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ARTICLE TYPE

New fluorinated fructose analogs as selective probes of the hexose transporter protein GLUT5†

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Facilitated hexose transporters (GLUTs) mediate the transport of hexoses and other substrates across the membranes of numerous cell types, and while some are expressed ubiquitously (e.g., GLUT1), others are more tissue specific (e.g., GLUT5). These properties have been exploited for the imaging of cancer cells by the use of hexose based probes, including fluorinated hexose derivatives for use with positron emission tomography (PET). However, design of new probes has been hampered by a limited understanding of how GLUT transporters interact with their substrates at the molecular level. Two fluorinated fructose surrogates designed for uptake by the GLUT5 transporter are described here: 3-deoxy-3-fluoro-D-fructose (3-FDF) and 1-deoxy-1-fluoro-2,5-anhydromannitol (1-FDAM). Synthesis (both cold and radiolabeled) and *in vitro* analysis of their transport characteristics in two breast cancer cell lines (EMT-6 and MCF-7) expressing GLUT5 are detailed. Both analogues are readily taken up into both cancer cell lines, with uptake mediated primarily by GLUT5. They also have low IC_{50} values, indicating a high affinity for the transporter, suggesting that the uptake of these probes would be unaffected by endogenously circulating fructose. Selective uptake by GLUT5 was also demonstrated in *Xenopus* oocytes. Finally, these results are the first demonstration that a hexose existing predominantly in the pyranose ring structure (3-FDF) is transported by GLUT5, strongly suggesting that this transporter can handle both furanose and pyranose forms of fructose.

Introduction

Facilitated hexose transporters (GLUTs), belonging to the gene family SLC2A, are responsible for the entry and exit of hexoses and other substrates into and out of numerous cell types. Some of these proteins are considered to be essential for basic cell metabolism and to that end mammalian cells express one or more of them in their plasma membranes at quite high levels. In addition, because of the differing metabolic needs of specific cell and tissue types the various members of this gene family have different kinetic properties, are regulated in different ways and are expressed at varying levels.[1] Thus, they have the potential to be used as specific portals into target cells for imaging or therapeutic approaches.[2] However, the current understanding of how they bind and transport their substrates is very limited. All of the GLUTs appear to have a basic structure of twelve transmembrane helices (TM's) arranged in two bundles of six connected by a long intracellular loop between TM's 6 and 7.[3] Cysteine scanning studies suggest that four TM's form an outer scaffold supporting the remaining eight, regions of which come together to form an aqueous pore containing the substrate binding site(s).[4] Comparison with other similar transport protein families, some of which have been crystallized, suggests that transport is achieved by structural rearrangement of the protein folding such that the binding site is sequentially exposed to the outside of the cell, then occluded and finally exposed to the inside.[5,6,7,8] However, despite a significant number of studies having been performed on this family of mammalian proteins, it is only very recently that the crystal structure of GLUT1 (SLC2A1) itself been determined for the inward facing conformation.[9] The arrangement of the TM's is almost identical to a previously reported structure for XLe, a bacterial homologue of the human GLUT family and together these structures will allow for a much better understanding of how substrates are bound and translocated by these proteins. A number of key amino acid motifs, contributed by several of the pore lining TM's, have been proposed to contribute to the binding site for the substrates. These include a QQLS sequence in TM7 which is conserved almost completely throughout the GLUT family. In addition, the pairing of hydrophobic residues across the exterior vestibule has been suggested to contribute to substrate selectivity.[6] However, in no case has the full substrate binding pocket(s) of a GLUT yet been defined.

Competition experiments employing substrate analogues have been successful in identifying key features of the hexose structures involved in binding to the exo- and endofacial elements of the pore. Pioneering studies done by Holman and colleagues examined the requirements for substrate binding to the fructose transporter GLUT5 at both sides of the membrane. Using the ability of their analogues to inhibit fructose transport they found that the fructofuranose ring structure, usually assumed for this hexose, was not essential for binding and that related structures locked in the fructopyranose form could also bind. This led them to propose that the hydroxyls at position C1, C2, C3 and C4 approach the exofacial binding site, while the C5 and C6 hydroxyls are less involved in substrate recognition. This in turn suggested that most of the interaction for recognition and binding involved hydrogen bonding between the hydroxyls on the hexose and specific amino acid residues lining the pore. They also proposed that C6 may lie within the pore and not normally interact with the exofacial binding site of GLUT5, and suggested that labeling fructose at position 6 would provide analogues that could be readily handled by GLUT5,[6,10,11] and explicitly raised the possibility fluorine substitution at position 6 could increase

the binding affinity of the compound. Importantly, though, this seminal work focused only on *binding* but not transport.

Using Holman's work as a starting point, our initial efforts focused on the synthesis and characterization of the fructose analogue 6-deoxy-6-fluoro-D-fructose (6-FDF) as a possible substrate for GLUT5 with the goal of employing the ^{18}F labeled compound as an imaging probe for use with PET. Subsequently, we determined this compound to be handled readily by GLUT5 in two breast cell lines. We also demonstrated that it could be utilized for PET imaging of two murine xenograft models of breast cancer, and that it had a favorable clearance and dosimetry profile in a rat model.[12,13] However, although fluorine substitution at the 6 position allowed the fructose derivative to enter the cells rapidly, this substitution also blocked the ability of hexokinase to phosphorylate this analogue at the 6 position. Moreover, the lack of expression of ketohexokinase (KHK) in these cells precluded phosphorylation at the 1 position and hence no metabolic trapping occured.[13]

Given the favourable uptake and lack of metabolic trapping seen with 6-FDF, we chose to explore labeling at another position on the ring structure, leaving C6 open for phosphorylation. Holman and coworkers suggested that replacement of the hydroxyl at the 3 position with a bulky group was not well tolerated by the transporter – and while this may be the case for the allyl derivates which they studied, we reasoned that a fluorine at position 3 may satisfy the size and hydrogen bonding requirements for proper recognition and possible translocation by GLUT5.[10,11,14] Therefore, determining the ability of 3-deoxy-3-fluoro-D-fructose (3-FDF) **12** to bind and be transported by the previously characterized GLUT5 expressing cell lines EMT-6 and MCF-7 was of great interest. These experiments were designed to establish the tolerance of GLUT5 to fructose substitution at C-3, using breast cancer cell lines known to express high levels of this hexose transporter.

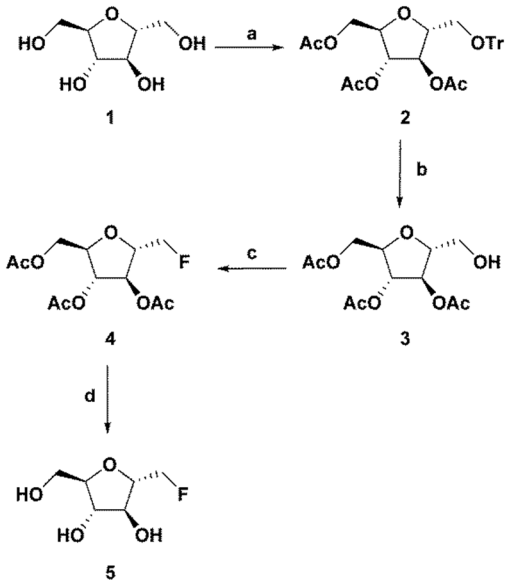
The work of Yang *et al*. was also intriguing, as it suggested the possibility of using fluorinated derivatives of 2,5-anhydromannitol (2,5-AM) **1** as GLUT5 substrates.[10] 2,5-AM **1** is a C_2-symmetric fructose mimic permanently held in the furanose form and which can inhibit the transport of fructose. However, its transport has never been directly measured, although, McQuade and co-workers recently showed that a 2,5-AM derivative labeled with a nitrobenzoxadiazole fluorescent tag is transported by GLUT5 and/or GLUT2.[15] A fluorinated 2,5-AM derivative could show key advantages over a fructose analogue for imaging of GLUT5 expressing tumors. 2,5-AM **1** possesses an affinity for GLUT5 comparable to that of fructose, suggesting that the furanose form might be a preferred structure for the binding pocket [6-FDF also exists primarily in the furanose ring form[13]]; and being C_2 symmetric, positions 1 and 6 are equivalent allowing for labeling at either position while preserving affinity for GLUT5 [15,12] and potentially permitting phosphorylation by either KHK or hexokinase.[10,16]

The 2,5-AM **1** and the 2,5-AM derivative 1-deoxy-1-fluoro-2,5-anhydro-D-mannitol (1-FDAM) **5** were both synthesized, as well as the fructose derivative 3-deoxy-3-fluoro-D-fructose (3-FDF) **12** and herein the *in vitro* analysis of their transport characteristics in two breast cancer cell lines expressing GLUT5 is described. These data demonstrate that these fluorinated probes were transported into breast cancer cell lines primarily by the fructose transporter GLUT5 and provide the first demonstration that a hexose existing primarily in the pyranose form can be transported by GLUT5. In addition, this work provides valuable information on the GLUT5 structural demands and suggests potential routes for the development of new molecular imaging probes for breast cancer.

