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The vanadyl chelate *bis*(acetylacetonato)oxovanadium(IV) increases the fractional uptake of 2-(fluorine-18)-2-deoxy-D-glucose by cultured human breast carcinoma cells

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Abstract

Detection of breast cancer by positron emission tomography (PET) imaging with 2-(fluorine-18)-2-deoxy-D-glucose (FDG) as the tracer molecule is limited in part by both tumor dimension and metabolic activity. While some types of aggressive breast cancers are associated with a high capacity for FDG uptake, more indolent breast cancers are characterized by low FDG uptake. Moreover, detection of malignant lesions in most clinical settings requires tumor dimensions > 10 mm. Development of a method to increase the fractional uptake of FDG by cancer tissue would provide a means to detect smaller tumors. However, there is no clinically available pharmacologic reagent known to enhance the preferential uptake of FDG by cancer tissue. Because the vanadyl (VO²⁺) chelate *bis*(acetylacetonato)oxovanadium(IV) [VO(acac)₂] is known to enhance cellular uptake of glucose, we have investigated whether VO(acac)₂ facilitates enhanced uptake of FDG by cultured human breast carcinoma cells. We observed that the fractional uptake of FDG by cultured human MDA-MB-231 carcinoma cells is increased in the presence of VO(acac)₂ in a dose dependent manner. Preliminary results with xenograft tumors generated in severely compromised, immunodeficient (SCID) female mice showed that VO(acac)₂ treatment of mice 3 – 4 hr prior to FDG injection enhanced FDG uptake by the malignant tissue by a factor > 2.0 compared with that by normal surrounding tissue.

Introduction

Positron emission tomography (PET[†]) with FDG as the tracer molecule has become an important imaging modality for the detection, diagnosis, and staging of cancer.

Mammography by magnetic resonance imaging or X-ray methods is not able to specify

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whether a tumor is malignant or benign. In contrast, the specificity of FDG PET imaging to identify malignancy is 90% – 100% in most studies¹. While mammography is anatomically based, FDG PET imaging is metabolically based, relying on the high rate of glucose uptake and glycolysis that is generally associated with cancer. Because hexokinase is not product inhibited in cancer cells as in normal tissue and because FDG phosphorylated at the C-6 position is not further metabolized through glycolysis, cancer cells accumulate more of the tracer molecule than normal tissue, providing the basis for intensity contrast between malignant and normal, surrounding tissue.

Imaging based on FDG as the tracer molecule relies on tumor size and the local, metabolic characteristics of the type of cancer. Some types of breast cancer are characterized by relatively low FDG uptake, giving rise to false negatives^{2–4}. In addition, glucose in the blood stream competes with FDG for cell entry. Under conditions of impaired glucose metabolism, as in poorly controlled diabetes mellitus, high blood glucose levels can attenuate intensity contrast in FDG PET imaging and result in uninterpretable images^{5, 6}. What is needed is a pharma-cological reagent that facilitates preferential FDG uptake by cancer tissue, thereby augmenting the sensitivity of FDG PET imaging. Hitherto, no such reagent has been available for clinical application.

The capacity of organic vanadyl (VO^{2+}) chelates to facilitate increased uptake of glucose *in vivo* as insulin-enhancing reagents has been long documented^{7–10}. We have shown that $\text{VO}(\text{acac})_2$ exhibits not only greater capacity to enhance the cellular uptake of glucose than many other VO^{2+} -chelates, but that it is also associated with significantly greater stability in biological milieu^{11, 12}. The much greater enhancement of cellular glucose uptake by $\text{VO}(\text{acac})_2$ than by structurally similar chelates suggested that $\text{VO}(\text{acac})_2$ may facilitate increased uptake of FDG for FDG PET imaging of cancer. To this end by application of PET imaging, we have investigated the capacity and dose dependence of $\text{VO}(\text{acac})_2$ to increase FDG uptake by cultured human MDA-MB-213 carcinoma cells. We have also determined whether, compared with normal tissue, $\text{VO}(\text{acac})_2$ augments FDG uptake by xenograft tumors generated with MDA-MB-231 cells in SCID mice as a laboratory, small animal model. The results indicate that $\text{VO}(\text{acac})_2$ promotes preferential FDG uptake by cancer tissue *in vivo*. Because of the generally low toxicity of VO^{2+} -chelates¹³, there may be translational potential for use of $\text{VO}(\text{acac})_2$ as a pharmacological reagent to increase the sensitivity of all PET imaging modalities in which FDG is used as the tracer molecule.

