Imaging, Diagnosis, Prognosis

Temporal and Spatial Evolution of Therapy-Induced Tumor Apoptosis Detected by Caspase-3–Selective Molecular Imaging

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Abstract

Purpose: Induction of apoptosis in tumors is considered a desired goal of anticancer therapy. We investigated whether the dynamic temporal and spatial evolution of apoptosis in response to cytotoxic and mechanism-based therapeutics could be detected noninvasively by the caspase-3 radiotracer \[^{18}\text{F}]\text{ICMT-11}\) and positron emission tomography (PET).

Experimental Design: The effects of a single dose of the alkylating agent cyclophosphamide (CPA or 4-hydroperoxycyclophosphamide), or the mechanism-based small molecule SMAC mimetic birinapant on caspase-3 activation was assessed in vitro and by \[^{18}\text{F}]\text{ICMT-11–PET}\) in mice bearing 38C13 B-cell lymphoma, HCT116 colon carcinoma, or MDA-MB-231 breast adenocarcinoma tumors. Ex vivo analysis of caspase-3 was compared to the in vivo PET imaging data.

Results: Drug treatment increased the mean \[^{18}\text{F}]\text{ICMT-11}\) tumor uptake with a peak at 24 hours for CPA (40 mg/kg; AUC\(_{40–60}\): 8.04 ± 1.33 and 16.05 ± 3.35 %ID/mL × min at baseline and 24 hours, respectively) and 6 hours for birinapant (15 mg/kg; AUC\(_{40–60}\): 20.29 ± 0.82 and 31.07 ± 5.66 %ID/mL × min at baseline and 6 hours, respectively). Voxel-based spatiotemporal analysis of tumor-intrinsic heterogeneity suggested that discrete pockets of caspase-3 activation could be detected by \[^{18}\text{F}]\text{ICMT-11}\). Increased tumor \[^{18}\text{F}]\text{ICMT-11}\) uptake was associated with caspase-3 activation measured ex vivo, and early radiotracer uptake predicted apoptosis, distinct from the glucose metabolism with \[^{18}\text{F}]\text{FDG–PET}\), which depicted continuous loss of cell viability.

Conclusion: The proapoptotic effects of CPA and birinapant resulted in a time-dependent increase in \[^{18}\text{F}]\text{ICMT-11}\) uptake detected by PET. \[^{18}\text{F}]\text{ICMT-11–PET}\) holds promise as a noninvasive pharmacodynamic biomarker of caspase-3–associated apoptosis in tumors. Clin Cancer Res; 19(14); 3914–24. ©2013 AACR.

Introduction

Dysregulated cell death apoptosis is a common feature of many human diseases, including neurodegeneration, stroke, and cancer, and modulation of this cellular response has proved to be an effective therapeutic strategy (1, 2). Conventional therapies for the treatment of human malignancies—largely comprising cytotoxic drugs such as topoiso-erase inhibitors, alkylating agents, and antimitabolites and radiation therapy—primarily aim at eliminating tumor cells by “crudely” interfering with the basic machinery of DNA synthesis and cell division, thus, preventing cellular expansion culminating in the commitment to cell death via apoptosis. In parallel, they induce significant toxicity in tissues that normally maintain a proliferative compartment, such as gut epithelium and the hematopoietic system, leading to a range of side effects inconvenient and deleterious for the patient. Alongside the conventional anticancer drugs, the past decade has seen emergence of a multitude of targeted therapeutics—including agents that specifically modulate the apoptotic signaling pathway—with the possibility of developing therapeutic management to suit individuals. These drugs present single-agent antitumor activity as well as synergistic effects in combination with several conventional or targeted therapies by enhancing the initial chemosensitivity subsequently leading to higher levels of apoptosis (3, 4).

In this context, the use of biomarkers of cell death apoptosis could provide important predictive pharmacodynamic and outcome information to identify patients for whom discontinuation of ineffective toxic treatment is...
Translational Relevance

In cancer, increased caspase-3 activation linked to apoptotic cell death provides a mechanistic readout of drug response. There is, however, a paucity of specific imaging strategies to annotate caspase-3 activity. We establish that noninvasive positron emission tomography imaging with the caspase-3 binding probe \([^{18}F]ICMT-11\) permits characterization of caspase-3 dynamics in vivo and the early assessment of apoptosis induced by both cytotoxic and mechanism-based drugs. As the development of noninvasive molecular imaging strategies to detect apoptosis continues to gain momentum, we show that this new imaging approach provides essential insights into the pharmacodynamics of anticancer agents and holds promise for predicting anticancer drug response in tumors. Advanced image analysis methodology has been applied, enabling the nonuniform topology of apoptosis to be mapped and followed over time. Because of the specific annotation of apoptosis documented in this study together with initial good safety profile, \([^{18}F]ICMT-11\) is now transitioning into clinical development.

