Fluorine-18-Labeled Thymidine Positron Emission Tomography (FLT-PET) as an Index of Cell Proliferation after Pharmacological Ascorbate-Based Therapy

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Pharmacological ascorbate (AscH[−]) induces cytotoxicity and oxidative stress selectively in pancreatic cancer cells compared with normal cells. Positron emission tomography (PET) with the thymidine analog 3'-deoxy-3'-[¹⁸F] fluorothymidine (FLT) enables noninvasive imaging and quantification of the proliferation fraction of tumors. We hypothesized that the rate of tumor proliferation determined by FLT-PET imaging, would be inversely proportional to tumor susceptibility to pharmacological AscH[−]-based treatments. Indeed, there was decreased FLT uptake in human pancreatic cancer cells treated with AscH[−] *in vitro*, and this effect was abrogated by co-treatment with catalase. In separate experiments, cells were treated with AscH[−], ionizing radiation or a combination of both. These studies demonstrated that combined AscH[−] and radiation treatment resulted in a significant decrease in FLT uptake that directly correlated with decreased clonogenic survival. MicroPET ¹⁸F-FLT scans of mice with pre-established tumors demonstrated that AscH[−] treatment induced radiosensitization compared to radiation treatment alone. These data support testing of pharmacological ascorbate as a radiosensitizer in pancreatic cancer as well as the use of FLT-PET to monitor response to therapy. © 2016

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INTRODUCTION

Pancreatic cancer is the fourth most common cause of cancer-related death in the United States with over 39,500 fatal cases reported annually in the U.S. alone

(1). Even after curative resection, the five-year survival rates achieved at specialized centers are less than 20% and the majority of patients die of metastatic cancer recurrence (2). Deregulation of cell proliferation is a hallmark of cancer (3). Recently, it has been reported that high-dose intravenous pharmacological ascorbate (AscH[−]) inhibits cell proliferation and tumor growth *in vivo* and induces cytotoxicity and oxidative stress selectively in pancreatic cancer cells compared with normal cells (3–6), by acting as a prodrug for the delivery of hydrogen peroxide (H₂O₂) (7–11). Furthermore, recent phase I clinical trials have demonstrated pharmacologic ascorbate to be safe and well tolerated in combination with standard-of-care chemotherapeutics (gemcitabine and erlotinib, and gemcitabine alone) for the treatment of pancreatic cancer (12, 13).

In recent years, the thymidine analog 3'-deoxy-3'-[¹⁸F] fluorothymidine (FLT) has been developed as a proliferation marker for cancer research. Imaging and measurement of proliferation with positron emission tomography (PET) provide a noninvasive tool to both stage and monitor the response to anticancer treatment (14), especially when targeted drugs are utilized. Interestingly, the rate-limiting enzyme of FLT metabolism, the pyrimidine metabolizing enzyme thymidine kinase-1 (TK-1), is overexpressed in pancreatic cancer cell lines and pancreatic cancer (15). While FLT has certain limitations compared with fluorodeoxyglucose (FDG), which is the most widely used PET tracer (FLT uptake is lower in most cancers), FLT was found to be the PET tracer with the highest and most consistent uptake in various human pancreatic tumor cell lines in SCID mice (even more so than ¹⁸F-FDG). Therefore, it has been suggested that FLT-PET scans are particularly useful in imaging pancreatic cancer (16).

In light of these data, we hypothesized that FLT-PET would be a useful technique for quantifying response to ascorbate-based therapies both *in vitro* and *in vivo*. Since