Results and Discussion

FDG PET Imaging of Human Cultured Carcinoma Cells

Figure 1 compares representative FDG PET images of cultured MDA-MB-231 breast cancer cells after the uptake of FDG was quenched with cold KRBH and physiological saline, as described in the Experimental section. Because only 9 wells of the 12-well plate could be

[‡]Abbreviations: BSA, bovine serum albumin; CT, computed tomography; DMEM, Dulbecco's modification of Eagle medium; DMSO, dimethylsulfoxide; FDG, 2-(fluorine-18)-2-deoxy-D-glucose; GLUT, glucose transporter protein; HEPES, N-(2-hydroxyethyl)-piperazine-N'-(4-butanedisulfonic acid); KRBH, HEPES-buffered Krebs-Ringer solution; PET, positron emission tomography; ROI, region of interest; SCID, severe compromised, immunodeficient; SPECT, single photon emission computed tomography; $\text{VO}(\text{acac})_2$, bis(acetylacetonato)oxovanadium(IV).

imaged with the plate secured to the gantry of the imaging system, the other 3 wells were not used. The concentration of VO(acac)₂ added to each well at the beginning of the incubation period is indicated in the figure legend. The coronal PET images show a radially non-uniform distribution of intensity due to FDG uptake. VO(acac)₂ and FDG solutions were added to wells, and cells were washed without disruption of the cellular monolayer on the bottom surface of the well. Microscopic examination of cells grown overnight in wells showed localized variations in population density and distribution of cells and occasional, small areas completely devoid of cells. Such regions are reflected, for instance, by the dark blue areas of wells 8 and 9 indicative of little or no FDG uptake. We, therefore, ascribe the radially non-uniform pattern of FDG uptake to variations in population density and distribution of cells. Our procedure for imaging FDG PET intensity of cultured cells was comparable to that described by Fischer and coworkers for cancer cells seeded and grown in six-well plates¹⁴. The intensity due to FDG uptake in their experiments similarly showed a radially non-uniform distribution of intensity.

To quantify PET signal intensity due to FDG uptake by MDA-MB-231 breast cancer cells, the ROI for each well was first defined as a circular disc of sufficient height to account for the thickness of the cellular monolayer, as shown in Figure 2. Because areas of sparse cell growth were variable from well to well, as reflected by the dark blue areas in each well in Figure 1, the total number of voxels with intensity above the threshold value was first evaluated within each ROI. The fractional distribution of voxels was then quantified according to intensity in the form of histograms, as illustrated in Figure 3. To determine the capacity of VO(acac)₂ to facilitate FDG uptake, the fraction of voxels in each well with intensity greater than or equal to the mean of the control well with no added VO(acac)₂ was then calculated. As seen in Figure 3, there is a shift of voxels to higher intensity and a broadening of their distribution in the presence of VO(acac)₂. By plotting the fraction of voxels in wells containing VO(acac)₂ with intensity above the mean of the control well vs. VO(acac)₂ concentration, as illustrated in Figure 4, an approximately linear dependence of enhanced FDG uptake was observed over the 1–8 μ M range. At VO(acac)₂ concentrations greater than 10 μ M, no further increase was observed. In the 10 – 25 μ M concentration range, 89% of cells on average had greater intensity than the mean value of control cells.