Results and discussion

Synthesis of 1-deoxy-1-fluoro-2,5-anhydro-D-mannitol (1-FDAM) 5. The synthesis of 2,5-anhydro-D-mannitol (2,5-AM) **1** was modified from the procedure reported by Horton and co-workers.[17] Briefly, treatment of D-glucosamine with sodium nitrite and acidic acid in water generated 2,5-anhydro-D-mannose which was subsequently reduced *in situ* with sodium borohydride to afford 2,5-AM in 71% over two steps.

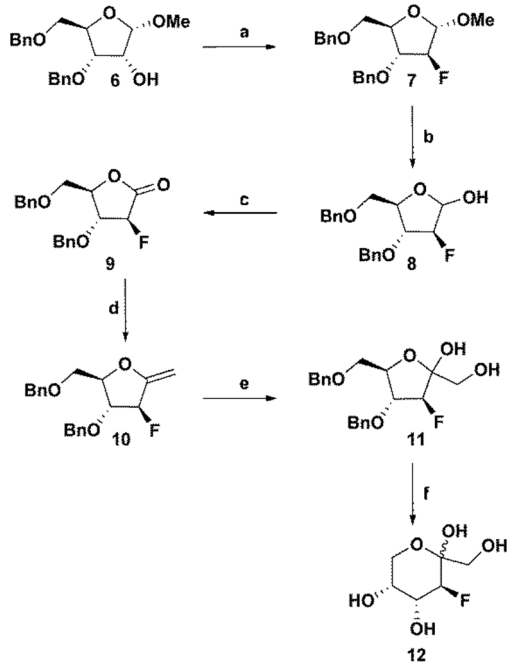
The synthesis of 1-deoxy-1-fluoro-2,5-anhydro-D-mannitol (1-FDAM) **5** was accomplished in four steps starting from **1** (Scheme 1).[18] Selective protection of the primary C-1 hydroxyl with trityl chloride (TrCl) in pyridine at 90 °C for 3 h, followed by global acetylation afforded the product **2** in moderate yield over the two steps. Subsequently, the trityl protecting group was removed selectively by treatment with trifluoroacetic acid and triethylsilane in dichloromethane to afford primary alcohol **3** in 84%. Triflation under standard conditions and treatment with cesium fluoride in *tert*-amyl alcohol at 90 °C gave the fluorinated compound **4** in 80% isolated yield.[19] Finally, deprotection of the acetates using sodium methoxide provided 1-FDAM **5** in 95% isolated yield.



Scheme 1 Synthesis of 1-deoxy-1-fluoro-2,5-anhydro-D-mannitol (1-FDAM). Reagents and conditions : (a) TrCl, Pyr, 90 °C, 3h then Ac_2O, pyr, 0 °C-r.t., 12 h, 40%; (b) TFA (4-5% in CH_2Cl_2), Et_3SiH, r.t., 20 min, 84%; (c) Tf_2O, pyr, CH_2Cl_2, -10 °C, 30 min then CsF, *t*-AmOH, 90 °C, 25 min, 80%; (d) NaOMe, MeOH, 30 min, 95%.

Synthesis of 3-deoxy-3-fluoro-D-fructose (3-FDF) 12. The synthesis of 3-deoxy-3-fluoro-D-fructose (3-FDF) **12** was achieved in six steps starting from the known intermediate methyl 3,5-di-*O*-benzyl-α-D-ribofuranoside **6** (Scheme 2).[20] Briefly, the compound **6** provided the fluorinated compound **7** in 77 % via triflation under standard conditions and treatment with cesium fluoride in *tert*-amyl alcohol at 90°C.[19] Glycoside hydrolysis with formic acid (80%) provided the compound **8** in 65%, which was subsequently oxidized in the presence of TPAP and NMO in CH_2Cl_2 to give the compound **9** in 94%. The latter was then

converted to its corresponding olefin **10** via Julia-Lythgoe olefination in 78% yield over two steps. Upjohn dihydroxylation in the presence of catalytic osmium tetroxide and NMO gave compound **11** in 85%. Finally, deprotection of the remaining benzyl protecting groups under hydrogenolysis conditions afforded 3-FDF **12** in 93% yield. Notably, NMR analysis indicated that **12** exists predominantly in the pyranose form, raising the interesting question of whether it would be transported effectively by GLUT5.



Scheme 2 Synthesis of 3-deoxy-3-fluoro-D-fructose (3-FDF). Reagents and conditions : (a) Tf_2O, pyr, CH_2Cl_2, -10 °C, 1 h then CsF, *t*-AmOH, 90 °C, 12 h, 77% ; (b) 80% HCO_2H, 60-70 °C, 24 h, 65%; (c) TPAP, NMO, 3 Å MS, CH_2Cl_2, r.t., 12h, 94%; (d) 2-methanesulfonylbenzothiazole, LiHMDS, THF, -78 °C, 45 min then DBU, THF, r.t., 1 h, 78% ; (e) OsO_4 (cat.), NMO, acetone:water (10:1), r.t., 12 h, 85% ; (f) $Pd(OH)_2/C$, H_2, MeOH, r.t., 12 h, 93%.

Radiochemical synthesis. Syntheses of [^{14}C]-labeled 1-FDAM **5** and 3-FDF **12** were carried out via variations on the routes described above. In the case of [^{14}C]-**5**, [^{14}C]-D-glucosamine was used as starting material to prepare first [^{14}C]AM, then [^{14}C]-**5** (in 14% overall yield). For [^{14}C]-**12**, lactone **9** was olefinated using [^{14}C]-2-(methylsulfonyl)benzothiazole labeled specifically at the methyl position, and the resulting [^{14}C]-**10** was carried through the remaining steps to afford [^{14}C]-**12** in 70% overall from **9**.

Inhibition of [^{14}C]D-fructose transport with 2,5-AM 1. In order to understand the ability of 2,5-AM **1** and its derivatives to bind to and be transported by GLUT5, 2,5-AM **1** was used as a competitive inhibitor against [^{14}C]D-fructose transport (100 μM) and its IC_{50} determined. Figure 1 shows inhibition of [^{14}C]D-fructose transport measured over 60 min in both of the GLUT5 expressing EMT-6 and MCF-7 cell lines.[12,13,21,22,23] In both cell types 0.1 – 10 mM 2,5-AM **1** showed significant inhibition of fructose with an IC_{50} of 1.06 ± 0.58 μM in EMT-6 (*n=3*) and 0.16 ± 0.94 μM in MCF-7 cells (*n=3*). At a concentration of 10 mM 2,5-AM **1**, transport of [^{14}C]D-fructose was inhibited by 62% in both EMT-6 and MCF-7 cells.

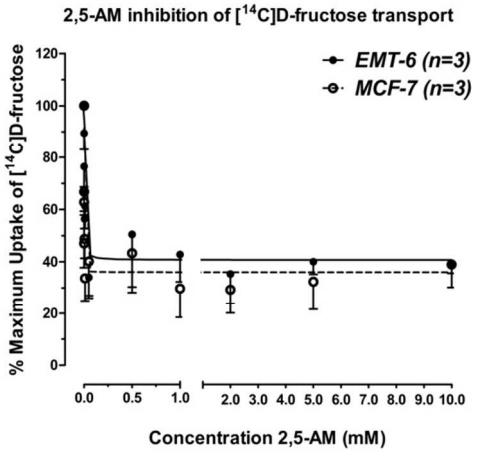


Figure 1. 2,5-AM inhibition of [^{14}C]D-fructose transport after a 60 min incubation at 37 °C with both EMT-6 and MCF-7 using increasing concentrations of 2,5-AM. Fructose transport was inhibited by increasing concentrations of 2,5-AM, and the IC_{50} obtained for EMT-6 (•) was 1.06±0.58 μM and 0.16±0.09 μM in MCF-7 (○). Error bars represent the SEM. (See ESI for semi-log plots of the same data.)