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pharmacological ascorbate generates H_2O_2 , we hypothesized that inhibition of peroxide removal (11, 23) or increased ascorbate oxidation with the use of mangano-porphyrins (17, 28) would synergize with ascorbate. In particular, we were interested in exploring how FLT would reflect response to AscH^- treatment alone, ionizing radiation exposure alone or a combination of the two (AscH^- and radiation). Specifically, we hypothesized that pharmacologic ascorbate would act as a radiosensitizing agent to synergistically increase the cytotoxicity of radiation. Indeed, therapeutic interventions designed to increase oxidant stress, such as radiation, combined with AscH^- , would be predicted to preferentially sensitize tumor cells compared to normal cells due to increased baseline metabolic oxidative stress in the tumors (3, 17). Furthermore, radiation therapy is the standard of care for locally advanced pancreatic cancer and when there are positive margins or positive lymph nodes after pancreatic resection (18). Therefore, AscH^- -induced sensitization of tumors to radiation in these scenarios could potentially be clinically beneficial. Our studies demonstrate that FLT uptake has a linear correlation to the effectiveness of ascorbate-induced cytotoxicity and that AscH^- radiosensitizes pancreatic cancer both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell Culture and Reagents

The human pancreatic cancer cell line MIA PaCa-2 was purchased from the American Type Culture Collection (Manassas, VA) and passaged for fewer than 6 months after receipt. Cells were maintained as previously described (19). Mn(III)tetrakis(N-methylpyridinium-4-yl) porphyrin pentachloride (MnT4MpyP), was purchased from Axxora, LLC (Farmingdale, NY). The solids were stored at -20°C or in solution at 4°C in colored vials to minimize photo-oxidation (20). A stock solution of 1.0 M ascorbate (pH 7.0) was made under argon and stored in screw-top sealed test tubes at 4°C . Ascorbate concentration was verified, using: $\epsilon_{265} = 14,500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (21). The solution can be kept for several weeks without significant oxidation due to the lack of oxygen (21). A 1 mM gemcitabine stock solution was prepared in Nanopure™ water and stored at 4°C . Dilutions were prepared as needed. ^{18}F fluorine was produced in-house with a 16.5 MeV cyclotron and synthesized using 5'-O-(4,4'-dimethoxytrityl)-2,3'-anhydrothymidine as precursor and an FLT synthesis module.

In Vitro FLT Uptake

Cells were treated with ascorbate (5 mM) with and without catalase (100 U/ml), ascorbate (5 mM) and 2DG (20 mM), ascorbate (5 mM) and radiation (2 Gy), or ascorbate (0.25 mM) and MnT4MpyP (0.2 μM) for 1 h. After treatment, approximately 74 MBq (2 mCi) of ^{18}F -FLT in 1 ml Dulbecco's modified Eagle media (DMEM) was added per 60 mm dish for 60 min. Incubation was stopped by quickly removing the supernatant and repeated washing with PBS. PBS (900 μl) was added to monolayer. Cells were harvested by scraping into microcentrifuge tubes. The radioactivities of samples were counted for 1 min using a well-type gamma counter (Canberra Industries Inc., Meriden, CT). Cellular tracer uptake was then expressed as percentage of total activity per culture vial or adjusted for cell protein.

Clonogenic Survival Assays

Clonogenic survival assays were performed as previously described (3). Briefly, treatments were performed for 1 h in DMEM and 10% fetal bovine serum (FBS) at 37°C and 21% O_2 , 48 h after initial seeding. Cells were treated with radiation (2 Gy) and ascorbate (5 mM) for 1 h, then plated 72 h after treatment. The dishes were maintained in a 37°C , 21% O_2 , 5% CO_2 incubator or a 37°C , 4% O_2 , 5% CO_2 incubator for 10–14 days to allow colony formation. The colonies were then fixed with 70% ethanol, stained with Coomassie™ Blue (10% acetic acid, 50% methanol and 0.1% Coomassie Blue G-250) and counted (colonies containing >50 cells were scored).