These experiments were repeated multiple times with variations in the VO(acac)₂ concentration, and the results are plotted in Figure 4. Although the initial level of radioactivity of FDG and the total number of cells per well varied for each experiment, the same pattern of enhanced fractional FDG uptake dependent on VO(acac)₂ concentration was observed repeatedly.

Glucose competes with FDG for entry into cells via glucose transporter proteins¹⁵. In our experiments the KRBH medium was supplemented with 5 mM glucose to simulate the average fasting blood glucose level in a normal adult. It is, therefore, important to note that VO(acac)₂ facilitated increased FDG uptake by MDA-MB-231 breast cancer cells in the presence of a background, physiologically-relevant glucose concentration. In preliminary experiments we have also observed VO(acac)₂ facilitated FDG uptake by MDA-MB-231 cells in the presence of a background concentration of 10 mM glucose (data not shown). Under the latter conditions, the enhanced fractional uptake of FDG compared to control cells

was lower than in the presence of 5 mM glucose but measurably detectable. Measurement of FDG uptake by cancer cells described by Fischer and coworkers was carried out in the absence of background glucose¹⁴.

The uptake of FDG, as shown in Figure 4, is linear with VO(acac)₂ concentration 8×10^{-6} M, with uptake approaching a plateau above this range. At present we do not know whether the plateau reflects the maximum rate at which glucose and FDG can enter cells or saturation of the target site of VO(acac)₂ in the cell through which glucose uptake is enhanced. The results demonstrate, nonetheless, that fractional uptake of FDG is enhanced linearly in a dose dependent manner within a physiologically attainable concentration range of the VO²⁺-chelate.

FDG PET Imaging of Xenograft Tumors in Mice

Because our ultimate objective is to test whether VO(acac)₂ can be employed clinically as a pharmacologic reagent to increase the sensitivity of FDG PET imaging, we have explored through preliminary experiments whether the VO²⁺-chelate enhances FDG uptake by xenograft cancer tumors in a small, laboratory animal model. Although the efficacy of VO(acac)₂ enhanced FDG uptake by cultured cells is demonstrated in Figures 1 – 4, the *in vivo* physiological factors regulating delivery of an agent to a specific tissue and site differ greatly from the *in vitro* conditions used in the laboratory for cells. Therefore, to this end we generated xenograft tumors in the hind limb of female SCID mice by subcutaneous injection of cultured MDA-MB-231 breast carcinoma cells¹⁶.

In initial experiments we tested different adjuvants and time intervals for intraperitoneal injection of the VO²⁺-chelate prior to FDG PET imaging. Because of its low solubility in aqueous media, VO(acac)₂ dissolved in physiological saline required upwards of 0.5 mL to introduce $\sim 10^{-6}$ mole VO(acac)₂ per mouse. Furthermore, solubilization required significant time and was accompanied by partial oxidation of the VO²⁺ moiety, as evaluated by the ultraviolet absorption spectrum. These conditions promoted diarrhea causing dehydration and making injection of FDG via the tail vein difficult. We then adapted the method of Lee and coworkers¹⁷ using neat DMSO to dissolve the VO²⁺-chelate. By this method small volumes (0.05 – 0.10 mL) of DMSO could be used because of the high solubility of the chelate in DMSO; also, oxidation of the VO²⁺ moiety was minimal. While injection of the chelate dissolved in physiological saline resulted in erratic results, particularly when injection of the chelate was carried out 12 hr prior to imaging, use of DMSO as the adjuvant caused no observable complications.

As illustrated in Figure 5, we observed maximum uptake of FDG by xenograft tumor tissue when mice were subjected to intraperitoneal injection of the VO²⁺-chelate 3 – 4 hr prior to imaging. Data collection, continued over time, showed gradual decay of FDG intensity. Because the results in Figure 5 were corrected for the half-life of the ¹⁸F radionuclide ($t_{1/2} \sim 109.75$ min), we conclude that the time-dependent decrease is due to washout of FDG from the tumor tissue. We also note that, as evident in Figure 5, the level of FDG radioactivity per unit volume of tumor tissue decreased to that observed in controls after 12 – 14 hr. It is notable that the maximum uptake of FDG in the presence of VO(acac)₂ was at least 2-fold greater than observed in the absence of VO(acac)₂.

