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[^{11}C]Ascorbic and [^{11}C]Dehydroascorbic Acid, An Endogenous Redox Pair for Sensing Reactive Oxygen Species Using Positron Emission Tomography

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Abstract

Here we report the radiosynthesis of an endogenous redox pair, [^{11}C]ascorbic acid ([^{11}C]VitC) and [^{11}C]dehydroascorbic acid ([^{11}C]DHA), the reduced and oxidized forms of vitamin C, and their application to ROS sensing. These results provide the basis for *in vivo* detection of ROS using positron emission tomography (PET).

Reactive oxygen species (ROS) are generated as a normal product of oxidative metabolism and are required signalling molecules in a diverse array of biological processes.[1] Dysregulation of ROS in common disease states including cancer,[2] neurodegeneration,[3] chronic inflammation,[4] and diabetes[5] provides a powerful motivation to develop non-invasive biomarkers of oxidative stress. Current ROS sensing techniques in living systems are largely limited to *in vitro* study. Advances toward *in vivo* ROS detection include approaches using electron spin trapping (ESR),[6] near-IR optical,[7] bioluminescent,[8] [^{13}C] magnetic resonance imaging (MRI),[9,21] chemiluminescent probes,[10] fluorescent probes[11] and positron emission tomography (PET).[12] Due to its high sensitivity, good spatial resolution and low toxicity,[13] PET has potential for detecting ROS in a clinical setting.

VitC is transported into cells via the sodium dependent vitamin C transporter (SVCT1–2).[14] In the presence of ROS, VitC undergoes a two-electron oxidation to DHA, which in aqueous solution exists predominantly in bicyclic hemiketal form and is a substrate for glucose transport (GLUT 1, 3, 4).[15] We hypothesized that by taking advantage of rapid GLUT

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transport,[16] this mechanism could be employed to detect extracellular ROS (Figure 1). Previously Yamamoto[17] and Kothari[18] have investigated 6-[^{18}F]-fluoro-6-deoxy-L-ascorbic acid as a PET analogue of VitC; however this probe cannot enter cells via GLUT, as the ^{18}F label in the 6 position prevents formation of the bicyclic species of DHA.[17b,17e,f] We have therefore developed a new pair of endogenous PET radiotracers [^{11}C] ascorbic acid ([^{11}C]VitC) and its oxidized partner, [^{11}C] dehydroascorbic acid ([^{11}C]DHA) and have used this redox pair to sense ROS *in vitro*.

[^{11}C]VitC was synthesized from L-xylosone based on a modification of the previously reported [$^{13/14}$C] enriched techniques (Scheme 1).[19] The methods employed and relevant analytical data are reported in full in the Supporting Information (Figures S1–S3). Table 1 summarizes radiochemical yields for synthesis with varying amounts of added KCN carrier. With no carrier added [^{11}C]VitC *in situ* oxidation to [^{11}C]DHA was observed at pH 7 (Figure S4) possibly related to generation of ROS by radiolysis. This phenomenon has been previously observed for 6-[^{18}F]-Fluoro-6-deoxy-L-ascorbic acid.[18] Thus, we used non-radioactive carrier VitC to protect against *in situ* oxidation. This was achieved by adding carrier KCN during the radiochemical preparation. With the presence of 0.6 – 1.0 mM (specific activity ≈ 3.0–10.0 mCi/μmol; 110–370 MBq) carrier in the final isolated product [^{11}C]VitC is stable at all time points tested (Figure S5). As sampling of our institution's clinical 2-deoxy-2-[^{18}F]fluoroglucose ([^{18}F]FDG) doses revealed that the administered [^{18}F]FDG solution contained 1.2 ± 0.1 mM (n = 3) non-radioactive glucose, we do not anticipate that addition of carrier at this level will significantly diminish the ability to image [^{11}C]DHA transport via GLUT. Other antioxidants were also considered to prevent [^{11}C]VitC *in situ* oxidation, but given that these also react with ROS they would likely confound interpretation of *in vitro* and *in vivo* data.