In Vivo Studies

All protocols were reviewed and approved by the Animal Care and Use Committee of the University of Iowa (Iowa City, IA). MIA PaCa-2 tumor cells (2×10^6) were delivered subcutaneously into the hind legs of 30-day-old athymic nude mice and allowed to grow until the tumor reached 5 mm in greatest dimension (~ 10 –14 days after injection). At that time, baseline microPET FLT scans were performed on all mice to determine baseline cellular proliferative activity (day 0). Mice were then treated with saline [1 M NaCl intraperitoneal (i.p.) daily], pharmacological ascorbate (4 g/kg $^{-1}$ /day $^{-1}$ i.p.), radiation (5 Gy on day 3), or combination ascorbate and radiation (saline and ascorbate administered on day 1–4). In mice randomized to receive radiation treatment, 5 Gy was given to the mice at a dose rate of 1.27 Gy/min. Before irradiation, the animals were anesthetized with 80–100 mg/kg ketamine/10 mg/kg xylazine i.p. and shielded in a lead block with only the tumor-bearing right hind flank unshielded. The lead block served as a shield so that only the tumor was directly irradiated. On day 5 FLT scans were repeated to determine tumor response to treatment. Treatment response was assessed using a proliferative index equal to the product of FLT tumor uptake (as measured by the standardized uptake value and the tumor volume). The ratio of post-treatment to pre-treatment proliferative index was determined for each treatment group.

MicroPET FLT scans were performed at the Small Animal Imaging Core (SAIC, University of Iowa). Animals were fasted for 12 h prior to FLT injection. Ten minutes prior to FLT injection, 2 mg/kg of 5-fluoro-2'-deoxy-uridine (FUDR) (Sigma-Aldrich LLC, St. Louis, MO) was injected into the left lateral tail vein. Then, under isoflurane anesthesia, the mice were injected via right lateral tail vein with 11 ± 3.6 MBq (0.3 ± 0.1 mCi) of FLT in 0.2 cc. The mice were allowed to awaken and were returned to their cage for a 60 min uptake period with access to drinking water. After the uptake period, the mice were anesthetized with isoflurane, which was maintained (1.5%) during the remainder of the imaging session. Mice were positioned supine on a temperature-controlled bed (m2m™ Imaging, Cleveland, OH), which was affixed to the pallet of an Inveon® multimodality system (Siemens Preclinical Systems, Knoxville, TN). Mice were remotely translated into the center of the PET axial field of view (FOV). After completion of the PET acquisition, mice were remotely moved to the CT gantry and a low-dose CT scan was performed for attenuation purposes. Image analysis was completed using PMOD v3.2 (PMOD Technologies, Zurich, Switzerland). Volumes of interest were manually drawn for the tumors using PET, CT and hybrid images and specific uptake of FLT was calculated. Standardized uptake values (SUV) were determined from PET (SUV-PET) measures of radioactivity (22). Treatment response was assessed using a proliferative index, as described above.

RESULTS

Ascorbate-Based Therapies Decrease FLT Uptake In Vitro

Pharmacological ascorbate has been well established as a prodrug for the delivery of H_2O_2 to tumors, increasing tumor

cytotoxicity alone and in combination with other drugs (3–5, 11). It was hypothesized that the ascorbate-induced cytotoxicity in pancreatic cancer cells would directly correlate with a decrease in FLT uptake in those cells. Furthermore, the combination of AscH⁻ with 2-deoxyglucose (2DG), ionizing radiation or the manganoporphyrin (MnP) MnT4MPyP was hypothesized to synergistically decrease FLT uptake. Compared to control cells, which received FLT alone, uptake was significantly decreased in cells treated with ascorbate (**P* < 0.05; Fig. 1A). Ascorbate is known to exert its effects through the production of H₂O₂. To determine if the reduction in FLT uptake observed in cells treated with AscH⁻ was also the result of the production of peroxide, the experiment was repeated with the addition of extracellular catalase (100 U/ml) to the media. This reversed the decrease in FLT uptake, supporting H₂O₂ as the mediator of the AscH⁻-induced decrease in FLT uptake. As an additional control, the experiment was repeated with boiled, inactivated catalase, and again demonstrated that FLT uptake was decreased compared to the controls (**P* < 0.05; Fig. 1A). Finally, to demonstrate that external catalase was not itself affecting FLT uptake, the experiment was repeated in cells treated with catalase alone demonstrating no change in uptake compared to controls. Taken together, these data suggest a direct correlation between ascorbate therapy and ¹⁸F-FLT uptake in pancreatic cancer cells *in vitro*.