Inhibition of [^{14}C]D-fructose transport by 1-FDAM 5. Figure 2 shows initial experiments ascertaining the viability of 1-FDAM **5** as a substrate for GLUT5. The previously characterized GLUT5 expressing breast cancer cell lines EMT-6 and MCF-7 were utilized for dose-dependent competitive inhibition of [^{14}C]D-fructose uptake by 1-FDAM **5** and to determine the relative affinity for the transporter. After 60 min incubation with 0.1 to 10 mM non-radiolabeled 1-FDAM **5** in the extracellular media, dose dependent inhibition of [^{14}C]D-fructose uptake was observed with an IC_{50} of 6.9 ± 3.0 μM (*n=4*) in EMT-6 and 3.96 ± 2.6 μM (*n=4*) in MCF-7 cells. At the highest examined concentration of 10 mM 1-FDAM **5**, there was 68% inhibition of total [^{14}C]D-fructose transport in EMT-6, and 76% in MCF-7 cells.

Inhibition of [^{14}C]D-fructose transport by 3-FDF 12. Figure 3 illustrates that 3-FDF **12** is also able to inhibit [^{14}C]D-fructose transport in a dose dependent manner (0.1 – 10 mM) and with a IC_{50} of 1.16 ± 0.67 μM in EMT-6 and 2.37 ± 1.5 μM in MCF-7. These values are comparable to those for 1-FDAM **5** and its parent compound 2,5-AM **1**, providing preliminary indication that it may be a high affinity substrate for GLUT5.

Uptake of [^{14}C]1-FDAM ([^{14}C]-5). While it was observed that 1-FDAM **5** was able to competitively inhibit the entry of [^{14}C]D-fructose into the cell in a dose dependent manner, it was not clear whether it was being translocated across the membrane, or simply binding to the extracellular binding site of GLUT5 and thus blocking [^{14}C]D-fructose from the binding site and preventing its translocation. To ascertain the ability of 1-FDAM **5** to be transported, uptake of tracer concentrations (~0.001 mM) of newly synthesized [^{14}C]1-FDAM (0.3 μCi ml^{-1}) [^{14}C]-**5** was studied over time (Figure 4). Cells were incubated for 0, 1, 5, 10, 20, 30 and 60 minutes, and then lysed to determine how much compound was internalized in each breast cancer cell line. After 60 min it was observed that EMT-6 had approximately 1.7 times

more uptake per mg of protein than MCF-7 (0.48 normalized CPM/mg protein, *n=3* vs 0.29 normalized CPM mg^{-1} protein, *n=3*). Studies with EMT-6 cells elicited an almost linear uptake while MCF-7 on the other hand began to plateau after 30 minutes.

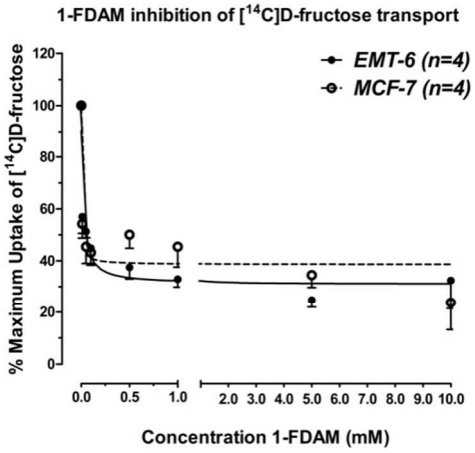


Figure 2. 1-FDAM inhibition of [^{14}C]D-fructose transport after a 60 min incubation at 37 °C with both EMT-6 and MCF-7 using increasing concentrations of 1-FDAM. Fructose transport was inhibited by increasing concentrations of 1-FDAM, and the IC_{50} obtained for EMT-6 (•) was 6.82 ± 3.0 μM and 3.96 ± 2.60 μM in MCF-7 (○). Error bars represent the SEM.

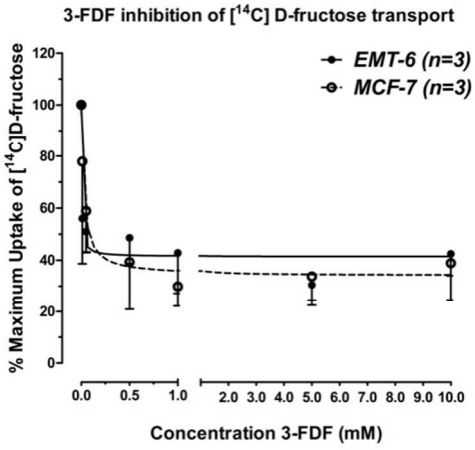


Figure 3. 3-FDF inhibition of [^{14}C]D-fructose transport after a 60 min incubation at 37 °C with both EMT-6 and MCF-7 using increasing concentrations of 3-FDF. Fructose transport was inhibited by increasing concentrations of 3-FDF, and the IC_{50} obtained for EMT-6 (•) was 1.16 ± 0.67 μM and 2.37 ± 1.5 μM in MCF-7 (○). Error bars represent the SEM.

Uptake of [^{14}C]3-FDF ([^{14}C]-12). Cell uptake experiments using 0.3 μCi ml^{-1} the [^{14}C] labelled 3-FDF ([^{14}C]-**12**) indicates that it is transported into both cell lines, and that EMT-6 has higher uptake than that of MCF-7 which is agreement with previous findings of relative uptake levels of [^{14}C] fructose in both cell lines (Figure 5). After 60 minutes EMT-6 had approximately 1.3 times greater uptake of [^{14}C]3-FDF ([^{14}C]-**12**) than that of MCF-7 (0.30 normalized CPM mg^{-1} protein, *n=4* vs. 0.23 normalized CPM mg^{1} protein, *n=4*). Both uptake curves began to display plateau behavior within 30 minutes.

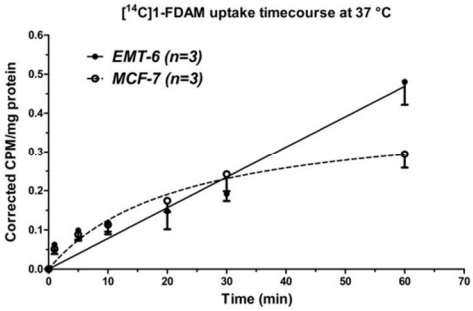


Figure 4. [^{14}C]1-FDAM 60 minute time course in both MCF-7 (○) and EMT-6 (•) at 37 °C. Uptake is observed in both cell types after a 60 min incubation. Uptakes were corrected for non-carrier mediated fluxes. Error bars represent the SEM.

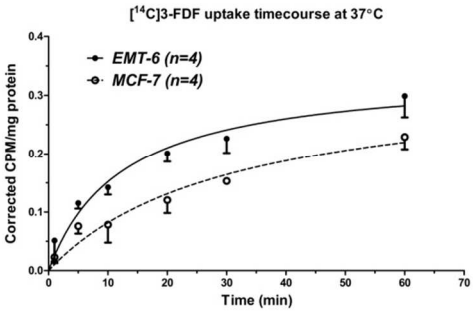
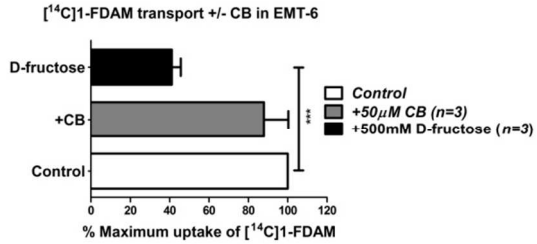


Figure 5. [^{14}C]3-FDF 60 minute time course in both MCF-7 (○) and EMT-6 (•) at 37 °C. Uptake is observed in both cell types after a 60 min incubation. Uptakes were corrected for non-carrier mediated fluxes. Error bars represent the SEM.

Inhibition of [^{14}C]1-FDAM ([^{14}C]-5) uptake by cytochalasin B and D-fructose. Cells were incubated in the presence of 0.3 μCi mL^{-1} of [^{14}C]1-FDAM [^{14}C]-**5** and a 50 μM concentration of the Class I (GLUTs1-4) GLUT inhibitor cytochalasin B (CB) for 60 minutes to measure the GLUT2 mediated [^{14}C]1-FDAM flux (Figure 6). After 60 min incubation, uptake into EMT-6 cells in the presence of CB was inhibited only 12% compared to the control (*n=3*), and uptake into MCF-7 cells was inhibited 11% (*n=3*) suggesting that GLUT5 mediates the majority of the flux (88-89%), with GLUT2 playing at most a minor role. A Student's t-test found no significant difference between the uptake under control and CB treated conditions in either cell line indicating that uptake of [^{14}C]1-FDAM [^{14}C]-**5** is primarily mediated by GLUT5.