indicated, for the detection of acquired drug resistance, and for monitoring the efficacy of a wide range of current and future anticancer drugs. The dynamics of apoptosis and apoptotic body clearance may vary depending on the type and mechanism of action of the selected anticancer agent, and between patients (5). Furthermore, exactly which biochemical events occur will depend on whether there is involvement of the intrinsic or extrinsic apoptotic pathway (6, 7). The time frame required to detect apoptosis induction can, therefore, vary according to cell type, mechanism of action of apoptosis-inducing agent, drug concentration or schedule, and exposure time. The monitoring of these activities before or after the optimal time frame could then result in little to no signal above background, leading to erroneous conclusion that the treatment did not induce apoptosis. Moreover, the conventional method to assess apoptosis biomarkers through tumor biopsies, when applicable, failed to provide detailed time-course or spatial relationship data on a pharmacodynamic response.

Among the different methods investigated to assess tumor apoptosis, noninvasive molecular imaging strategies continue to gain momentum (5, 8). In this study, we show that the caspase-3–specific apoptosis radiotracer, \([^{18}F]ICMT-11\) (9–11) can be used to define and resolve the time-course of tumor apoptosis in response to cytotoxic and mechanism-based agents. Spatial characterization of the caspase-3 PET signal through voxel-wise analysis further revealed tumor-intrinsic heterogeneity of apoptosis induction, consistent with histologic evidence. We suggest that noninvasive assessment of caspase-3 activation using \([^{18}F]ICMT-11\) PET imaging constitutes a viable strategy for detecting the spatiotemporal evolution of therapy-induced tumor apoptosis, and can be used optimally to detect early drug efficacy for treatment planning and drug development.

Materials and Methods

Cells and reagents

38C13 cells, derived from a B-cell lymphoma that arose in a C3H mouse treated with 7,12-dimethylbenz[a]anthracene, were kindly provided by R. Levy (Division of Oncology, Stanford School of Medicine, CA). The human cells HCT116 colon carcinoma and MDA-MB-231 breast adenocarcinoma were purchased from the American Type Culture Collection (Manassas, VA) and Caliper Life Sciences (MDA-MB-231-luc-D3H2LN), respectively. 4-Hydroperoxycyclophosphamide [4-HC, the primary active metabolite of cyclophosphamide ([CPA]), CPA, and TNF-α were purchased from Niochem, Sigma-Aldrich, and R&D Systems, respectively. The small molecule second mitochondriaderived activator of caspases (SMAC) mimetic birinapant was provided by TetraLogic Pharmaceuticals. The cell lines were selected based on sensitivities to the drugs used with 38C13 being sensitive to CPA (10) and HCT116 and MDA-MB-231 showing intermediate and high sensitivity to birinapant (unpublished data). Furthermore, clinically, CPA is used in lymphoma, colon, and breast cancer as initial indication areas for birinapant.

In vitro caspase-3 activation assay

Luminescent caspase-3/7 activation assay was conducted according to the manufacturer’s instructions (Promega Caspase-Glo 3/7 assay). Briefly, cells were transferred into a white opaque 96-well plate, incubated for 1 hour with Caspase-Glo reagent and the enzymatic activity of caspase-3/7 was measured using a TopCount luminometer (PerkinElmer). To enable normalization of data to total cellular protein content, the bichinchoninic acid protein assay was conducted for all samples according to the manufacturer’s instructions. Data were expressed as relative light unit per μg of protein (RLU/μg).

Small animal experimental models

All animal experiments were done by licensed investigators in accordance with the UK Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act 1986 (HMSO, London, UK, 1990) and within guidelines set out by the UK National Cancer Research Institute Committee on Welfare of Animals in Cancer Research (12). The in vivo experimental xenograft models were established by subcutaneous injection of \(5 \times 10^3\) 38C13 cells in C3H mice and \(5 \times 10^6\) HCT116 or \(2 \times 10^6\) MDA-MB-231 cells in BALB/c nude mice. All mice were 6- to 8-week-old females from Charles River. When xenografts reached approximately 80 mm³ [tumor dimensions were measured using a caliper and tumor volumes were calculated using the ellipsoid formula that is best for estimating tumor mass; volume \(V = \frac{4}{3} \pi \times a \times b \times c\), where \(a\), \(b\), and \(c\) represent 3 orthogonal axes of the tumor], mice were injected a single dose of CPA (40 mg/kg i.p.) or birinapant (15 mg/kg i.p.) and...
subjected to positron emission tomography-computed tomography (PET-CT) imaging in a longitudinal setting where the same animal serves as its own control.