We first evaluated the transport of both [^{11}C]VitC and [^{11}C]DHA in U87 human glioblastoma cells using [^{18}F]FDG as a standard radiotracer for GLUT transport. GLUT blocking studies were carried out with application of 10μg/ml cytochalasin B, a potent inhibitor of GLUT transport.[15a,23] SVCT transport was interrogated by modulating (+)/(−) co-transport of Na^+ as per Vera et al.[15b–d] While uptake of [^{11}C]VitC is not affected by blocking of the GLUT receptor, uptake is notably decreased in the absence of Na+ (Figure 2a). However, uptake of [^{11}C]DHA is not affected by availability of Na^+ for co-transport, and is effectively blocked by application of cytochalasin B (Figure 2b) mirroring the trend observed for [^{18}F]FDG (Figure 2c). This data confirms previously reported trends for uptake of the non-radioactive compounds.[15] Since the uptake of [^{11}C]DHA via GLUT is 10 fold higher than uptake of [^{11}C]VitC we anticipated that intracellular accumulation of [^{11}C]VitC would be primarily via an oxidation-dependent process. Indeed, transport of [^{11}C]VitC via SVCT occurs more slowly than transport of the oxidized species, [^{11}C]DHA via GLUT in many tissues.

Having shown the expected behavior of [^{11}C]VitC and [^{11}C]DHA *in vitro*, we performed a proof of concept *in vivo* experiment to demonstrate the differential transport of the two tracers. It has been well-established that DHA (but not VitC) crosses the blood-brain barrier transported primarily by GLUT1.[23] Approximately 200 μCi of [^{11}C]VitC (n = 3) and [^{11}C]DHA (n = 3) each were administered to normal rats via tail vein injection and a 40 min

dynamic scan was obtained using a microPET/CT scanner. As anticipated the brain accumulation of [^{11}C]DHA was markedly higher than that of [^{11}C]VitC (Figure 3), confirming our hypothesis that changes in uptake based on oxidized vs. reduced forms of ascorbic acid can be detected using PET.

Finally we investigated ROS-dependent [^{11}C]VitC accumulation in cells. This was first accomplished in U87 cells by addition of exogenous H_2O_2 to the media,[12a] resulting in a greater than 2-fold increase in [^{11}C] accumulation (*p = 0.0006) as shown in Figure 4a. We next applied [^{11}C]VitC to a model of endogenous ROS production, namely stimulated neutrophil-lineage cells undergoing oxidative burst. For this study we used the HL60 cell-line, a human leukemia neutrophilic precursor, and human neutrophils, which had been freshly isolated from whole blood. The mechanism of VitC uptake in human neutrophils has been well established in literature.[24,15c] During phagocytosis, neutrophils undergo Nox-mediated generation of ROS to destroy bacteria and simultaneously oxidize extracellular VitC.[25] [^{11}C]VitC was oxidized to [^{11}C]DHA by the major ROS produced during the oxidative burst, H_2O_2, O_2^- and ClO^- (Figure S6). To investigate tracer uptake via this mechanism, cells were incubated with 10 μCi (0.37 MBq) [^{11}C]VitC +/− activation with 2 μM phorbol 12-myristate 13-acetate and +/− 20 μg/mL cytochalasin B blocking.[24,15c] For both HL60 cells and neutrophils a significant increase in cell-associated activity, approximately 2-fold, was noted with activation (**p = 0.0025, ***p = 0.00041) (Figure 4b,c). For HL60 cells administration of cytochalasin B decreased the uptake of [^{11}C]VitC to approximately the amount observed for (−) PMA. For neutrophils partial blocking was observed. This partial blocking effect in activated neutrophils could be explained by nitric oxide mediated expression of SVCT, as previously described.[26] As expected % cell associated activity of [^{11}C]DHA with co-administration of cytochalasin B did not differ significantly between +/− PMA in human neutrophils due to blocking of GLUT transport (Figure S7). These results provide the basis for detection of endogenously produced ROS using [^{11}C]VitC PET.