2DG is a relatively nontoxic analog of glucose that competes with glucose for uptake. 2DG creates a chemically induced state of glucose deprivation resulting in inhibition of hydroperoxide detoxification by compromising the cell's ability to regenerate NADPH as well as pyruvate, which can all potentially contribute to hydroperoxide detoxification. Recently, we have demonstrated that combinations of these chemical inhibitors of hydroperoxide metabolism increase intracellular accumulation of H₂O₂, resulting in selective enhancement of ascorbate toxicity in pancreatic tumor cells (23). It has been suggested that 2DG is an anticancer agent, which has been examined in clinical trials (24). We hypothesized that 2DG might induce similar decreases in FLT uptake as observed in AscH⁻-treated cells, and that in combination a synergistic decrease might be observed. In both cells treated with AscH⁻ (5 mM) and 2DG (20 mM) alone, FLT uptake was significantly decreased compared to controls, with 2DG resulting in a more significant decrease than ascorbate alone (**P* < 0.05; Fig. 1B). However, when cells were treated with combination AscH⁻ (5 mM) and 2DG (20 mM), no additional decrease was observed.

Ionizing radiation has long been known to induce DNA damage and generate reactive oxygen species (ROS) that can damage proteins, lipids and DNA, inducing both single- and double-strand DNA breaks (25). Ascorbate-mediated H₂O₂ formation also causes DNA damage through the production of a site-specific hydroxyl radical (HO[•]) (26, 27). Therefore, it was hypothesized that treatment with ascorbate might act as a radiosensitizer, further decreasing FLT uptake *in vitro*.

Again, in cells treated with AscH⁻ (5 mM) alone, a significant decrease in FLT uptake compared to controls was observed. Cells irradiated with a single 2 Gy dose also showed a significant decrease in FLT uptake compared to controls, although less so than the cells that received ascorbate alone. However, when used in combination, cells treated with AscH⁻ (5 mM) and radiation (2 Gy) showed a significant decrease in the amount of FLT uptake compared to controls and either treatment alone (**P* < 0.05 vs. controls; #*P* < 0.05 vs. either treatment alone; Fig. 1C), supporting the hypothesis of ascorbate as a radiosensitizing agent.

Recently, we showed that ascorbate in combination with manganoporphyrins synergistically increases cytotoxicity in several pancreatic cancer cell lines both *in vitro* and *in vivo* through a mechanism in which the MnPs act as catalysts to increase AscH⁻-induced H₂O₂ flux into cells (17, 28). To demonstrate that this synergism could also be observed using FLT uptake, the *in vitro* FLT experiment was repeated. Neither AscH⁻ (0.25 mM) nor the manganoporphyrin MnT4MPyP (0.2 μM) treatment alone resulted in a significant change in FLT uptake compared to controls (Fig. 1D). However, when cells were treated with a combination of AscH⁻ (0.25 mM) and MnT4MPyP (0.2 μM), a significant decrease in FLT uptake was observed (**P* < 0.05; Fig. 1D). Ascorbate combined with MnT4MPyP is extremely cytotoxic, as previously demonstrated (17, 28). To demonstrate additive effects, a significantly reduced dose of ascorbate was given compared to the previous experiments shown in Fig. 1. To confirm that this was a result of H₂O₂ production, the experiment was performed in the presence of catalase, which abrogated the decrease in FLT uptake. As a whole, the experiments shown in Fig. 1 suggest that FLT uptake can indeed be used as a marker for pancreatic cancer response to ascorbate-based treatment *in vitro*.

Clonogenic Survival Correlates with FLT Uptake In Vitro

Based on these initial experiments, 2DG and AscH⁻ treatment did not produce any further inhibition of FLT uptake. Furthermore, because of recently reported studies on the synergism of ascorbate and MnPs, we decided to focus our efforts on the use of combined AscH⁻ and radiation treatment to study how FLT might correlate to the response to treatment in MIA PaCa-2 cells *in vitro*. Each of the experiments (Fig. 2A and B) was performed in parallel; that is for each condition tested, one dish was used to set up a clonogenic survival assay and another to measure FLT uptake, as shown in Fig. 1. Clonogenic survival assays can be used to assess the cytotoxicity of the treatment of interest, and are an assessment of the cancer cell's ability to reproduce itself and propagate new clones after treatment (3). The experiments were performed under both room air (21% O₂) and reduced oxygen conditions (4% O₂).