[^18F]ICMT-11 radiosynthesis

The activated-caspase-3-specific radiotracer,[^18F]ICMT-11, was synthesized as described previously (13). Approximately 1.8 to 2 GBq of no-carrier-added aqueous[^18F]fluoride solution was purchased from PETNET Solutions and used following dilution with water. An aliquot, typically 1.5 mL, 740 MBq, was transferred to a FASTLabTM automated synthesis module (GE Healthcare). The activity was trapped on a Waters QMA-carbonate Sep-Pak SPE cartridge and eluted into a cation exchange polymer plastic reaction vessel with 700 μL of an eluent solution (3.9 mg Kryptofix 2.2.2, 0.5 mg potassium hydrogen carbonate, 560 μL acetonitrile, 140 μL H2O). The[^18F]fluoride solution was evaporated to dryness by a combination of vacuum and nitrogen flow at a temperature of 110 °C more than 8 minutes. Following evaporation, the tosylate precursor (2.85 mg and 3.75 μmol) in 1 mL of anhydrous acetonitrile was added into the reactor and the labeling reaction was conducted at 110 °C for 12.5 minutes resulting in formation of the acetal-protected isatin in 78% C6 more than 8 minutes. The activated-caspase-3-specific radiotracer,[^18F]ICMT-11 was purified using a Phenomenex UltraCarb ODS 250 × 10 mm (7 μm) HPLC column with an isocratic mobile phase of 0.05 M ammonium acetate and ethanol (58:42, v/v) at a flow rate of 5 mL/min. Following preparative HPLC purification, the isolated product was diluted 10-fold and extracted on to a tC18 light Sep-Pak cartridge (Waters) to achieve formulated product with a radiochemical purity of ≥98% at end of synthesis (EOS), and a specific activity of 685 ± 237 GBq/μmol. Reformulation of the product was achieved through manual elution with ethanol resulting in a product concentration of 0.83 ± 0.17 MBq/μL.

PET-CT imaging

At the indicated time-points, mice were anesthetized through isoflurane inhalation and scanned on a dedicated small animal PET-CT scanner (Siemens Multimodality Inveon). Low-dose CT scans were first acquired (80 kVp, 0.5 mA, 220° rotation, 600 ms per degree exposure time, 80 μm reconstruction pixel size) for PET data attenuation correction and to obtain an anatomical reference. The experimental models used throughout the study consist of subcutaneous xenografts, which allow reliable and reproducible delineation of tumor burden and selection of tumor volumes of interest (VOI) on CT images. The tumor VOIs were then applied to PET images for radioactivity measurements. Following a bolus intravenous injection of 3.7 MBq of[^18F]ICMT-11 or[^18F]fluorodeoxyglucose ([^18F]FDG, purchased from PETNET Solutions), dynamic PET emission scans were then acquired in list-mode format over 60 minutes and corrected for decay and dead time. The acquired data were then sorted into 0.5-mm sinogram bins and 19 time frames (4 × 15, 4 × 60, and 11 × 300 seconds) for image reconstruction; filtered back projection (FBP; Fourier rebinning, 256 × 256 × 159 matrix size, 0.39 × 0.39 × 0.80 mm3 pixel size, ramp filter with cut-off at Nyquist frequency, FWHM 1.45 mm), ordered subset expectation maximization in 2 dimensions (OSEM2D; Fourier rebinning, 256 × 256 × 159 matrix size, 0.39 × 0.39 × 0.80 mm3 pixel size, 4 iterations, FWHM 1.03 mm), and ordered subset expectation maximization in 3 dimensions followed by MAP reconstruction (OSEM3D/MAP; 256 × 256 × 159 matrix size, 0.43 × 0.43 × 0.80 mm3 pixel size, 2 OSEM3D iterations, 18 MAP iterations, b = 0.1 optimized for uniform resolution, FWHM 1.29 mm). OSEM3D reconstructions were used for visualization of radiotracer uptake and display of representative PET images (dynamic data 30–60 minutes framing) with Siemens Inveon Research Workplace software. The radioactivity concentrations within tissues were obtained (FBP and OSEM2D reconstructions) from mean voxel intensity values within the VOI and then converted to megabecquerels per milliliter using the calibration factor determined for the Inveon PET system. These values were then divided by the administered activity in megabecquerels to obtain an image VOI-derived percent injected dose per mL (%ID/mL) at each of the 19 time frames to give the time versus radioactivity curve (TAC) and the area under the TAC (AUC). AUCs 0 to 60 minutes, 30 to 60 minutes, and 40 to 60 minutes (%ID/mL) were computed to account for radiotracer initial uptake and washout.