In conclusion, we have developed a new PET radiotracer [^{11}C]VitC, which exhibits ROS-dependent cellular accumulation. [^{11}C]VitC and its redox partner [^{11}C]DHA behaved as anticipated *in vitro* and *in vivo*, consistent with their markedly different transport mechanisms. [^{11}C]VitC is capable of detecting endogenously produced ROS in activated neutrophil-lineage cells, suggesting potential clinical utility in studying inflammation and/or monitoring immunotherapy. We hypothesize that the ascorbate recycling mechanism may be used to image a myriad of ROS-driven disease states. Furthermore, as high-dose vitamin C has been studied as an anticancer therapy for decades,[27] low toxicity in patients has already been well-established.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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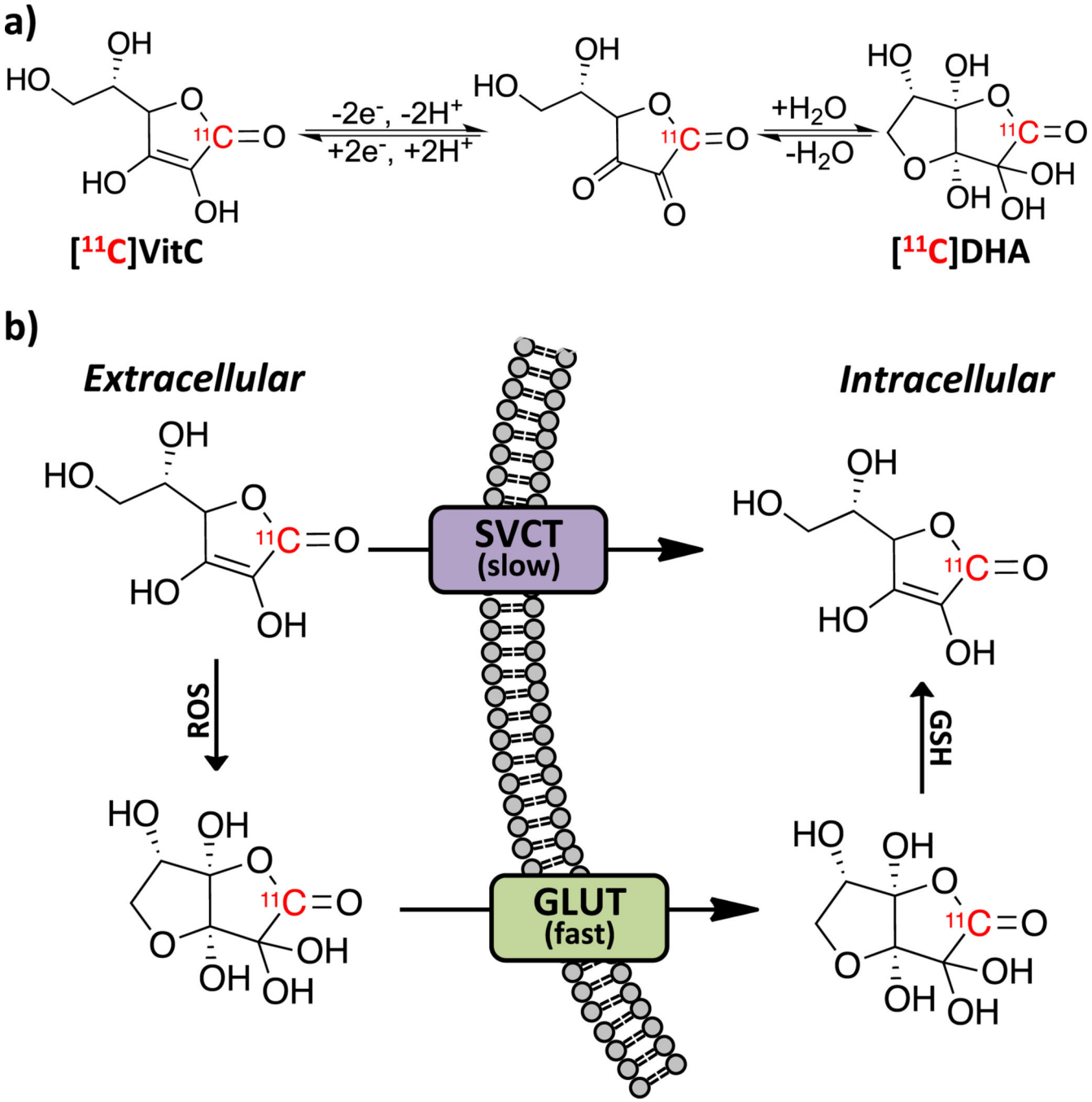


Figure 1.
(a) Oxidation of and hydration of VitC forming bicyclic DHA hydrate. (b) Transport mechanisms of VitC and DHA.