In order to further examine the characteristics of the ability of [^{14}C]1-FDAM [^{14}C]-**5** to bind to GLUT5, transport was measured in the presence of 500 mM D-fructose to compare the relative affinity of both substrates (Figure 6). After a 60 minute incubation, the uptake of [^{14}C]1-FDAM [^{14}C]-**5** into EMT-6 was inhibited 59% (*n=3*) and into MCF-7 by 60% (*n=3*) of control values. The relative ineffectiveness of D-fructose to inhibit transport of this substrate suggests that like previously described fluorinated GLUT5 substrates; they appear to have a much higher affinity for the transporter than the natural substrate D-fructose.



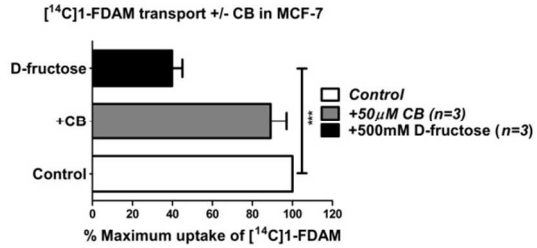
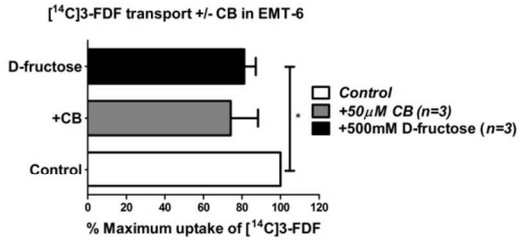


Figure 6. Effect of 50 μM cytochalasin B (CB) and 500 mM D-fructose on the uptake of [^{14}C]1-FDAM **5** into EMT-6 and MCF-7 cells. 37 °C incubations lasted 60 min and uptakes were corrected for non-mediated fluxes. Error bars represent the SEM. An unpaired t-test between was found to be extremely significant between the uptake under control and fructose treated conditions in both EMT6 and MCF-7 cell lines (n=3, unpaired t-test, p= 0.0002(***)).

Inhibition of [^{14}C]3-FDF ([^{14}C]-12) uptake by cytochalasin B and D-fructose. EMT-6 and MCF-7 cells were incubated in the presence of 0.3 μCi mL^{-1} of [^{14}C]3-FDF ([^{14}C]-**12**) and a 50 μM concentration of the Class I GLUT inhibitor cytochalasin B (CB) for 60 minutes to measure the GLUT2 mediated component of [^{14}C]3-FDF flux (Figure 6B). After 60 min incubation, uptake into EMT-6 cells in the presence of CB was inhibited 26% compared to the control (*n=3*), and 28 % in MCF-7 cells (*n=3*). A student's t-test found no significant difference between the two conditions suggesting that the majority of the flux is mediated by GLUT5 and not by any of the class I GLUTs (i.e. GLUTs 1, 2 , 3 or 4). After 60 minutes incubation, 500 mM D-fructose only inhibited uptake in both EMT-6 and MCF-7 cells by approximately 19% and 22% respectively, suggesting that 3-FDF **12** appears to have a much higher affinity for binding to GLUT5 than D-fructose, (Figure 7).

The objective of this study was to examine the ability of the fluorinated 2,5-AM derivative 1-FDAM **5** and the fructose analogue 3-FDF **12** to be transported into two models of breast cancer, and characterize and compare the profile of 1-FDAM **5** to that of 6-FDF *in vitro*. This work has identified that i) both are transported into human MCF-7 and murine EMT-6 cells, ii) both 1-FDAM **5** and 3-FDF **12**, like 2,5-AM **1**, are high affinity substrates for the facilitative hexose transporter GLUT5 or other Class II GLUTs, iii) a hexose existing predominantly in the pyranose ring structure (3FDF) is readily transported by GLUT5.

2,5-AM **1** was synthesized to provide a benchmark to 1-FDAM **5** and compare how replacement of hydroxyl by fluorine at position C-1 impacts transport and handling of 2,5-AM analogues via GLUT5. Inhibition studies show a marked decrease in the affinity of 1-FDAM **5** versus that of 2,5-AM **1**. 2,5-AM showed an ability to inhibit fructose uptake at the very low micromolar range (EMT-6: 1.06 ± 0.58 μM and MCF-7: 0.16 ± 0.09 μM) compared



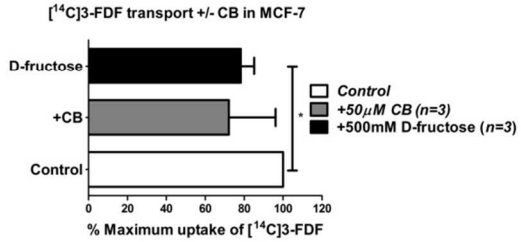


Figure 7. Effect of 50 μM cytochalasin B (CB) and 500 mM D-fructose on the uptake of [^{14}C]3-FDF **12** into EMT-6 and MCF-7 cells. 37 °C incubations lasted 60 min and uptakes were corrected for non-mediated fluxes. Error bars represent the SEM. An unpaired t-test between was found to be significant between the uptake under control and fructose treated conditions in both EMT6 and MCF-7 cell lines (n=3, unpaired t-test, p= 0.03 (*)).

to an almost one order of magnitude larger IC_{50} for 1-FDAM **5** (EMT-6: 7.11 ± 3.2 μM and MCF-7: 3.96 ± 2.6 μM).

The variability of the IC_{50} values between cell lines may result from structural differences between murine and human GLUT5 (and hence their binding to the probe molecule), as well as the possible expression of other fructose transporting GLUTs that may also show some variable affinity for the substrate. Due to the lack of specific inhibitors for the Class II and III GLUTs (GLUTs 6,8,12 & HMIT), it is not possible to fully assess their contribution to overall transport. The superior ability of 2,5-AM **1** to inhibit fructose transport compared to 1-FDAM **5** suggests that the presence of hydroxyl groups at both positions 1 and 6 increases the relative affinity for binding.

As shown in Figures 1, 2 and 3, a significant proportion of [^{14}C]D-fructose (30-40%) remained even in the presence of high concentrations of all of the probe compounds (2,5-AM **1**, 1-FDAM **5** and 3-FDF **12**) suggesting that other fructose uptake mechanisms might be present in these cells. The breast cancer cell lines EMT6 and MCF-7 have been characterized to express GLUT2 (CB sensitive) and GLUT7, 9 and 11 (CB insensitive), all of which, in addition to GLUT5, are able to transport fructose into the cells.[6,13] As illustrated in Figures 6 and 7, the cellular influx of [^{14}C]1-FDAM [^{14}C]-**5** and [^{14}C]3-FDF [^{14}C]-**12** was minimally influenced by the co-incubation with CB (specific inhibitor for Class I GLUTs), suggesting that GLUT-2 is minimally contributing to the 1-FDAM **5** and 3-FDF **12** transport. Therefore, the remaining [^{14}C]D-fructose (30-40%) could be mediated by other fructose transporters such as GLUT7, 9 and 11.

Transport studies of the [^{14}C] labelled substrates indicate that 1-FDAM **5** is taken up into murine EMT-6 cells (0.48 ± 0.06 corrected CPM mg^{-1} protein), as well as the human MCF-7 cells (0.25 ± 0.03 corrected CPM mg^{-1} protein). The higher level of uptake in EMT-6 compared to MCF-7 cells is consistent to what

has been observed in previous studies on the transport of 6-FDF and D-fructose.[11,12]

To verify the hypothesis that 1-FDAM **5** and 3-FDF **12** uptake is mediated by GLUT5, co-incubation of the Class I GLUT inhibitor cytochalasin B (CB) with [^{14}C]1-FDAM [^{14}C]-**5** and [^{14}C]3-FDF [^{14}C]-**12** indicates that the majority of cellular influx is for the most part CB insensitive, suggesting GLUT5 is the major mediator of 1-FDAM and 3-FDF's transport. This correlates with data for 6-FDF as described previously.[13] Interestingly, and in agreement with previous data looking at 6-[^{18}F]FDF transport inhibition with D-fructose,[12] inhibiting [^{14}C]1-FDAM [^{14}C]-**5** and [^{14}C]3-FDF [^{14}C]-**12** transport with high concentrations of D-fructose is ineffective compared to 1-FDAM **5** or 3-FDF **12** inhibition of [^{14}C]D-fructose transport. In fact, it was necessary to use a very high concentration (0.5 M) of D-fructose to observe significant inhibition of [^{14}C]1-FDAM [^{14}C]-**5** transport (see supporting information). This suggests that the binding affinity of both substrates for the transporter is much higher than the naturally occurring substrate.