PET-based voxel intensity sorting histograms and statistical box plot diagrams

For each VOI, all the voxels ([^18F]ICMT-11 40–60 minutes,[^18F]FDG 30–60 min) and their associated intensity were extracted, and sorted as per their intensity frequency to give the PET-based voxel intensity sorting (PVIS) histograms. The voxel intensities distributions were further processed through a statistical analysis (Prism 5.0 software, GraphPad) and represented graphically with box plot diagrams depicting minimum, 25th percentile, median, 95th percentile, and maximum voxel intensity values. Within the narrow range of apoptosis seen, we arbitrary selected the 95th percentile cut-off to biologically describe the 5% highest intensity voxels—likely to contain apoptotic cells—rather than, for example, on the basis of receiver operating characteristic analysis.

Diffusion-weighted magnetic resonance imaging

Diffusion-weighted magnetic resonance imaging (DW-MRI) was conducted in a 4.7 T horizontal-bore DirectDrive Varian MRI system equipped with 40 G/cm actively shielded gradient coils (software version: Vmnr) 3.1). Animals were anesthetized throughout the whole scan duration. A 45-mm-diameter saddle coil was used for excitation and a 20-mm-diameter stripline resonator was used as a receive-only coil to minimize setup times. Animals were positioned supine with the tumor placed at the center of the...
PET Imaging of Tumor Apoptosis

Excised and snap frozen tumor tissue samples were homogenized in RIPA lysis buffer with the PreCellys 24 homogenizer and CK14 beads-containing tubes (2 cycles of 25 seconds—6,500 rpm). Equal amounts of protein (30 μg) were denatured in sample buffer, subjected to SDS-polyacrylamide gel electrophoresis on 4% to 12% gels, and were denatured in sample buffer, subjected to SDS-polyacrylamide gel electrophoresis on 4% to 12% gels, and transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted with specific primary antibodies, horseradish peroxidase– conjugated secondary antibodies, and visualized by enhanced chemiluminescence. The following antibodies were used: anti-cIAP1, anti-cIAP2, anti-XIAP rabbit polyclonal antibodies, and anti-cleaved caspase-3 (Cell Signaling Technology); anti-PARP rabbit monoclonal antibody (Abcam); and anti-α-tubulin and anti-rabbit horseradish peroxidase secondary antibody (Santa Cruz Biotechnology).

Statistical analysis
Data were expressed as mean ± SEM and the significance of comparison between 2 datasets was determined using Student t test (Prism v5.0 software, GraphPad) and defined as significant (*, 0.01 < P < 0.05), very significant (**, 0.001 < P < 0.01), and extremely significant (***, P < 0.001).

Results
Temporal evolution of cytotoxic drug-induced tumor caspase-3 activation with \[^{18}\text{F}\]ICMT-11 PET imaging
The rapid evolution of caspase-3 activation presents challenges for detection. We selected the murine B-cell lymphoma 38C13 cell line treated with the alkylating agent CPA (10) as a typical experimental model of cytotoxic drug-induced apoptosis to define the optimal posttreatment time-point for the monitoring of tumor apoptosis in vivo. CPA-based therapy is used extensively for lymphomas and is often curative for aggressive non-Hodgkin lymphoma, with Burkitt’s lymphoma being particularly sensitive (14). The cytotoxic property of CPA results from its activation to nitrogen mustard that induces intrastrand and interstrand DNA crosslinks.