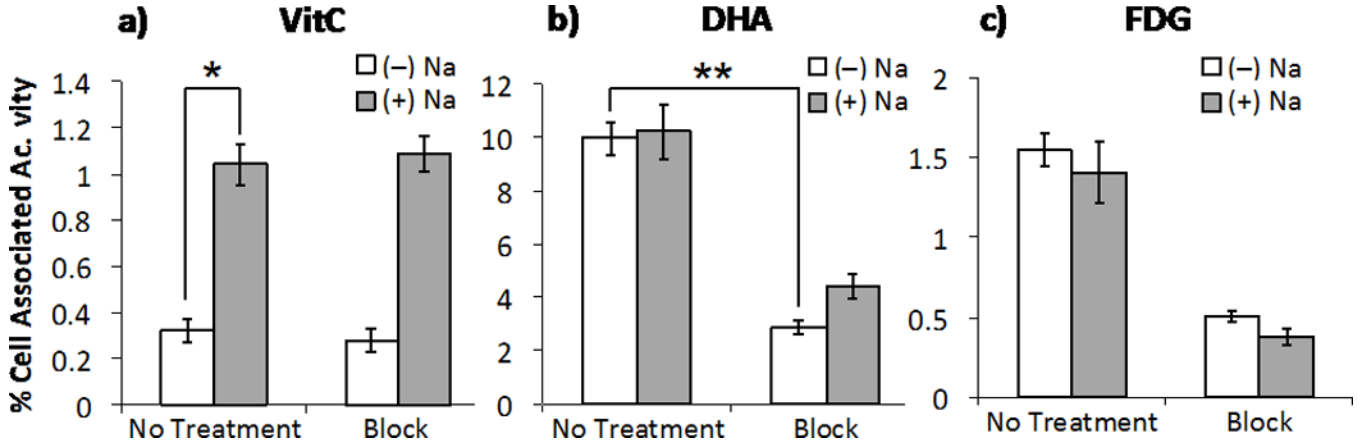


Figure 2.

Uptake of (a) [^{11}C]VitC (*p = 0.0002), (b) [^{11}C]DHA (**p < 0.0001) and (c) [^{18}F]FDG (+)/(−) availability of Na^+ in media for co-transport via SVCT, (+)/(−) 10 μg/mL cytochalasin B blockade of GLUT in U87 human glioblastoma cancer cells.

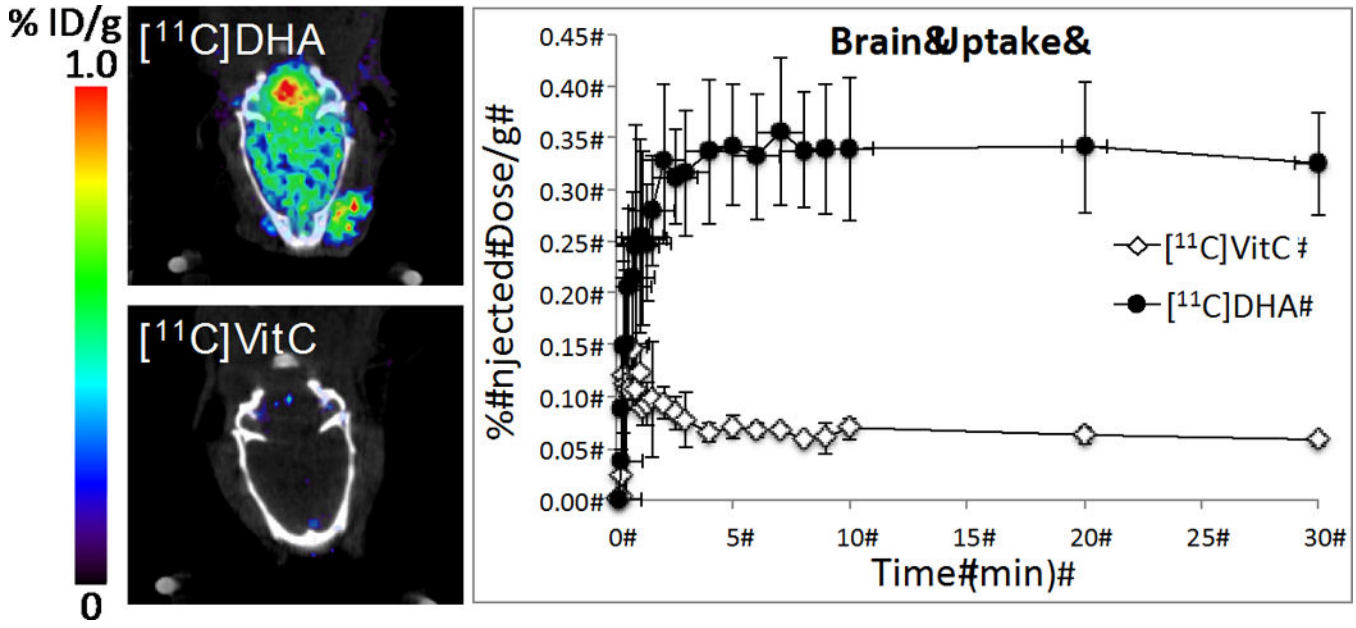


Figure 3.