To gain further insight into the uptake mechanism of these probes into cells, we performed the relevant uptake experiment of [^{14}C]3-FDF [^{14}C]-**12** and [^{14}C]D-fructose into *Xenopus laevis* oocytes expressing GLUT5 (after pretreatment with GLUT5 mRNA) and control oocytes (Figure 8, *see supporting information for more details*). The data clearly show significantly accelerated rate of uptake for both substrates in the eggs expressing the transporter protein (red and blue curves). These results indicate that a major portion of the uptake is GLUT5 dependent.

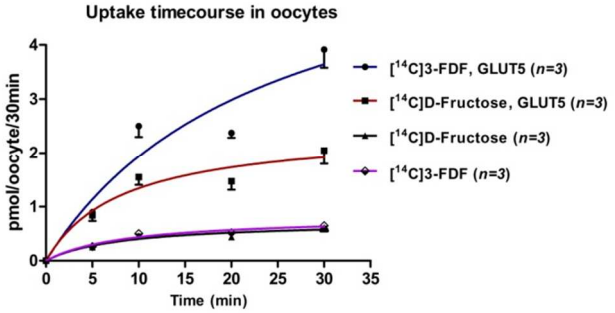


Figure 8. [^{14}C]-3FDF and [^{14}C]D-fructose 30 minute time courses in oocytes injected with GLUT5 mRNA or with water as a control experiment. Each data point represents the average of 10 oocytes. Error bars indicate SEM.

1-FDAM **5** is very effective in binding to the extracellular vestibule in the initial steps of transport, as indicated by its very low IC_{50}, but despite this favorable characteristic it is not being transported at the levels we previously observed with 6-[^{14}C]FDF.[13] These observations are in agreement with the preliminary evaluation of [^{18}F]1-FDAM [^{14}C]-**5** as a PET radiotracer for breast cancer imaging, recently reported by Sun and co-workers.[18] This result offers an important reminder that high affinity extracellular binding to the transporter is not in itself a good indicator of how rapidly a substrate will be translocated into the cell. 6-FDF on the other hand may have less affinity for the first steps of translocation, but is seems to be more amenable to transport in the latter stages during conformational change and facilitative transport via GLUT5. These *in vitro* results may indicate that 1-FDAM **5** would have somewhat lower uptake in GLUT5 expressing tumors *in vivo* compared to 6-FDF, but its uptake would not be impeded by the presence of fructose in the plasma.

Conclusion

In conclusion, 1-FDAM **5** and 3-FDF **12** are novel compounds with the potential for imaging of GLUT5 expressing breast cancer tissue using PET. GLUT5 mediated uptake with very high affinity into both human and murine tissues has been observed, suggesting their ability to be potential radiotracers for imaging of these tissues. Their kinetic characteristics suggest that low concentrations of these probes would be readily taken up into tumors. Further work needs to be done to analyze their transport characteristics in a human model of breast cancer and their metabolic fate both *in vitro* and *in vivo* to determine how effective their use with PET could be.[12]

Fructose transport via GLUT5 and its metabolism appear to be important players in breast cancer growth and proliferation.[12,13,21,22,23] The characterization of 1-FDAM **5** and 3-FDF **12** has shown that fructose based probes can be readily taken up into breast cancer cells via this and possibly other fructose transporters, and in the case of 3-FDF **12** may not require the presence of a furanose ring structure to be a suitable substrate. The effects of further structural modifications of the fructose scaffold, based upon these results, are under current study and will be reported in due course.

Experimental section

General information. Reactions were carried out in flame-dried glassware under a positive argon atmosphere unless otherwise stated. Transfer of anhydrous solvents and reagents was accomplished with oven-dried syringes or cannula. Solvents were distilled before use: methylene chloride (CH_2Cl_2) and *tert*-amyl alcohol from calcium hydride, and pyridine from KOH. Thin layer chromatography was performed on glass plates precoated with 0.25 mm Kieselgel 60 F_{254}. Flash chromatography columns were packed with 230–400 mesh silica gel. Optical rotations were measured at 22 ± 2 °C. Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded at 300MHz, 400 MHz or 500 MHz and coupling constants (*J*) are reported in hertz (Hz). Standard notation was used to describe the multiplicity of signals observed in ^{1}H NMR spectra: broad (br), multiplet (m), singlet (s), doublet (d), triplet (t), etc. Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 100 MHz or 125 MHz and are reported (ppm) relative to the centre line of the triplet from chloroform-d (77.00 ppm). Fluorine nuclear magnetic resonance spectra (^{19}F NMR) were recorded at 377 MHz and are reported (ppm) relative to trifluoroacetic acid (-76.55 ppm). Infrared (IR) spectra were measured with a Mattson Galaxy Series FT-IR 3000 spectrophotometer. Mass spectra were determined on a PerSeptive Biosystems Mariner high-resolution electrospray positive ion mode spectrometer.

Procedure for the synthesis of 1-trityl-3,4,6-tri-*O*-acetyl-2,5-anhydro-D-mannitol (2). 2,5-anhydro-D-mannitol **1** (0.57 g, 3.4 mmol) was dissolved in pyridine (6 mL) and trityl chloride (0.98 g, 3.5 mmol) was added. Subsequently, the solution was equipped with a reflux condenser and heated at 90 °C for 3 hours. The resulting yellow solution was cooled to 0 °C and acetic anhydride (5 mL) was added dropwise. The reaction was slowly warmed to room temperature overnight and was quenched by the addition of water (15 mL) and extracted with CH_2Cl_2 (3 x 20

mL). The combined organic layers were washed with 5% aqueous H_2SO_4 (20 mL) and water (20 mL) and dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo* afforded a oil residue that was purified via column chromatography (9:1 to 1:1 Hexane: EtOAc) to afford **2** as a clear colourless oil that matched previously reported characterization data [2] (0.75 g, 1.4 mmol, 40% yield): ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.48 (d, J = 7.5 Hz, 6H), 7.32 (t, J = 7.8 Hz, 6H), 7.25 (t, J = 7.3 Hz, 3H), 5.14 (t, J = 3.2 Hz, 1H), 5.14 (dd, J = 2.7, 4.3 Hz, 1H), 4.37-4.21 (m, 4H), 3.34 (dd, J = 4.4, 9.7 Hz, 1H), 3.30 (dd, J = 5.6, 9.8 Hz, 1H), 2.12 (s, 3H), 2.09 (s, 3H), 2.02 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.7, 169.9, 169.9, 143.7, 128.7, 127.8, 127.1, 86.9, 82.5, 80.9, 78.7, 78.5, 63.5, 63.4, 20.8, 20.8, 20.7.

Procedure for the synthesis of 3,4,6-tri-*O*-acetyl-2,5-anhydro-D-mannitol (3). Trifluoroacetic acid (40 mL, 5% solution in CH_2Cl_2) was added to a mixture of compound **2** (2.00 g, 3.74 mmol) and triethylsilane (0.72 mL, 4.5 mmol). The solution was stirred for 30 min and quenched with aqueous saturated $NaHCO_3$. . The resulting mixture was extracted with CH_2Cl_2 (3 x 25 mL) and the combined organic layers were dried over anhydrous $MgSO_4$. Filtration and concentration *in vacuo*, followed by flash column chromatography (9:1 to 1:1 Hexane: EtOAc) afforded alcohol **3** as a clear colourless oil that matched previously reported data[25] (0.90 g, 3.1 mmol, 84% yield): ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.25-5.18 (m, 2H), 4.25 (s, 3H), 4.10 (app q, J = 4.9 Hz, 1H), 3.85-3.76 (m, 2H), 2.28 (t, J = 6.1 Hz, 1H), 2.11 (s, 6H), 2.10 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.6, 170.3, 170.0, 83.4, 80.7, 78.4, 77.8, 63.1, 62.0, 20.8, 20.7.