At 21% oxygen, when MIA PaCa-2 cells were treated with AscH⁻ (5 mM) or radiation (2 Gy) alone, clonogenic

FIG. 1. Ascorbate-based therapies decrease FLT uptake *in vitro*. Panel A: MIA PaCa-2 cells that received FLT only (2 mCi) act as a positive control and have a baseline FLT uptake of $(35.4 \pm 2.1) \times 10^3$ counts/μg protein. After treatment with AscH^- (5 mM), FLT uptake was significantly decreased to $(19.3 \pm 1.4) \times 10^3$ counts/μg protein ($*P < 0.05$ vs. FLT). When external catalase (100 U/ml) was added to the cells in addition to AscH^- (5 mM), FLT uptake was restored to near control levels [$(30.0 \pm 3.2) \times 10^3$ counts/μg protein] supporting H_2O_2 as the mediator of ascorbate-induced reduction in FLT uptake. When boiled and inactivated external catalase was added along with AscH^- (5 mM), there was again a significant decrease in FLT uptake to $(20.4 \pm 18.3) \times 10^3$ counts/μg protein ($*P < 0.05$ vs. FLT). The addition of catalase alone had no effect on FLT uptake; $n = 3$ for each condition. Panel B: MIA PaCa-2 cells that received FLT only (2 mCi) acted as a positive control and had a baseline FLT uptake of $(53.9 \pm 3.3) \times 10^3$ counts/μg protein. Again, after treatment with AscH^- (5 mM), FLT uptake was significantly decreased to $(37.5 \pm 8.0) \times 10^3$ ($*P < 0.05$ vs. FLT). After treatment with 2DG (20 mM), FLT uptake was again significantly decreased to $(21.0 \pm 3.4) \times 10^3$ counts/μg protein ($*P < 0.05$ vs. FLT). Combination treatment with AscH^- (5 mM) and 2DG (20 mM) did significantly decrease FLT uptake relative to controls [$(18.7 \pm 0.7) \times 10^3$ counts/μg protein; $*P < 0.05$], however, there was no significant difference compared to either ascorbate or 2DG treatment alone and no synergism was observed; $n = 3$ for each condition. Panel C: MIA PaCa-2 cells that received FLT only (2 mCi) acted as a positive control and had a baseline FLT uptake of $(70.0 \pm 3.1) \times 10^3$ counts/μg protein. After treatment with AscH^- (5 mM), FLT uptake was significantly decreased to $(58.2 \pm 2.8) \times 10^3$ counts/μg protein ($*P < 0.05$ vs. FLT). After 2 Gy irradiation, FLT uptake was significantly decreased to $(62.7 \pm 1.4) \times 10^3$ counts/μg protein ($*P < 0.05$ vs. FLT). However, when cells were treated with both AscH^- and radiation, there was a significant and synergistic decrease in FLT uptake to $(50.1 \pm 5.2) \times 10^3$ counts/μg protein ($*P < 0.05$ vs. FLT; $*P < 0.05$ vs. AscH^- or radiation treatment alone); $n = 3$ for each condition. Panel D: MIA PaCa-2 cells that received FLT only (2 mCi) acted as a positive control and had a baseline FLT uptake of $50 \pm 15.2 \times 10^3$ counts/μg protein. Treatment AscH^- (0.25 mM), MnT4MPyP (0.2 μM) or catalase (100 U/ml) alone did not significantly decrease FLT uptake compared to controls. Combination of AscH^- (0.25 mM) and MnT4MPyP (0.2 μM) significantly reduced FLT uptake to $(10 \pm 0.82) \times 10^3$ counts/μg protein ($*P < 0.05$ vs. FLT). The addition of external catalase to the combination treatment returned FLT uptake to levels comparable to that of MnT4MPyP alone; $n = 3$ for each condition.