To establish the cell-intrinsic effect of CPA, we first determined the time-course of caspase-3 activation in vitro in 38C13 cells treated with 4-HC (active metabolite of CPA), and showed that peak caspase-3 activation occurred at 24 hours posttreatment (Fig. 1A). This observation highlights the transient nature of caspase-3 activation, and thus the critical requirement to select the optimal time frame for biomarker detection in vivo. We next investigated the dynamics of apoptosis in vivo in the longitudinal setting where the same animal was studied before drug treatment (baseline) and at 24 and 48 hours after a single dose of CPA (40 mg/kg). Mice bearing 38C18 tumors were studied with either \[^{18}\text{F}\]ICMT-11 PET (9, 10) or \[^{18}\text{F}\]FDG PET, an important clinical standard for assessing response in lymphoma (15), that provides a measure of glucose metabolism and cell viability (Fig. 1B and D, respectively; Supplementary Fig. S1B). Tumor uptake of \[^{18}\text{F}\]ICMT-11 (and associated imaging variables including the time vs. activity curve, TAC, and the area under the TAC, AUC40–60) was unremarkable at baseline, increased by 2-fold after 24 hours CPA treatment, then decreased at 48 hours posttreatment to levels greater than that of baseline (AUC40–60 = 8.04 ± 1.33, 16.05 ± 3.35 and 14.38 ± 4.12%ID/mL × min at baseline, 24 and 48 hours posttreatment, respectively; Fig. 1E). The AUC40–60 have been computed to account for initial radionuclide uptake and washout, and showed similar results to \[AUC_{0–60}\] (Supplementary Fig. S1A). In contrast, tumor \[^{18}\text{F}\]FDG PET uptake (Fig. 1F) was high at baseline and, as expected, decreased progressively at 24 hours (1.6-fold) and 48 hours (2.5-fold) posttreatment (AUC40–60 = 380.82 ± 30.47, 238.07 ± 18.57, and 152.88 ± 8.83%ID/mL × min at baseline, 24 and 48 hours, respectively). Changes in \[^{18}\text{F}\]ICMT-11 and \[^{18}\text{F}\]FDG tumor uptake occurred in
parallel with a small reduction of the tumor volume at 24 hours (to 94.3% ± 6.0), and a more drastic shrinkage at 48 hours posttreatment (to 29.9% ± 3.6) as monitored caliper measurements (Fig. 1C) and qualitatively on X-ray CT images (Fig. 1B and D; Supplementary Fig. S1B). We, therefore, showed that although both [18F]ICMT-11 and [18F]FDG PET imaging are sensitive to a CPA dose level that induce minimal changes in tumor size at 24 hours, the former reflects apoptosis induction and the latter is indicative of overall metabolism reduction. The remarkable tumor shrinkage at 48 hours posttreatment was, however, associated with residual low [18F]ICMT-11-associated tumor uptake and almost no [18F]FDG-related tumor metabolic activity. Notably, induction of [18F]ICMT-11 tumor retention preceded changes of tumor volumes assessed by anatomical measurements.

Systemic toxicity due to CPA treatment is well-documented including bone marrow suppression and liver toxicity (14, 16). We investigated the potential CPA-associated liver toxicity in our experimental model and showed a 1.4-fold increased [18F]ICMT-11 liver uptake at 24 hours posttreatment compared to baseline (AUC 40–60 = 249.44 ± 14.73 and 180.93 ± 14.36%ID/mL × min, respectively), which returned to baseline levels at 48 hours posttreatment (Supplementary Fig. S2A). In contrast, no changes in [18F]FDG liver uptake were detected following treatment (Supplementary Fig. S2B). Further characterization of liver apoptosis induction was obtained by protein and immunohistologic

![Figure 1](image-url)

**Figure 1.** Temporal evolution of CPA-induced tumor caspase-3 activation by [18F]ICMT-11 PET imaging. A, in vitro time-course of caspase-3 activation in response to vehicle or 3 μg/mL 4-HC treatment in 38C13 lymphoma cells. Data shown are mean ± SEM of RLU/μg from triplicates of 3 independent experiments. B to D, longitudinal CT/[18F]ICMT-11 and [18F]FDG PET studies, using 38C13 tumor-bearing mice (n = 3 and n = 6, respectively) treated with 40 mg/kg CPA and imaged pre- and posttreatment as indicated. Coronal PET and CT images of a representative animal at baseline and 24/48 hours after CPA treatment (white arrowheads indicate the tumor) are shown. C, tumor volumes recorded by caliper measurements of 38C13 tumor-bearing mice (n = 9) before and after CPA treatment as indicated. Data shown are mean ± SEM of % volume compared to baseline. E and F, [18F]ICMT-11 and [18F]FDG tumor TAC [time vs. activity curves, % injected dose per mL (%ID/mL)] and PET extracted variables AUC40–60 and AUC30–60 [area under the TAC, % injected dose per mL (%ID/mL)/min] are shown ([18F]ICMT-11 AUC40–60, P = 0.0451; [18F]FDG AUC40–60, **P = 0.0013, ***P < 0.0001; one-tailed paired Student t test).
analyses of tissue samples excised at 24 hours after CPA treatment (increased cleaved PARP and cleaved caspase-3 protein expression, decreased total PARP protein expression, and increased cleaved caspase-3 and TUNEL staining on liver tissue samples; Supplementary Fig. S3A and S3B). As no overt signs of toxicity were recorded along the course of the study, these data suggested the potential utility and sensitivity of [18F]ICMT-11 PET imaging to monitor drug-associated subclinical toxicity in nontumor tissues.

Spatial distribution of caspase-3 activation following cytotoxic drug-induced tumor apoptosis

Most tumors display biological heterogeneity including variations in genomic subtypes, growth factors expression, and surrounding microenvironment (17, 18). We suggest that the sensitivity of PET image analysis of caspase-3 activation could benefit from a systematic voxel-wise methodology.