These preliminary results demonstrate that the VO^{2+} -chelate facilitates significantly increased fractional FDG uptake by malignant tumor tissue compared to that of the surrounding normal tissue. Further studies are in progress to determine the optimal dosage of $\text{VO}(\text{acac})_2$ to maximize signal-to-noise in data collection and to determine the optimal time interval for the onset of FDG PET imaging post-injection of $\text{VO}(\text{acac})_2$. Furthermore, there is a significant correlation in clinical studies of the extent of FDG uptake on estrogen-receptor status¹⁸. Because human breast carcinoma MDA-MB-231 cells, as an estrogen-receptor-negative cell line, are associated with high FDG uptake in clinical studies, experiments need to be carried out in which xenograft tumors are generated with a more indolent, estrogen-receptor-positive cell line. Thus, while our preliminary results are encouraging, further investigations need be carried out to determine the translational potential for clinical application of $\text{VO}(\text{acac})_2$ in cancer detection.

Experimental

Materials

Fatty-acid-free BSA, HEPES, and 50% (w/v) sterilized D-glucose were obtained from Sigma-Aldrich (St. Louis, MO); crystalline $\text{VO}(\text{acac})_2$ and DMSO (ACS spectrophotometric grade > 99.9%) were purchased from Sigma-Aldrich (Milwaukee, WI). DMEM, Trypsin-Versene Mixture, and Penicillin/Streptomycin (10,000 U/mL of each) were obtained from BioWhittaker (Walkersville, MD). Heat inactivated fetal calf serum was purchased from Gemini Bio-products (West Sacramento, CA). FDG was obtained from IBA Molecular Radiopharmaceuticals and Isotopes USA (Romeoville, IL). All other chemicals were of analytical reagent grade or of the highest purity commercially available. The concentration of BSA in KRBH was estimated on the basis of the extinction coefficient of $4.36 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm¹⁹. The concentration of $\text{VO}(\text{acac})_2$ was determined gravimetrically. All solutions were either sterilized by autoclaving or passed through 0.2 micron filters before use with cultured cells or mice.

Cell culture

Human breast carcinoma MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA 20110). Cell cultures were conducted in an incubator maintained at 37 °C under an atmosphere of 5% CO_2 and 95% air. Cell cultures were grown in DMEM, containing 25 mM D-glucose, essential amino acids and L-glutamine, vitamins, and salts, and supplemented with 10% (v/v) fetal calf serum, 1% (v/v) penicillin-streptomycin (hereafter called *complete DMEM*). Cells were split by trypsinizing when 85–90% confluent and resuspended in complete DMEM. The culture medium was replenished with fresh medium every 72 hrs.

PET Imaging

MDA-MB-231 cells grown as described above were trypsinized and resuspended in serum-free DMEM. Approximately 1.0×10^5 cells were transferred to each of nine wells in 12-well plates (Thermo-Fisher Scientific, Hanover, IL). Serum-free DMEM was added to each well to a final volume of 2.0 mL for overnight incubation at 37 °C. Prior to imaging and addition of FDG, the medium was aspirated gently and replaced with 1.00 mL of KRBH containing