Representative *in vivo* microPET images of [^{11}C]VitC and [^{11}C]DHA in a normal rat brain (t = 0 – 30 min) and brain ROI data (n = 3) for dynamic scans.

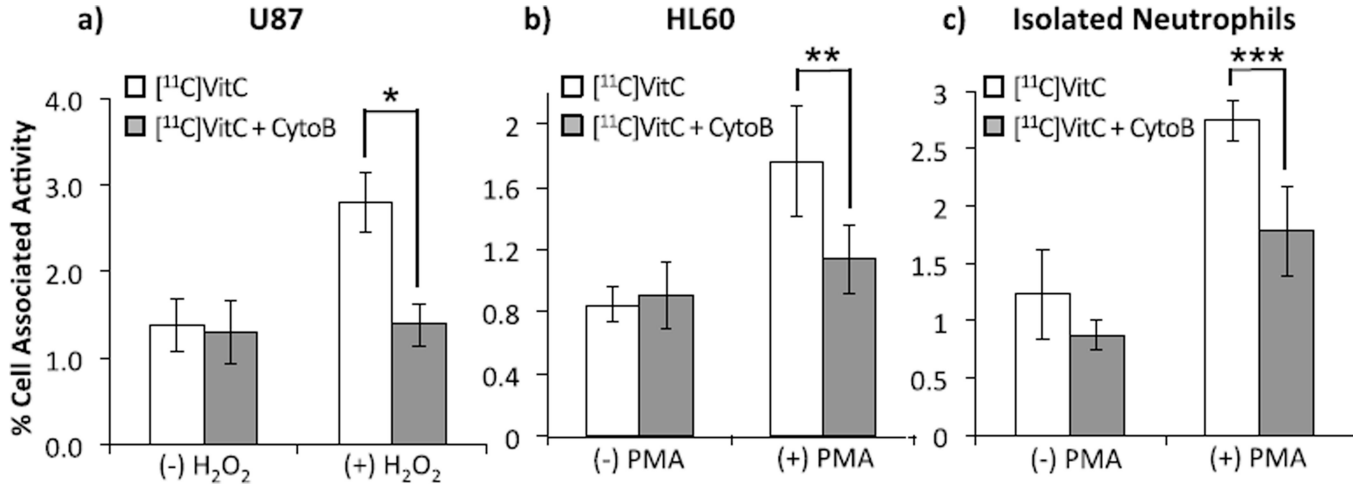
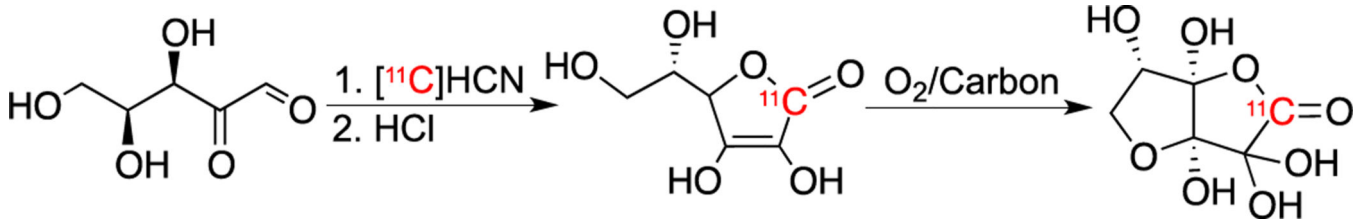


Figure 4.

Uptake of [^{11}C]VitC in (a) in U87 glioma cells (+)/(−) 100 μm H_2O_2, b) HL60 human promeylocyctic leukemia cells (+)/(−) 2 μM PMA activation and (c) freshly isolated human neutrophils (+)/(−) 2 μM PMA and (+)/(−) 20 μg/mL cytochalasin B blockade.



Scheme 1.
Radiochemical syntheses of [^{11}C]VitC and [^{11}C]DHA.

Table 1

Summary of radiochemical yields and specific activities for [^{11}C]VitCradiosyntheses with varying amounts of carrier added.

KCN carrier added	% Radiochemical Yield	Specific Activity (mCi/μmol)	number of trials
1 mg/mL	45.4 ± 9.8	4.0 ± 1.2	n = 6
0.75 mg/mL	46.1 ± 9.31	6.4 ± 0.66	n = 3
0.5 mg/mL	29.7 ± 8.8	9.0 ± 3.7	n = 5
0 mg/mL	14.3 ± 10.4	267 ± 148	n = 5