Radiotracer uptake is often assessed at the whole tumor level. We showed heterogeneous spatial distribution of tumor [18F]ICMT-11 uptake by PET, which occurred in parallel with discrete patches of active caspase-3 and TUNEL staining observed histologically (Supplementary Figs. S4A and S4B). We, therefore, established further characterization of tumor PET image data by a voxel-wise analysis method that entailed sorting all voxels within the VOI according to their individual intensities (PVIS; ref. 10). The evolution of [18F]ICMT-11 PVIS histograms—right shift (to higher intensity voxels) at 24 hours and returning to baseline at 48 hours—suggested a transient caspase-3 activation/apoptosis response to CPA treatment (Fig. 2A). In contrast, decreased tumor glucose metabolism was sustained—left-shifted [18F]FDG PVIS (to lower intensity voxels) at 24 hours and a further left-shift at 48 hours posttreatment (Fig. 2B). Although PVIS histograms were established for both radiotracers, the voxel-wise analysis may be more relevant to [18F]ICMT-11/caspase-3 activation that shows marked tumor-intrinsic heterogeneity. To quantitatively describe the PVIS histogram, we rationalized that comparison of the highest intensity voxels within a VOI, indicative of high radiotracer retention, could be more sensitive than the traditional comparison of voxel intensity mean, thus more representative of the underlying biological process of apoptosis. We conducted global statistical analyses of the tumor voxel intensity distribution at each time-point. Notably, the [18F]ICMT-11-derived median and 25th percentile PVIS statistical parameters mirrored the [18F]ICMT-11 TACs and PVIS histograms.

Early detection of caspase-3 activation by [18F]ICMT-11 PET imaging is associated with cell death detected by DW-MRI

DW-MRI detects the diffusion of water molecules in biological tissues and can be quantitated with the ADC (μm²/s), which reflects cellular integrity and density. A number of preclinical and clinical studies have reported utility of ADC as a pharmacodynamic biomarker of tumor cell death—whether induced through apoptosis, necrosis, or autophagy—for the monitoring of early response to treatment (19, 20). In this context, effective therapy-induced tumor cell death is characterized by increased tumor ADC. We showed that 24 hours CPA treatment induced a significant 1.4-fold increase in the tumor ADC values, compared to baseline (645 ± 47 and 459 ± 67 μm²/s, respectively; Fig. 3A and B). By analogy with PET PVIS histograms analysis, the ADC intensities for each individual voxel have been extracted and sorted, and indicate a right shift of the ADC histograms at 24 hours posttreatment compared to baseline (Fig. 3C). The increased ADC after CPA treatment was associated with decreased cell density as assessed through histologic analysis of tumor tissue sections (Fig. 3D). The histologic cell density pattern following treatment was characterized by the appearance of small discrete regions of intercellular space, which seems to represent an apoptosis profile rather than a necrotic core profile. Thus, [18F]ICMT-11 PET imaging is associated with ADC detectable cell death by DW-MRI.

Early detection of response to a mechanism-based apoptosis inducer with [18F]ICMT-11 PET imaging

Alongside conventional cytotoxic drugs, an increased number of anticancer therapeutic regimens incorporate molecular-targeted agents. The development of such agents is a lengthy and costly process and the establishment of their clinical indication to suit individuals can benefit from intensive tumor biological characterization using...
biomarkers (21, 22). Particular classes of mechanism-based agents aim to reactivate apoptosis in cancer cells by directly interacting with and inhibiting key antiapoptotic proteins.

In this context, we investigated the putative utility of [18F]ICMT-11 as a molecular-imaging response (proof of mechanism or proof of concept) biomarker for mechanism-based apoptosis inducing agents.

We investigated the ability of [18F]ICMT-11 to detect response to birinapant, a small molecule SMAC mimic that selectively antagonizes inhibitor of apoptosis proteins (IAP), thus, specifically triggers caspase activation and apoptosis with single-agent activity—cytostasis and tumor regression—in multiple animal models (23). The in vitro time-course of caspase-3 activation was characterized by a peak at 24 hours after birinapant treatment in 2 models of cancer cells, HCT116 human colon carcinoma and MDA-MB-231 human breast adenocarcinoma cells. Birinapant rapidly degrades cIAP1/2 and antagonizes XIAP enabling microenvironment cytokines (including TNF-α) to activate the extrinsic apoptosis pathway. In this context, we showed that TNF-α enhanced birinapant-induced caspase-3 activation with maximum enhancement at 6 hours and peak caspase-3 activation of the combination at 24 hours post-treatment (Fig. 4A).