0.1 mM BSA and 5 mM glucose. The supernatant was supplemented with 1 – 25 μ M VO(acac)₂ dissolved in neat DMSO, followed by incubation of cells at 33 °C[§] for 2 hrs. The radioactivity of an aliquot of stock FDG solution was calibrated with a Capintec Radioisotope CRC-7 Calibrator upon dilution with the KRBH/BSA/glucose medium to a final volume of 9.0 mL. A 1.0 mL aliquot of the diluted FDG solution was then added to each well with continued incubation for 1 hr at 33 – 34 °C. FDG uptake was quenched by placing the 12-well plate on ice, gently aspirating the supernatant, and washing the cells with cold KRBH followed by washing with cold physiological saline. For each 9-well plate, the incubation supernatant and the washings were removed with a pipet and combined. After aspirating the second washing, the liquid adherent to the bottom surface of the well was removed by wicking with narrow strips of Beckman No. 1 filter paper by placing the end of the paper strip against the side wall of the well without disturbing the monolayer of cells. The radioactivity of the washings containing the strips of filter paper was measured with a Capintec Radioisotope CRC-7 Calibrator. Comparison of the total radioactivity recovered, corrected for time-dependent decay, with the initial value measured at the start of the incubation period indicated that approximately 0.18 mCi FDG per 9 wells had been taken up by cells. The intensity of FDG uptake by cells as a function of VO(acac)₂ concentration was then quantified with a small-animal GMI FLEX Triumph tri-modal microPET/SPECT/CT imaging system (Gamma Medica, Northridge, CA).

For imaging of xenograft tumors in mice, MDA-MB-231 cells were harvested by trypsinization and washed with physiological saline. The hind legs of 4–5 week old, B17SC SCID female mice (Taconic Farms, Hudson, NY) were shaved, and xenograft tumors were generated in one hind limb by subcutaneous injection of *ca.* 1.0×10^7 cells suspended in a total volume of 0.05 – 0.10 mL physiological saline, as described by Pang and coworkers¹⁶. Injection of cells was carried out with the mice under Isoflurane anesthesia. Tumors with largest dimension of 8 – 10 mm developed within 5 – 6 weeks, at which point the tumor was imaged with the same small-animal GMI FLEX Triumph tri-modal microPET/SPECT/CT imaging system. Mice were subjected to overnight fasting with *ad libitum* access to water prior to data collection. To facilitate FDG injection via the tail vein, mice were “hydrated” by subcutaneous injection of a total volume of 1.0 mL physiological saline in the wall of the rib cage 12 hours prior to data acquisition. During imaging mice were kept supine under anesthesia (3% Isoflurane at 3 L O₂ flow/min). A temperature probe was placed dorsally against the animal skin and monitored by an SAI animal monitoring system. Body temperature was controlled by heating the CT/PET animal bed which was turned off or on manually depending on readings from the temperature probe. Imaging data were collected for 30 min at 1 hr after injection of approximately 0.1 mCi FDG via the tail vein.

The tumor boundaries were defined on the basis of CT images, and a region of interest (ROI) was drawn around the tumor according to the tumor outline in the CT images. The maximum and average uptake values were calculated within the ROI normalized to the body

[§]During incubation of cells with FDG, the 12-well plate was kept in as tight thermal contact as possible with a stainless steel platform through which water heated to 45 °C was circulated by a constant temperature bath. Measurement of the temperature of the medium in the wells with a thermocouple probe showed that the highest temperature that could be achieved for the cells was 33 – 34 °C because the construction of the 12-well plates prevented the bottom surface of the wells from having immediate contact with the steel platform. Raising the bath temperature higher did not result in a higher temperature for the medium in wells.

weight and the standardized FDG uptake in the liver²⁰. The data were analyzed with use of the software package Amira (Visualizations Sciences Group, Burlington, MA).

Crystalline VO(acac)₂ was freshly dissolved in neat DMSO so that a 0.05 – 0.10 mL intraperitoneal injection (given under anesthesia) corresponded to a dosage of 0.13 g/Kg body weight or approximately 1.0×10^{-6} mole VO(acac)₂/20 g mouse. Optimal enhancement of FDG uptake by tumor tissue was observed when injection of VO(acac)₂ was carried out 3–4 hr prior to FDG PET imaging. All procedures were carried out in accordance with the U. S. Public Health Service *Policy on Humane Care and Use of Laboratory Animals* and approved by the University of Chicago Institutional Animal Care and Use Committee.

