than naïve animals in liver, small intestine, and large intestine, but lower in muscle (P < 0.05). Bone-associated radioactivity was significantly higher in treated versus naïve animals with 99mTc-Annexin V (P < 0.05).

Ex vivo γ-counting of excised tumors revealed a significant increase in tumor-associated radioactivity in drug-treated versus naïve tumor-bearing mice for both 99mTc-Annexin V and 18F-C-SNAT, at 1.4- and 2.1-fold, respectively (P < 0.05; n = 4–7 mice/group). There was no change in 18F-FDG or 18F-ML-10 tumor uptake and retention following drug treatment 90 minutes after injection of radiotracer (Fig. 4D and Supplementary Table S1). A positive tumor-to-muscle ratio was measured with all radiotracers and treatment conditions, whereas a positive tumor-to-blood ratio was only measured with 18F-FDG at 90 minutes after radiotracer injection (Supplementary Table S2).

Correlation of radiotracer binding to histology. The specificity of the radiotracers to image tumor response to therapy was achieved through correlation of their retention with TUNEL staining, an independent marker of drug efficacy, and the current clinical gold standard (Fig. 5). EL-4-tumor-bearing animals injected with 18F-C-SNAT showed a good correlation (R² = 0.778, P = 0.02) between whole tumor mean radioactivity at 90 minutes after radiotracer injection and TUNEL staining in histologic sections obtained post mortem, whereas there was no correlation for either 18F-FDG, 99mTc-Annexin V, or 18F-ML-10 (R² = 0.249, 0.231, and 0.001, respectively; n = 3 animals/group; P > 0.05).

Radionuclide imaging of tumor cell death. Dynamic PET images were acquired from animals with implanted EL-4 tumors (Fig. 6). Similarly to ex vivo biodistribution data, 18F-FDG distribution was characterized by high uptake in the tumor, heart, and bladder (Fig. 6A). 18F-FDG-PET/CT fusion images, highlighting the tumor xenograft and rendered in 3D, are...
shown in Fig. 6A, ii (full, uncropped versions are displayed in Supplementary Videos SM1 and SM2). 18F-FDG tumor uptake was characterized by a steady increase in tumor retention in both naïve and drug-treated animals, with a plateauing of radioactivity recorded between 60 and 90 minutes. By 90 minutes, a significant increase in tumor-associated 18F-FDG uptake was measured in drug-treated animals in comparison with naïve animals at 15.1 ± 1.2%ID/g and 12.3 ± 1.1%ID/g, respectively (P = 0.011; n = 4/group). There was no difference in the area under the tumor time–activity curves (AUC) between the two treatment groups (P > 0.05).

Tumor uptake and retention was visible above background in both naïve and drug-treated animals with 18F-ML-10 PET (Fig. 6B), with increased uptake noticeable around the periphery of the tumor (Fig. 6B, ii and Supplementary Videos SM3 and SM4). The kinetic profile of tumor retention varied considerably for 18F-ML-10 in comparison with 18F-FDG, as shown by the tumor time–activity curves (TAC). Rapid tumor uptake of 18F-ML-10, peaking at 10 minutes, preceded a slow washout of radioactivity over the remaining 80 minutes. There was no difference in image-derived tumor uptake values (%ID/g or AUC) between naïve and drug-treated cohorts.

In contrast to what was observed with 18F-ML-10 and 18F-FDG-PET, a clear difference in tumor-associated 18F-C-SNAT uptake between treatment groups was measured by PET (Fig. 6C). In drug-treated mice, background activity was increased when compared with naïve mice, possibly reflecting the trend toward increased blood retention observed in biodistribution studies (Supplementary Table S1). In naïve mice, tumor uptake was confined to the margins (Fig. 6C, ii and Supplementary Video SM5), corresponding to elevated regions of DNA damage observed by histochemical staining of excised tumors (Fig. 4C), whereas uniform tumor distribution was evident in tumors of drug-treated mice (Fig. 6C, ii and Supplementary Video SM6). The profile of tumor uptake varied considerably between drug-treated and naïve animals (Fig. 6C, iii). In drug-treated animals, C-SNAT rapidly accumulated in tumors, peaking at 10 minutes, with subsequent stabilization of tumor-associated radioactivity. For naïve animals,
18F-C-SNAT peaked to similar levels 10 minutes after injection, followed by a slow washout from the tumor over the remaining 80 minutes. By 90 minutes after injection, tumor uptake was 2.2 ± 0.2%ID/g in drug-treated tumors versus 0.8 ± 0.2%ID/g when left untreated, a 2.7-fold increase (P < 0.001, n = 3-4 animals/group).