survival was significantly decreased with reductions to 25 and 64% of control levels, respectively ($*P < 0.05$; Fig. 2A). However, when treated with combination AscH^- and radiation, there was an even greater decrease in clonogenic survival compared to controls or either treatment alone (4% of control levels; $*P < 0.05$; Fig. 2A). At reduced oxygen conditions (4% O_2), a similar pattern of cytotoxicity was observed. Interestingly, due to the increased stress on the cells at low oxygen tension, treatment with AscH^- alone and radiation alone resulted in even greater decreases in clonogenic survival compared to controls with reductions in clonogenic survival to 12 and 25%, respectively ($*P < 0.05$; Fig. 2B). A synergistic increase in cytotoxicity was again observed when AscH^- and radiation treatment were combined, reducing clonogenic survival to 1% of controls,

representing a significant decrease compared to both AscH^- alone and radiation alone ($*P < 0.05$; Fig. 2B).

Next, FLT uptake activity was plotted compared to the actual percentages of clonogenic survival for both the 21% O_2 and 4% O_2 experiments. At 21% O_2 there was a robust positive linear correlation between FLT uptake and clonogenic survival ($R^2 = 0.97$; Fig. 2C). This suggests that FLT uptake can indeed be used as a marker for cellular response to treatment *in vitro*, with low FLT uptake after treatment corresponding to increased cytotoxicity, and higher FLT uptake corresponding to resistance to treatment. The same relationship was observed at 4% O_2 , albeit with a weaker correlation ($R^2 = 0.77$; Fig. 2D). This reduced correlation was likely due to the increase in stress on the cells being cultured under reduced oxygen conditions and

FIG. 2. Combined ascorbate and radiation treatment decreases clonogenic survival and directly correlates with FLT uptake *in vitro*. Panel A: Clonogenic survival assay performed on MIA PaCa-2 cells at 21% O₂. Clonogenic survival was normalized to positive control cells that received 2 mCi FLT for 1 h and then re-plated 72 h later. Cells treated with AscH⁻ (5 mM) had a significant decrease in clonogenic survival to $32.1 \pm 12.3\%$ of control levels ($*P < 0.05$). Again, 2 Gy irradiated cells also had a significant decrease in clonogenic survival compared to controls to $69.2 \pm 9.6\%$ ($*P < 0.05$). However, when treated with AscH⁻ and radiation, there was a significant and synergistic decrease in clonogenic survival compared to controls, as well as AscH⁻ and radiation treatment alone to $8.8 \pm 2.7\%$ of control levels ($*P < 0.05$ and, $*P < 0.05$ vs. AscH⁻ and radiation treatment alone); $n = 3$ for each condition. Panel B: Clonogenic survival assay performed on MIA PaCa-2 cells at 4% O₂. Clonogenic survival was normalized to positive control cells that received 2 mCi FLT for 1 h and then re-plated 72 h later. Cells treated with AscH⁻ (5 mM) had a significant decrease in clonogenic survival to $15.0 \pm 7.1\%$ of control levels ($*P < 0.05$). Again, cells treated with 2 Gy irradiation also had a significant decrease in clonogenic survival compared to controls to $24.5 \pm 8.8\%$ ($*P < 0.05$). However, when treated with combined AscH⁻ and radiation, there was a significant and synergistic decrease in clonogenic survival compared to controls, as well as AscH⁻ and radiation alone to $1.5 \pm 1.6\%$ of control levels ($*P < 0.05$ vs. controls, $*P < 0.05$ vs. AscH⁻ and radiation treatment alone); $n = 3$ for each condition. Panel C: Positive linear correlation between FLT uptake and clonogenic survival at 21% O₂ ($R^2 = 0.97$). Panel D: Positive linear correlation between FLT uptake and clonogenic survival at 4% O₂ ($R^2 = 0.77$).

its interplay with treatment, but further studies will be required to verify this conclusion.