Procedure for the synthesis of 3,4,6-tri-*O*-acetyl-1-deoxy-1-fluoro-2,5-anhydro-D-mannitol (4). 3,4,6-tri-*O*-acetyl-2,5-anhydro-D-mannitol **3** (0.61 g, 2.1 mmol) was dissolved in freshly distilled CH_2Cl_2 (21 mL) and cooled to -10 °C. Pyridine (0.24 mL, 2.9 mmol) and triflic anhydride (0.39 mL, 2.3 mmol) were added dropwise and the resulting solution was mixed at -10 °C. After one hour, water (10 mL) was added and the solution was extracted with CH_2Cl_2 (3 x 20 mL). The combined organic layers were washed with 10% aqueous H_2SO_4 (2 x 20 mL), dried over anhydrous $MgSO_4$, filtered and concentrated to afford the triflate intermediate as a viscous yellow oil that was used without further purification in the next reaction: $[\alpha]_D^{20}$ = +15.81 (c 0.95, $CHCl_3$); IR (film) 1746, 1415, 1373, 1227, 1147, 1049, 956 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.20 (t, J = 2.5 Hz, 1H), 5.12 (dd, J = 2.5, 4.1 Hz,1H), 4.68 (d, J = 4.2 Hz, 2H), 4.36-4.16 (m, 4H), 2.13 (s, 3H), 2.11 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.5, 170.1, 169.9, 118.4 (q, $J_{C\text{-}F}$ = 319.6 Hz), 81.6, 80.9, 78.0, 77.7, 74.2, 62.6, 20.7, 20.6, 20.6; HRMS (ESI, [M+Na$^+$]) for $C_{13}H_{17}O_{10}SF_3Na$ calcd 445.0387, found: m/z 445.0380.

Crude triflate (≤ 2.09 mmol) was dissolved in distilled *tert*-amyl alcohol (6.3 mL) and cesium fluoride (0.93 g, 6.2 mmol) was added as a single portion. The reaction was heated at 95 °C for 25 minutes and then cooled to room temperature. Water (10 mL) was added and the mixture was extracted with CH_2Cl_2 (3 x 10 mL). The combined organic layers were then washed with water (10 mL), dried over anhydrous $MgSO_4$, filtered and concentrated. Flash column chromatography (9:1 to 1:1 Hexane: EtOAc) afforded the fluorinated product **4** as a clear, colourless oil (0.49 g, 1.7 mmol, 80% yield over two steps): $[\alpha]_D^{20}$ = +18.39 (c 0.82, $CHCl_3$); IR (film) 1744, 1436, 1232, 1041 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.20 (t, J = 3.8 Hz, 1H), 5.15 (t, J = 3.4 Hz, 1H), 4.55 (dd, J = 3.8, 46.8 Hz, 2H), 4.27-4.12 (m, 4H), 2.07 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.5, 170.0, 169.9, 82.3 (d, $J_{C\text{-}F}$ = 174.8 Hz), 81.8 (d, $J_{C\text{-}F}$ = 15.3 Hz), 81.0, 77.9, 77.4 (d, $J_{C\text{-}F}$ = 6.5 Hz), 63.0, 20.7, 20.6; ^{19}F NMR (400 MHz, $CDCl_3$): δ -230.1 (ddd, J= 25.1, 46.9, 46.9 Hz); HRMS (ESI, [M+Na$^+$]) for $C_{12}H_{17}O_7FNa$ calcd 315.0851, found: m/z 315.0851.

Procedure for the synthesis of 1-deoxy-1-fluoro-2,5-anhydro-D-mannitol (5). 3,4,6-tri-*O*-acetyl-1-deoxy-1-fluoro-2,5-anhydro-D-mannitol **4** (0.49 g, 1.7 mmol) was dissolved in anhydrous MeOH (15 mL) and NaOMe in MeOH (1.5 M, 0.35 mL) was added dropwise. The solution was mixed at room temperature for 30 minutes and neutralized with the addition of Amberlite IR-120 (H^+). The resin was filtered off and the filtrate was concentrated *in vacuo* to afford **5** as a clear colourless oil that crystallized over time (0.27 g, 1.6 mmol, 95% yield): mp 84-86 °C; $[\alpha]_D^{20}$ = +39.99 (c 0.78, MeOH); IR (film) 3365, 2924, 1456, 1119, 1058 cm^{-1}; ^{1}H NMR (400 MHz, D_2O): δ 4.71-4.50 (m, 2H), 4.14-3.99 (m, 3H), 3.92 (m, 1H), 3.78 (dd, J = 2.7, 12.6 Hz, 1H), 3.69 (dd, J = 5.5, 12.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 83.5 (d, $J_{C\text{-}F}$ = 168.9 Hz), 83.3, 81.4 (d, $J_{C\text{-}F}$ = 14.6 Hz), 76.8, 76.2 (d, $J_{C\text{-}F}$ = 8.2 Hz), 61.7; ^{19}F NMR (400 MHz, $CDCl_3$): δ -229.8 (ddd, J = 24.5, 47.6, 47.6 Hz); HRMS (ESI, [M+Na$^+$]) for $C_6H_{11}O_4FNa$ calcd 189.0534, found: m/z 189.0532.

Radiochemical synthesis of [^{14}C]FDAM 5. The procedures described above were repeated using [^{14}C]-D-glucosamine as starting material. Final product **[^{14}C]-5** (SA ~ 0.3 μCi mL^{-1}) was obtained in 14% yield overall, and was found to be identical by TLC to the cold material and homogeneous.

Procedure for the synthesis of methyl 3,5-di-*O*-benzyl-2-deoxy-2-fluoro-α-D-arabinofuranoside (7). Methyl 3,5-di-*O*-benzyl-α-D-ribofuranoside [19] **6** (0.28 g, 0.81 mmol) was dissolved in CH_2Cl_2 (8 mL) at room temperature. The temperature of the reaction mixture was dropped to -10 °C. Pyridine (0.10 mL, 1.20 mmol) and trifluoromethanesulfonic anhydride (0.15 mL, 0.89 mmol) were subsequently added at low temperature *via* syringe. After 1 hour stirring at -10 °C, water was added to quench the reaction. After dilution with CH_2Cl_2, the organic and aqueous layers were separated. The organic layer was washed with 10% H_2SO_4 aq. (2 x 20 mL) and saturated aqueous $NaHCO_3$. The organic layer was then dried over anhydrous $MgSO_4$ and filtered before removing the solvent *in vacuo*. The 2-*O*-triflyl product was obtained as a yellow oil and used in the next step without further purification.

The crude material was re-dissolved in *tert*-amyl alcohol (2.5 mL).[18] Cesium fluoride (0.37 g, 2.4 mmol) was added in a single portion. The reaction mixture was then heated to 90 °C and allowed to stir overnight. After cooling to room temperature, water was added to quench the reaction. CH_2Cl_2 was added to dilute the reaction mixture. The organic layer was washed with water (3 x 10 mL), dried ($MgSO_4$) and filtered before removing the solvent *in vacuo*. The crude material was purified by column chromatography (8:2 Hexane: EtOAc) to provide pure methyl 3,5-di-*O*-benzyl-2-deoxy-2-fluoro-α-D-arabinofuranoside **7** (0.21 g, 0.62 mmol, 77% over two steps) as a pale yellow oil. $[\alpha]_D^{20}$ = +96.78 (c 0.8, $CHCl_3$); IR (film) 3031, 2918, 2865, 1454, 1365, 1103, 1056, 990 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38-7.28 (m, 10H), 5.07 (d, J = 12.0 Hz, 1H), 4.96 (dd, J = 2.0, 51.5 Hz, 1H), 4.68 (d, J = 12.0 Hz, 1H), 4.57 (ABq, $\Delta\nu_{AB}$ = 14.7 Hz, J_{AB} = 12.0 Hz, 2H), 4.55 (d, J = 12.0 Hz, 1H), 4.23 (app dt, J = 4.0, 5.5 Hz, 1H), 4.00 (br dd, J = 5.5, 24.0 Hz, 1H), 3.64 (dd, J = 3.5,

10.5 Hz, 1H), 3.60 (dd, J = 5.5, 11.0 Hz, 1H), 3.42 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 137.9, 137.2, 128.4, 128.3, 127.9 (2C), 127.7, 127.6, 106.5 (d, $J_{C\text{-}F}$ = 35.9 Hz), 99.6 (d, $J_{C\text{-}F}$ = 180.1 Hz), 82.9 (d, $J_{C\text{-}F}$ = 25.4 Hz), 81.3 (d, $J_{C\text{-}F}$ = 3.8 Hz), 73.5, 72.5, 69.3, 54.9; ^{19}F NMR (469 MHz, $CDCl_3$): δ -188.8 (ddd, J = 12.2, 24.4, 51.1 Hz); HRMS (ESI, $[M+Na]^+$) for $C_{20}H_{23}FNaO_4$ calcd 369.1473, found: m/z 369.1481.