Dynamic SPECT imaging was not possible with the experimental setup available. Instead, static 60 to 90 minutes SPECT images were obtained for 99mTc-Annexin V (Supplementary Fig. S4). These images revealed typical 99mTc-Annexin V tissue distribution, with high liver, kidney, and bladder radioactivity, which was consistent with biodistribution data. Although tumor uptake was evident above background, we were unable to distinguish between drug-treated tumors and naive controls using qualitative imaging data alone, reflecting the small differences observed by ex vivo γ-counting.

Discussion

Resistance to chemotherapy and molecularly targeted therapies provides a major hurdle for cancer treatment due to the underlying genetic and biochemical heterogeneity of tumors. An early read-out of drug efficacy, through the measurement of tumor cell death, would provide valuable insights into resistance status, allowing the selection of the most appropriate therapy (or combination) for the individual and termination of ineffectual treatments at an early stage.

There have been considerable efforts to develop methods to noninvasively measure tumor cell death (recently reviewed in ref. 6), but as yet, no cell-death imaging agent has made it into routine clinical practice. There is therefore a need to develop new imaging agents for the assessment of therapy response. A number of important considerations are required to assess the suitability for any novel cancer imaging agent: namely, the temporal and spatial distribution of the imaging biomarker in tumors, the sensitivity and specificity of detection, tumor-to-background contrast, and the pharmacokinetic profile of the radiotracer. Here, we systematically compared the novel imaging agent, 18F-C-SNAT, to clinically evaluated radiotracers, 18F-FDG, 18F-ML-10, and 99mTc-Annexin V, for the detection of tumor cell death. We selected the well-characterized EL-4 murine lymphoma model (11–13) for the assessment of drug-induced cell death. Lymphomas typically respond well to first-line therapy in comparison with the majority of solid tumor types (30), accurately reflected by extensive drug-induced cell death observed here in EL-4 cells and in implanted tumors (Figs. 2A and 4A–C, respectively). We then further assessed therapy response in two additional NSCLC lines to confirm that the variability in results between the different radiotracers was not specific to this one cell line.

In cell culture experiments, EL-4 cell–associated radioactivity for the different radiotracers varied over 3 orders of magnitude following drug treatment (Fig. 2), highlighting large differences in their sensitivity and mechanisms to detect cell death. For 99mTc-Annexin V, cell-associated radioactivity was 173.2% ± 15.7% radioactivity/mg protein at 60 minutes after addition of radiotracer, in comparison with just 0.10% ± 0.04% radioactivity/mg for 18F-ML-10. Furthermore, 18F-ML-10 uptake failed to correlate to drug-induced cell death in this model, either in cells or in tumors (Figs. 3 and 5, respectively); a pattern also replicated in the two NSCLC cells tested here. This is in contrast to previous reports in a preclinical model of neuronal cell death (26) and in drug-treated tumor cells in culture, in which a tritiated derivative of ML-10 was used (31).

It is possible that tissue culture media buffering of extracellular...
pH may reduce cell binding of $^{18}$F-ML-10, whose binding is purportedly mediated through the acidification of the external plasma membrane leaflet (31). Experimental evidence linking apoptosis-specific alterations in asymmetric membrane distribution to $^{18}$F-ML-10 uptake is limited however, and not supported by our findings in vivo, where the acidic tumor microenvironment likely remains largely un-buffered (13).

Drug treatment induced high levels of DNA damage and loss of cellularity in vivo, characteristic of an apoptotic response. Despite the high levels of cell death, accumulation in EL-4 tumors was moderate (<3%ID/g) for all radiotracers except $^{18}$F-FDG (Figs. 4C and 6A). A significant decrease in $^{18}$F-FDG uptake is indicative of a positive response to therapy, as measured here in EL-4 cells (Figs. 2B and 3), as a consequence of glucose transporter redistribution from the plasma membrane to the cytosol (12). Despite levels of drug-induced cell death correlating excellently with a reduction in $^{18}$F-FDG uptake in cells, no response was detectable by $\gamma$-counting of excised tumors ex vivo (Figs. 4D and 5), with a small but statistically significant increase observed by semiquantitative imaging parameters in vivo. $^{18}$F-FDG uptake is not confined to tumor cells, with drug-induced alterations in other constituents of the tumor microenvironment potentially confounding measurements in vivo. For example, infiltration of FDG-avid immune cells following drug treatment, detected here by H&E staining (Fig. 4B), may mask tumor cell-specific reductions in glucose metabolism in this immunocompetent murine model, as has been observed elsewhere (32, 33). These data are in line with the general advice for nuclear medicine physicians to wait a minimum of 10 days after chemotherapy before performing $^{18}$F-FDG-PET scans to bypass potential immune effects (20). A reduction in $^{18}$F-FDG in lymphoma patients has, however, been observed just 24 hours after initiation of treatment (34), highlighting a potential limitation of preclinical mouse models of cancer, where the nature and timing of immune responses are known not to precisely match those found in the clinic (35).