Hybrid MicroPET FLT/CT Imaging of Pancreatic Tumor Xenografts

Based on the observation that FLT uptake correlated directly with response to AscH⁻-based therapies *in vitro*, we extended these experiments to an *in vivo* model. MIA PaCa-2 xenografts were placed into the hind limbs of athymic nude mice and pre-treatment microPET FLT scans were performed to establish a baseline proliferating fraction of the tumor using the metric of proliferative index. A representative baseline PET/CT fusion image is shown as both a 3D reconstruction and a 2D coronal slice through the plane containing the tumor in Fig. 3A (left and right sides, respectively). After baseline scans on day 0,

mice were divided into the four treatment groups ($n = 3$ mice per group) of controls, AscH⁻ only, radiation only, and AscH⁻ and radiation. Treatment was initiated immediately on day 1 (5 Gy radiation administered on day 3) and continued for 4 days. On day 5 mice received post-treatment scans and post-treatment proliferative index was determined.

As shown in Fig. 3B, control mice receiving saline had a mean post- to pre-treatment proliferative index ratio of 2.8 ± 0.5 . This ratio indicates that the tumor continued to proliferate rapidly over the five-day experiment when saline was administered, resulting in both a higher standardized uptake value and larger tumor volume, and therefore, an increased proliferative index. Mice who received AscH⁻ alone had a significantly decreased proliferative index ratio (PI_{ratio}) compared to control mice ($*P < 0.05$; Fig. 3B),

FIG. 3. Noninvasive hybrid microPET ^{18}F -FLT/CT imaging of MIA PaCa-2 pancreatic tumor xenografts in nude mice. Panel A: MIA PaCa-2 xenografts were placed into the hind limbs of athymic nude mice and noninvasive hybrid microPET ^{18}F -FLT/CT imaging was performed. A representative baseline PET/CT fusion image is shown as both a 3D reconstruction and a 2D coronal slice through the plane containing the tumor (left and right sides, respectively); $n = 3$ mice per group. Panel B: Image analysis of pre- and post-treatment FLT/CT scans was completed using PMOD v3.2. Volumes of interest were manually drawn for the tumors using PET, CT and hybrid images and specific uptake of FLT was calculated. Standardized uptake values (SUV) were determined from PET (SUV-PET) measures of radioactivity. Treatment response was assessed using a proliferative index equal to the product of FLT tumor uptake (as measured by the standardized uptake value) and the tumor volume. The ratio of post-treatment to pre-treatment proliferative index was determined for each treatment group. Control mice had a proliferative index ratio of 2.83 ± 0.54 , indicating continued proliferation of the tumor. Treatment with AscH⁻ (4 g/kg/day^{-1}) significantly reduced the proliferative index ratio to 1.52 ± 0.48 , indicating continued, yet inhibited proliferation (* $P < 0.05$ vs. controls). Treatment with radiation alone (5 Gy) further reduced the proliferative index ratio to 1.12 ± 0.14 , suggesting further inhibition of tumor growth (* $P < 0.05$ vs. controls). Combined treatment with AscH⁻ and radiation synergistically decreased the proliferative index ratio to 0.71 ± 0.16 , suggesting both a tumoristatic and tumoricidal mechanism (* $P < 0.05$ vs. control, # $P < 0.05$ vs. AscH⁻ and radiation alone); $n = 3$ mice per group.

although the post- to pre-treatment ratio was still >1 ($\text{PI}_{\text{ratio}} = 1.5 \pm 0.5$). This indicates that although tumor proliferation was inhibited by ascorbate, the tumors continued to grow, albeit at a slower rate compared to controls. Next, mice exposed to a single 5 Gy dose were examined. These mice also had a significant decrease in the proliferative index compared to control mice (* $P < 0.05$; Fig. 3B), with a value of 1.1 ± 0.1 . Again, this proliferative index ratio >1 indicates that treatment with radiation alone was tumoristatic, i.e., that tumor growth rate was inhibited or slowed after treatment compared to controls. Finally, in mice that received combined AscH⁻ and radiation treatment, there was both a significant reduction in the proliferative index compared to control mice (* $P < 0.05$; Fig. 3B) and a significant reduction in the proliferative index in mice treated with AscH⁻ or radiation alone (* $P < 0.05$; Fig. 3B). Furthermore, the value of the proliferative index in AscH⁻ and radiation-treated mice was now less than one ($\text{PI}_{\text{ratio}} = 0.7 \pm 0.2$), suggesting that the combination of AscH⁻ and radiation treatment was tumoricidal; i.e., the treatment not only inhibited tumor growth but also induced tumor cell death. This was consistent with ascorbate as a radiosensitizer, which was also observed in the *in vitro* studies. The *in vivo* microPET FLT scans correlated well with previous studies from our group, which demonstrated that pharmacological ascorbate alone (3, 17, 28) or combined with

ionizing radiation (29) decreases tumor growth and increases survival. The results shown here strongly support the use of FLT-PET imaging as an indicator of response to ascorbate-based therapies.