Procedure for the synthesis of 3,5-di-*O*-benzyl-2-deoxy-2-fluoro-α/β-D-arabinose (8). Methyl 3,5-di-*O*-benzyl-2-deoxy-2-fluoro-α-D-arabinofuranoside **7** (0.50 g, 1.4 mmol) was dissolved in 80% aqueous formic acid (31 mL) at room temperature. The reaction flask was equipped with a reflux condenser and heated to 60-70 °C. After overnight stirring, the reaction was cooled to room temperature and neutralized with the addition of aqueous NaOH (2N). The reaction was then diluted with CH_2Cl_2 and the organic/aqueous layers separated. The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers washed with saturated aqueous $NaHCO_3$. The organic layer was dried ($MgSO_4$) and filtered before removing the solvent *in vacuo*. The crude material was purified by column chromatography (9:1 to 8:2 Hexane: EtOAc), providing a 2:1 mixture of α/β-anomers of 3,5-di-*O*-benzyl-2-deoxy-2-fluoro-D-arabinose **8** (0.31 g, 0.91 mmol, 65%) as a clear, colorless oil. $[\alpha]_D^{20}$ = +42.7 (c 0.96, $CHCl_3$); IR (film) 3412, 3031, 2924, 2869, 1454, 1367, 1097, 1028 cm^{-1}; HRMS (ESI, $[M+Na]^+$) for $C_{19}H_{21}FNaO_4$ calcd 355.1316, found: m/z 355.1316.

***α*-anomer**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40-7.28 (m, 10H), 5.48 (d, J = 10.5 Hz, 1H), 4.96 (dd, J = 1.0, 50.5 Hz, 1H), 4.66 (d, J = 12.0 Hz, 1H), 4.60 (d, J = 12.0 Hz, 1H), 4.56 (s, 2H), 4.47 (ddd, J = 4.5, 5.5, 5.5 Hz, 1H), 4.03 (dd, J = 4.5, 20.0 Hz, 1H), 3.61 (dd, J = 5.5, 10.5 Hz, 1H), 3.53 (dd, J = 5.5, 10.5 Hz, 1H), 3.10 (br s, 1H); Partial ^{13}C NMR (125 MHz, $CDCl_3$): δ 100.4 (d, $J_{C\text{-}F}$ = 34 Hz), 97.8 (d, $J_{C\text{-}F}$ = 182.9 Hz), 82.3 (d, $J_{C\text{-}F}$ = 25.7 Hz), 82.1 (d, $J_{C\text{-}F}$ = 1.9 Hz), 73.4, 72.4, 69.7 (d, $J_{C\text{-}F}$ = 0.75 Hz); ^{19}F NMR (376 MHz, $CDCl_3$): δ -189.7 (ddd, J = 10.3, 19.7, 50.6 Hz).

***β*-anomer**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40-7.28 (m, 10H), 5.31 (br s, 1H), 4.97 (dd, J = 4.5, 53.0 Hz, 1H), 4.66 (d, J = 12.0 Hz, 1H), 4.56 (s, 2H), 4.54 (dd, J = 12.0, 2.5 Hz, 1H), 3.32 (app td, J = 5.0, 17.5 Hz, 1H), 4.12 (app td, J = 3.5, 5.0 Hz, 1H), 3.92 (br s, 1H), 3.62-3.58 (m, 1H, obscured by α-anomer), 3.50 (dd, J = 3.5, 10.5 Hz, 1H); Partial ^{13}C NMR (125 MHz, $CDCl_3$): δ 96.0 (d, $J_{C\text{-}F}$ = 172.7 Hz), 95.3 (d, $J_{C\text{-}F}$ = 39.4 Hz), 80.3 (d, $J_{C\text{-}F}$ = 23.5 Hz), 80.2 (d, $J_{C\text{-}F}$ = 7.5 Hz), 73.7, 72.1, 69.7; ^{19}F NMR (376 MHz, $CDCl_3$): δ -202.8 (dd, J = 17.8, 52.5 Hz).

Procedure for the synthesis of 3,5-di-*O*-benzyl-2-deoxy-2-fluoro-D-arabinolactone (9). 3,5-Di-*O*-benzyl-2-deoxy-2-fluoro- α/β-D-arabinose **8** (2.40 g, 7.20 mmol) was dissolved in CH_2Cl_2 (60 mL) at room temperature. 3Å molecular sieves (1.8 g) were added, followed by *N*-methylmorpholine *N*-oxide (1.30 g, 11.0 mmol) at room temperature. The reaction mixture was stirred for 10 minutes prior to the addition of tetrapropylammonium perruthenate (0.13 g, 0.36 mmol) in a single portion. The black reaction mixture was stirred at room temperature and monitored by TLC (silica gel, 2:1 Hex : EtOAc). After overnight stirring, the crude reaction mixture was filtered through a short plug of silica gel to remove particulate material (EtOAc). After rotary evaporation, a pale yellow oil was obtained. The material was purified by column chromatography (silica gel, 2:1 Hexane: EtOAc) to afford 3,5-di-*O*-benzyl-2-deoxy-2-fluoro-D-arabinolactone **9** (2.23 g, 6.76 mmol, 94%) as a white solid: m.p. 53-56 °C; $[\alpha]_D^{20}$ = +82.83 (c 0.83, $CHCl_3$); IR (film) 3064, 3021, 2922, 2869, 1802, 1454, 1378, 1323, 1177, 1115, 1028 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40-7.26 (m, 10H), 5.31 (dd, J = 7.2, 51.6 Hz, 1H), 4.78 (d, J = 11.6 Hz, 1H), 4.60 (d, J = 11.2 Hz, 1H), 4.57 (d, J = 11.2 Hz, 1H), 4.53 (ddd, J = 7.2, 7.6, 17.6 Hz, 1H), 4.50 (d, J = 12.0 Hz, 1H), 4.37 (ddd, J = 2.4, 3.6, 8.0 Hz, 1H), 3.76 (app td, J = 2.0, 11.6 Hz, 1H), 3.61 (dd, J = 4.0, 11.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.2 (d, $J_{C\text{-}F}$ = 22.3 Hz), 137.2, 136.3, 128.4, 128.3, 128.2, 127.9, 127.7, 127.6, 91.4 (d, $J_{C\text{-}F}$ = 197.5 Hz), 78.1 (d, $J_{C\text{-}F}$ = 9.8 Hz), 77.6 (d, $J_{C\text{-}F}$ = 19.9 Hz), 73.3, 72.7, 67.0; HRMS (ESI, $[M+Na]^+$) for $C_{19}H_{19}FNaO_4$ calcd 353.1160, found: m/z 353.1160.

Procedure for the synthesis of 4,6-di-*O*-benzyl-1,2,3-trideoxy-3-fluoro-2-methylene-D-arabinofuranose (10). 3,5-Di-*O*-benzyl-2-deoxy-2-fluoro-D-arabinolactone **9** (1.20 g, 3.60 mmol) and 2-(methylsulfonyl)benzothiazole [26,27] (0.94 g, 4.4 mmol) were dissolved in THF (15 mL). The temperature of the reaction mixture was then dropped to -78 °C. LiHMDS in THF (14.7 mL, 8.70 mmol, 0.590 M) was subsequently added to the cold reaction *via* cannula over 10 minutes. The reaction was stirred for 45 minutes before the addition of acetic acid (0.65 mL). Upon warming to room temperature, the mixture was diluted with EtOAc and water. The organic and aqueous layers were separated and the aqueous layer extracted with EtOAc (3 x 20 mL). The combined organic layers were dried ($MgSO_4$) and filtered before removing the solvent *in vacuo*. The cloudy yellow oil was then re-dissolved in THF (75 mL) and DBU (1.08 mL, 7.24 mmol) was added *via* syringe. The reaction was stirred for 1 hour before removing the solvent *in vacuo* to provide a dark yellow oil. The crude material was purified by column chromatography (silica gel, 4:1 Hexane: EtOAc) to afford the product **10** (0.92 g, 2.8 mmol, 78%) as a yellow oil : IR (film) 3031, 2925, 2865, 1685, 1665, 1454, 1367, 1092, 1028 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.41-7.28 (m, 10H), 5.36 (tdd, J = 1.5, 3.9, 54.9 Hz, 1H), 4.63 (AB quartet, $\Delta\nu_{AB}$ = 30.0 Hz, J_{AB} = 12.0 Hz, 2H), 4.64-4.58 (m, 3H), 4.39 (app q, J = 5.1 Hz, 1H), 4.35 (ddd, J = 1.2, 2.4, 3.6 Hz, 1H), 4.23 (ddd, J = 3.9, 5.1, 17.1 Hz, 1H), 3.63 (dd, J = 1.0, 5.1 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 158.0 (d, $J_{C\text{-}F}$ = 18.3 Hz), 137.7, 136.9, 128.4, 128.3, 127.9, 127.7, 127.6, 127.6, 94.1 (d, $J_{C\text{-}F}$ = 185.3 Hz), 86.7 (d, $J_{C\text{-}F}$ = 6.1 Hz), 82.0 (d, $J_{C\text{-}F}$ = 5.1 Hz), 80.8 (d, $J_{C\text{-}F}$ = 22.4 Hz), 73.3, 72.0, 68.9 (d, $J_{C\text{-}F}$ = 1.9 Hz); ^{19}F NMR (376 MHz, $CDCl_3$): δ -183.2 (dd, J = 54.5, 16.5 Hz); HRMS (ESI, $[M+Na]^+$) for $C_{20}H_{21}FNaO_3$ calcd 351.1370, found: m/z 351.1373.