With this knowledge, we designed a longitudinal in vivo characterization of birinapant-induced apoptosis, where the same mouse is subjected to baseline, 6, 24, and 48 hours posttreatment PET imaging. The experimental model used was an HCT116 tumor-bearing mouse treated with 15 mg/kg single dose of birinapant. [18F]ICMT-11 PET detected a 1.5-fold increased apoptosis following a single dose of birinapant as early as 6 hours posttreatment, compared to baseline (AUC_{0–60}: 31.07 ± 5.66 and 20.29 ± 0.82%ID/mL × min, respectively; Fig. 4B and E; Supplementary Fig. S5B). At 24 and 48 hours posttreatment, tumor [18F]ICMT-11 uptake had decreased to baseline levels indicating a rapid and transient dynamic of the caspase-3 activation in response to birinapant treatment. Similar results were obtained with AUC_{0–60} (Supplementary Fig. S5A). Interestingly, at the dose of birinapant used, tumor [18F]FDG uptake was characterized by a modest 1.18-fold decrease at 6 hours posttreatment, compared to baseline (AUC_{0–60} = 124.65 ± 4.77 and 147.47 ± 7.46%ID/mL × min, respectively; Fig. 4D and F; Supplementary Fig. S5B), with a similar recovery to baseline levels at 24 and 48 hours posttreatment, which seemed to indicate that [18F]ICMT-11 detection of caspase-3 activation could constitute a more sensitive approach than [18F]FDG in that particular experimental setting. Both radiotracers showed a clear shift of the PVS histograms at 6 hours posttreatment compared to baseline (right shift and left shift for [18F]ICMT-11 and [18F]FDG, respectively; Fig. 4G and H). Statistical analysis of both [18F]ICMT-11 and [18F]FDG tumor voxel intensity distribution, as depicted by the median and 95th percentile, showed similar profiles to the TAC curves. The rapid and transient [18F]ICMT-11 detectable caspase-3 activation induced by birinapant was associated with a small delay in tumor growth at the 24 and 48 hours time-points; later time-points were not examined (Fig. 4B–D). This profile is expected for birinapant, which requires multiple dosing to elicit antitumor activity as monotherapy. Further characterization of tumor tissue excised at 6 hours (peak [18F]ICMT-11 time-point) showed lower cIAP-1 protein expression following birinapant treatment compared to vehicle, most probably due to degradation of cIAP-1 (Supplementary Fig. S6A). As expected from the specificity of birinapant, no variations in cIAP-2 and XIAP were observed at the 6 hours time-point examined. Histologic analysis of formalin fixed tumor tissues showed that birinapant treatment significantly increased apoptosis compared to vehicle treatment, illustrated by increased cleaved caspase-3 and TUNEL staining (3.5- and 4.5-fold induction, respectively; Supplementary Fig. S6B). Unlike CPA, no biomarker-related liver toxicity changes could be detected with [18F]ICMT-11 or [18F]FDG PET (Supplementary Fig. S7A and S7B). Similar results were
obtained in the breast adenocarcinoma experimental model (Fig. 5A–D; Supplementary Fig. S8A and B and S9), thus supporting the utility of [18F]ICMT-11 PET imaging for the early assessment of tumor caspase-3 activation/apoptosis induced by a single dose of the mechanism-based SMAC mimetic birinapant.

Discussion

As the development of noninvasive molecular imaging strategies to detect apoptosis continue to gain momentum, we have characterized the biological determinants of signal change with a caspase-3–specific PET radiotracer, [18F]ICMT-11, for the early detection of cytotoxic and mechanism-based drug-induced tumor apoptosis. This represents an attractive alternative to histologic assessment of apoptosis on tumor biopsies (6, 7). Ex vivo methods are only applicable to resectable cancers and may fail to predict therapy response because of biopsy sampling errors, intratumoral biological heterogeneity, or nonuniform drug delivery. Another recently developed approach to assess...
apoptosis entails detection of circulating caspase-cleaved cytokeratin 18 (CK18) fragments. The assay has been optimized for routine clinical use and validated as a proof of concept biomarker in patients receiving conventional chemotherapy with different tumor types (24, 25). A preclinical study indicated that early (6 hours) increases in cleaved CK18 in response to the small molecule Bcl-2 inhibitor ABT-737, correlated with intratumoral cleaved caspase-3 (26). In the subsequent clinical trial, rapid (within 6 hours) increases in circulating cleaved CK18 were observed (27). Unlike circulating apoptosis biomarkers that are unable to distinguish tumor response from host tissue toxicity, we showed that the [18F]ICMT-11 PET signal is sensitive to CPA induced subclinical liver toxicity, whereas no such signal changes were seen with the mechanism-based birinapant treatment, probably due to differential expression of target tumor versus host tissue and the lack of synergistic combination from the tumor microenvironment proapoptotic signals [e.g., TNF-α]. Furthermore, the liver represents a challenging site for [18F]ICMT-11 imaging given the high background uptake, due at least in part to tracer elimination (10, 11). Despite this fact other investigators have shown utility of alternative isatin radiotracers for detecting liver apoptosis (28, 29). In keeping with those reports, we suggest that CPA-associated liver toxicity was responsible for the increase in [18F]ICMT-11 (compared to baseline) in liver supported by biochemical and histologic data. [18F]ICMT-11 uptake in other models of drug-induced normal tissue toxicity is warranted to support this finding.