Although changes in $^{18}$F-FDG uptake act as an indirect measure of treatment response, Annexin V and its labeled derivatives have been shown to measure bona fide cell death, through binding to exposed PS with nanomolar affinity (36). Annexin V continues to be the gold standard for measurements of cell death in vitro. In culture, $^{99m}$Tc-Annexin V binding excellently correlated to cell death, with high sensitivity and specificity in both the lymphoma and two NSCLC cell lines. Despite encouraging performance in cell culture, a poor pharmacokinetic profile and low tumor binding was observed in vivo. The half-life of $^{99m}$Tc (6 hours) permits imaging at time points over 12 hours after radiotracer injection. The rapid clearance of $^{99m}$Tc-Annexin V from circulation and the absence of tissue redistribution just 30 minutes after

![Figure 6](image_url)

Dynamic PET imaging of cell death in vivo. PET image analysis of EL-4 tumors in naïve and drug-treated mice was performed for $^{18}$F-FDG (A), $^{18}$F-ML-10 (B), and $^{18}$F-C-SNAT (C). Corresponding axial and sagittal PET-CT images (60–90 minutes summed activity) of naïve and drug-treated EL-4 tumor-bearing mice are shown in panels (i). Arrowheads indicate the tumor, identified from the CT image. (ii) Representative PET-CT (60–90 minutes summed activity) volume rendering technique (VRT) images of tumor-bearing naïve and drug-treated mice. Arrows indicate the tumor. (iii) The tumor TAC representing average counts from a dynamic 90-minute scan 24 hours after drug treatment and in naïve animals. Data are mean ± SD (n = 3–4 animals per group). * P < 0.05, ** P < 0.001. B = bladder, H = heart, L = liver, SI = small intestine.
injection (37), however, supports an imaging protocol, whereby response is assessed at 90 minutes after intravenous injection. At this time point, 99mTc-Annexin V tumor uptake did not correlate significantly to levels of DNA damage (Fig. 5), with a modest 40% increase in radioactivity accumulation following drug treatment detectable by average counts in excised tumors. Nonspecific tumor retention of Annexin V in the vascular space has recently been attributed to changes in enhanced permeability and retention (EPR) effects at baseline and after therapy (38). Despite advances in both site-specific and PET labeling strategies little has been achieved to improve the in vivo profile and tumor binding of this radiotracer (39).

Given the relatively poor performance of existing radiotracers, alternative approaches are required for the measurement of cell death. PET-labeled and fluorescent derivatives of a peptidic caspase-3/7 substrate, C-SNAT and C-SANF, respectively, have recently emerged as promising agents for cell death imaging (7, 40). Specificity of C-SNAT for caspase-3/7 was previously shown, and through abrogation of signal in the presence of a caspase-3 inhibitor, and with a control probe containing the D-DEVD sequence, resistant to caspase-3 cleavage (7, 40). Here, 18F-C-SNAT uptake was significantly elevated between 2.9- and 4.5-fold in EL-4 and NSCLC cells following treatment. In EL-4 xenograft tumors, 18F-C-SNAT uptake correlated strongly with the levels of drug-induced DNA damage ($R^2 = 0.778$), with 18F-C-SNAT uptake increased 2.7-fold in drug-treated tumors versus naïve controls just 24 hours after therapy. This early readout of treatment response was shown to be an accurate predictor of outcome, assessed by almost complete tumor regression 72 hours after therapy (Fig. 4A). At 90 minutes after radiotracer injection, washout of unbound radiotracer in muscle resulted in a positive tumor-to-muscle ratio in both drug-treated and naïve controls (Fig. 4A).

Complete tumor regression 72 hours after therapy was detectable by average counts in excised tumors. Nonspecific increase in radioactivity accumulation following drug treatment could not be replicated in the EL-4 tumor model in the current work. Although progress has been made with the development of novel PET agents, such as 18F-C-SNAT, there is an urgent need to continue to develop new radiotracers that display an improved pharmacokinetic profile and tumor uptake in order to measure treatment response of tumors located in the abdomen and surrounding regions. Furthermore, detailed clinical studies will be needed to truly identify a radiotracer that can have utility in monitoring treatment efficacy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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