Conclusions

This is the first investigation to demonstrate that a VO²⁺-chelate, namely VO(acac)₂, known for its capacity to promote uptake of glucose by insulin-sensitive tissues²¹, facilitates uptake of FDG by cancer cells. While glucose enters insulin-sensitive cells primarily via the glucose transporter 4 (GLUT4) protein activated upon insulin binding to its membrane receptor, cancer cells, in general, exhibit little or no insulin sensitivity and primarily overexpress the GLUT1 protein system for glucose uptake^{22, 23}. Because 2-deoxyglucose enters cells via the same glucose transporter protein systems as D-glucose¹⁵, the target enzyme of VO(acac)₂ must lie downstream of the glucose transporter protein.

It is particularly noteworthy that VO(acac)₂ not only promotes increased fractional uptake of FDG by cultured breast carcinoma cells *in vitro* but also facilitates increased uptake of FDG by malignant tumor tissue *in vivo*, as illustrated through Figure 5. We have previously demonstrated that VO(acac)₂ is accumulated intact by xenograft tumor tissue over a 3–4 hr period post-washout of the VO²⁺-chelate from surrounding normal tissue²⁴. The increased uptake of FDG by tumor tissue is to be attributed, therefore, to the longer retention of VO(acac)₂ in the xenograft tumor tissue over that by normal surrounding tissue. At present we do not know how the VO(acac)₂ gains entry into cells and whether its uptake differs between normal and cancer cells. Identification of the mechanism of translocation of the VO²⁺-chelate is likely, therefore, to be an important research objective to maximize intensity contrast in FDG PET imaging.

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Figure 1.

Coronal images of FDG uptake by MDA-MB-231 cells cultured in 9 wells of a 12-well plate. Vanadyl concentrations: Well 1 (0 μM), Well 2 and 5 (5 μM), Well 3 and 6 (10 μM), Well 4 and 7 (15 μM), Well 8 and 9 (25 μM). Color is proportional to uptake of FDG (Blue, least intense; Red, most intense). The ROI for each well was restricted to the inner diameter defined by the cylindrical plastic wall of the well. Regions where color due to FDG uptake “bleeds over” into space between wells were excluded from the ROI and are likely due to decreased resolution of the PET scanner.

Figure 2.

Diagram illustrating regions of Interest (ROI) selected for each well. The yellow, cylindrical disc-like areas illustrate the ROI within which the intensity of voxels was measured. The vertical plane represents one “slice” that was swept across the 9 wells from the nearest edge of wells 1, 4, and 7 to the more distant edge of wells 3, 6, and 9 (*cf.*, Figure 1) to measure voxel intensities. The faint green, red, and yellow colored areas at the bottom edge of the plane represent cells adherent as a monolayer to the bottom of each well. Regions of color outside of the boundaries of the discs were excluded from measurements of voxel intensity.

Figure 3.

Comparison of fraction of voxels (y-axis) vs. voxel intensity (x-axis) for Well 1 [control, no VO(acac)₂] and Well 3 [10 μM VO(acac)₂] in Figure 1, showing a shift in voxel intensities to higher values because of increased FDG uptake.

Figure 4. Scatter plot depicting the fractional population of voxels with intensity due to FDG uptake above the mean of cells in the control well (absence of VO(acac)₂) as a function of VO(acac)₂ concentration.

Figure 5.

Comparison of relative FDG uptake per unit volume of xenograft tumor tissue in SCID mice as a function of the time interval between intraperitoneal $\text{VO}(\text{acac})_2$ injection and initiation of FDG PET scanning. The relative FDG uptake was measured as described in the Experimental section normalized to the body weight and the standardized FDG concentration in the liver²⁰. In this experiment, all mice were injected within a 10–15 min period with the VO^{2+} -chelate dissolved in DMSO, the control mouse receiving the same volume of DMSO. Each histogram bar represents the FDG uptake by the tumor tissue of a mouse measured at the indicated time interval post- $\text{VO}(\text{acac})_2$ injection. We estimated an approximate uncertainty of $\pm 20\%$ in the measured relative FDG uptake values.