There is also increasing evidence that quantitative imaging biomarkers can provide additional information related to tumor-intrinsic heterogeneity, with the potential to add valuable information and increase the sensitivity of the technique. In this context, a growing number of studies have showed changes in treatment response using histogram analysis that were not seen on whole tumor measurements (18, 30). The biological presentation of apoptosis fits this paradigm. We showed that [18F]ICMT-11 PET data quantified by voxel intensity sorting and statistical analysis permits detection of activated caspase-3 foci. Further studies will strengthen the interpretation and utility of this approach, in particular, characterization of the contribution of partial-volume errors will strengthen the interpretation and utility of this approach as purely reflecting heterogeneity; neighboring pixels may not be truly independent and features of the pixel histogram may be dominated by the size and shape change of the tumors.

Response of lymphoma to CPA is detectable by [18F]FDG PET (31). Comparison of [18F]ICMT-11 with [18F]FDG PET highlighted interesting biological dynamics in the CPA

Figure 5. Early detection of birinapant-induced tumor apoptosis with [18F]ICMT-11 PET imaging in a breast carcinoma experimental model. A, in vitro time-course of caspase-3 activation in response to vehicle, 100 nmol/L birinapant, 100 ng/mL TNF-α, or combined birinapant and TNF-α (combination) treatment in MDA-MB-231 breast adenocarcinoma cells. Data shown are mean ± SEM of RLU/mg from triplicates of 3 independent experiments. B, longitudinal CT/[18F]ICMT-11 PET study using MDA-MB-231 tumor-bearing mice (n = 5) treated with a 15 mg/kg single-dose birinapant and imaged pre- and posttreatment as indicated. Coronal CT and PET images of a representative animal at baseline and 6 hours after birinapant treatment (white arrowheads indicate the tumor) are shown. C, [18F]ICMT-11 tumor TAC (time vs. activity curves, % injected dose per mL [%ID/mL]) and PET extracted variables AUC40–60 (area under the TAC, % injected dose per mL × min) are shown (%ID/mL × min). D, [18F]ICMT-11 PVIS histograms and box plot diagrams.


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model. \[^{18}F\text{ICMT-11}\] kinetics were characterized by uptake and washout of nonspecific localization over time rather than progressive retention, as is seen with \[^{18}F\text{FDG}\]. This was responsible for selection of imaging variables such as AUC\textsubscript{40–60} for analysis. A peak in caspase-3 activation detectable with \[^{18}F\text{ICMT-11}\] occurred in parallel with a reduction in glucose metabolism, however, further loss of metabolic activity at 48 hours did not lead to further accentuation of the \[^{18}F\text{ICMT-11}\] signal. The early \[^{18}F\text{ICMT-11}\]-detectable activated caspase-3 signal occurred even earlier with a mechanistic inducer of apoptosis likely due to the more direct effect of birinapant on caspase-3 activation pathway analogous to effect of Bcl-2 inhibition on caspase-3 activation (26). We show that the optimal time-point for detection of caspase-3 activation with \[^{18}F\text{ICMT-11}\] depends on the type of drug used and its associated mechanism of action in relation to caspase-3 activation rather than a nonspecific detection of necrosis for example showed in comparative DW-MRI studies. Although 6, 24, and 48 hours were selected as convenient time points for analysis, other time points should be explored in the future to fully define the kinetics of uptake in apoptotic lesions.

In conclusion, \[^{18}F\text{ICMT-11}\] PET imaging permits assessment of the spatiotemporal evolution of caspase-3 activation and, thus, tumor apoptosis in response to cytotoxic or mechanism-based agents. These interesting findings, along with our previous reports indicating rapid radiotracer tissue distribution/dosimetry, rapid renal/hepatic clearance, and relatively good metabolic stability (10, 11), support clinical development of \[^{18}F\text{ICMT-11}\] in cancer patients that are planned. Of relevance to use of \[^{18}F\text{ICMT-11}\] for clinical management, future studies designed to compare the predictive power of apoptosis imaging versus \[^{18}F\text{FDG-PET}\] or DW-MRI on subject survival/tumor response should be considered as such studies will provide further clarity on the clinical value of apoptosis imaging.

Disclosure of Potential Conflicts of Interest

M.A. Graham has ownership interest (including patents) in Tetralogic Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Q.-D. Nguyen, I. Lavdas, J. Gubbins, I. Carroll
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