DISCUSSION

Currently, only FDG is widely accepted and used in clinical practice for oncology imaging. Although it can detect areas of increased cellular metabolism that inform clinicians about possible cellular proliferation and cell death, FDG does not convey an actual measurement of cancer cell proliferation (30). However, in the past few years FLT uptake has been gaining interest as clinically useful for proliferation imaging and several studies have validated its use in a variety of cancers including lymphomas, breast and even lung (31–38). However, FLT has traditionally had limited utility in abdominal imaging due to high physiological hepatic uptake of the tracer. A recent study evaluated 20 patients with pancreatic cancer and determined that FLT-PET could in fact detect changes in proliferation, predicting progressive disease with a high specificity, when combined with a temporal-intensity information-based voxel-clustering approach termed kinetic spatial filtering (FLT-PET/CTKSF) (39). The authors also concluded that

FLT-PET could be used as an early response biomarker for gemcitabine-based chemotherapy to select a poor prognostic group who might benefit from novel therapeutic agents in advanced and metastatic pancreatic cancer.

In the current study, it was first demonstrated that an inverse association existed between treatment with various therapies (2DG, AsC^H⁻, radiation, MnT4MPyP and AsC^H⁻, and AsC^H⁻ and radiation) and the amount of FLT activity after treatment. The studies on AsC^H⁻, radiation, and AsC^H⁻ and radiation treatments were extended to demonstrate a strong, positive correlation between FLT uptake and clonogenic survival of pancreatic cancer cells after treatment suggesting that FLT can indeed be used as a predictor of response to treatment *in vitro*. Furthermore, it was demonstrated that when AsC^H⁻ was used in combination with radiation, there was a significant increase in cellular cytotoxicity and concomitant decrease in FLT uptake suggesting radiosensitization. These results were consistent with prior reports of AsC^H⁻-induced radiosensitization in both glioblastoma multiforme and leukemia cell lines (40, 41).

Finally, it was demonstrated that FLT uptake as measured by the proliferative index can be used to monitor response to therapies for pancreatic cancer xenografts *in vivo*, specifically the post-therapy to pre-therapy ratio of the proliferative indices for each treatment group. Whereas all treatments resulted in significantly decreased FLT uptake compared to control mice, the proliferating fraction of the tumor seemed to decrease incrementally from treatment with AsC^H⁻ alone, to radiation alone and finally to a combination of both. Although these decreases were all significant, treatment with AsC^H⁻ or radiation alone resulted in proliferative index ratios >1, suggesting that the tumor continued to proliferate, although at a slower rate. Because tumor growth was inhibited, it was concluded that these therapies are tumoristatic. However, when AsC^H⁻ was combined with radiation, there was a significant decrease in the proliferative index ratio compared to each individual treatment, dropping below 1, suggesting that the combined treatment was tumoricidal and that AsC^H⁻-induced radiosensitization may be responsible.

However, given the limitations of these studies, these data support further testing of pharmacological ascorbate as an adjuvant treatment for radiotherapy in pancreatic cancer patients as well as the use of FLT-PET to monitor response to therapy in clinical trials. Pharmacologic ascorbate induces cytotoxicity selectively in pancreatic cancer cells *in vitro* and *in vivo* and has been determined as safe and well-tolerated in clinical trials (4, 5, 10, 11, 13, 39). Currently, an additional clinical trial [NCT01852890, Cullen (PI)] is recruiting patients to determine efficacy of pharmacologic ascorbate in combination with both gemcitabine and radiation therapy.

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