Procedure for the synthesis of 4,6-di-*O*-benzyl-3-deoxy-3-fluoro-D-fructose (11). 4,6-Di-*O*-benzyl-1,2,3-trideoxy-3-fluoro-D-arabino-hex-1-enofuranose **10** (0.92 g, 2.8 mmol) was dissolved in acetone (19 mL) and water (1.9 mL). *N*-Methylmorpholine *N*-oxide (0.48 g, 4.1 mmol) was added in a single portion at room temperature. Osmium tetroxide solution (0. 35 mL, 4% wt. solution) was then added *via* syringe and the reaction was allowed to stir at room temperature for 24 hours. Water (10 mL) and EtOAc (10 mL) were then added and the organic/aqueous layers separated. The aqueous layer was extracted with EtOAc (2 x 20 mL) and the combined organic layers were subsequently washed with water (2 x 20 mL). The organic layer was dried ($MgSO_4$) and filtered before removing the solvent *in vacuo* to afford a yellow oil. The crude material was purified by column chromatography (silica gel, 2:3 Hexane: EtOAc) to provide a 1:1.1 mixture of α/β-anomers of 4,6-di-*O*-benzyl-3-deoxy-3-fluoro-D-fructose **11** (0.86 g, 2.4 mmol, 85%) as a clear, colorless oil: $[\alpha]_D^{20}$ = +44.32 (c 0.67, $CHCl_3$); IR (film) 3403, 3032, 2926, 2870, 1454, 1366, 1072, 1028 cm^{-1};

HRMS (ESI, $[M+Na]^+$) for $C_{20}H_{23}FNaO_5$ calcd 385.1421, found: m/z 385.1420.

α-anomer: Partial ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38-7.27 (m, 10H), 5.09 (dd, *J* = 5.5, 53.5 Hz, 1H), 4.44 (td, *J* = 5.5, 18.0 Hz, 1H), 4.14 (ddd, *J* = 2.5, 2.5, 5.5 Hz, 1H), 3.62 (ddd, *J* = 1.5, 3.0, 10.5 Hz, 1H), 3.48 (dd, *J* = 2.5, 10.5 Hz, 1H), 2.09 (dd, *J* = 5.5, 8.5 Hz, 1H); Partial ^{13}C NMR (125 MHz, $CDCl_3$): δ 104.5 (d, J_{C-F} = 25.8 Hz), 98.3 (d, J_{C-F} = 187.1 Hz), 82.5 (d, J_{C-F} = 25.5 Hz), 80.7, 73.4, 72.4, 69.3, 63.5 (d, J_{C-F} = 6.5 Hz); ^{19}F NMR (469 MHz, $CDCl_3$): δ -195.2 (dd, *J* = 21.1, 51.6 Hz).

β-anomer: Partial ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38-7.27 (m, 10H), 4.99 (dd, *J* = 2.0, 51.5 Hz, 1H), 4.38 (app q, *J* = 5.0 Hz, 1H), 4.10 (ddd, *J* = 2.0, 5.0, 21.0 Hz, 1H), 3.58 (dd, *J* = 5.5, 10.5 Hz, 1H), 3.54 (ddd, *J* = 1.0, 5.5, 10.5 Hz, 1H), 1.91 (dd, *J* = 5.5, 8.0 Hz, 1H); Partial ^{13}C NMR (125 MHz, $CDCl_3$): δ 101.6 (d, J_{C-F} = 18.3 Hz), 95.3 (d, J_{C-F} = 195.5 Hz), 80.6 (d, J_{C-F} = 19.3 Hz), 80.0 (d, J_{C-F} = 9.3 Hz), 73.7, 72.1, 69.7, 63.8; ^{19}F NMR (469 MHz, $CDCl_3$): δ -201.3 (dd, *J* = 17.8, 53.4 Hz).

Procedure for the synthesis of 3-deoxy-3-fluoro-D-fructose (12). 4,6-Di-*O*-benzyl-3-deoxy-3-fluoro-D-fructose **11** (0.71 g, 2.0 mmol) was dissolved in MeOH (50 mL). Palladium hydroxide on carbon (0.15 g, 20% wt $Pd(OH)_2$) was added in a single portion at room temperature. The round bottom flask was then equipped with a hydrogen-filled balloon and the reaction progress monitored by TLC (9:1 EtOAc: MeOH). After 24 hours stirring at room temperature, the reaction mixture was filtered through a short Celite pad to remove particulates. The crude material was purified by column chromatography (9:5 EtOAc: MeOH) to afford 3-deoxy-3-fluoro-D-fructose **12** (0.34 g, 1.86 mmol, 93%) as a white solid. In aqueous solution 3-FDF exists as a mixture of 3 isomers in a ratio of 17:1:1. $[\alpha]_D^{20}$ = -91.88 (c 0.61, MeOH); IR (film) 3387, 2945, 1646, 1420, 1163, 1052 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) for the major isomer: δ 4.67 (dd, *J* = 10.0, 50.5 Hz, 1H), 4.15 (ddd, *J* = 3.5, 9.5, 13.0 Hz, 1H), 4.05-4.03 (m, 2H), 3.71 (d, *J* = 13.0 Hz, 1H), 3.69 (d, *J* = 12.0 Hz, 1H), 3.58 (d, *J* = 12.0 Hz, 1H); ^{13}C NMR (125 MHz, D_2O): δ 97.2 (d, J_{C-F} = 19.0 Hz), 89.3 (d, J_{C-F} = 183.1 Hz), 70.6 (d, J_{C-F} = 8.5 Hz), 68.9 (d, J_{C-F} = 17.5 Hz), 64.4, 64.3; ^{19}F NMR (469 MHz, D_2O): δ -195.1 (dd, *J* = 24.4, 51.6 Hz, ~5%), -205.9 (dd, *J* = 18.8, 53.5 Hz, ~5%), -209.1 (dd, *J* = 13.0, 50.6 Hz, ~90%); HRMS (ESI, $[M+Na]^+$) for $C_6H_{11}FNaO_5$ calcd 205.04827, found: m/z 205.04807.

Radiochemical synthesis of [^{14}C]3-FDF 12. [^{14}C]-2-(methylsulfonyl)benzothiazole was prepared by treatment of 2-mercaptobenzothiazole with [^{14}C]-methyl nosylate via a variation of the published procedures.[26,27] This methylenating reagent was then used as per the procedures above to provide **[^{14}C]-12** (SA ~ 0.3 μCi mL^{-1}) in 70% over 3 steps, found to be identical by TLC to the cold material and homogeneous.

Cell culture and transport experiments. EMT-6 and MCF-7 cells were grown in a CO_2 incubator at 37 °C in Gibco DMEM/F12 media supplemented with 15 mM HEPES, L-glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin with media renewal every 2 days. Uptake studies were performed once allowing the cells to reach confluence in 12 well plates. One hour before the initiation of an experiment, the media was removed, the plate rinsed twice with PBS and then replaced with glucose-free Krebs-Ringer solution (120 mM NaCl, 4 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM, $MgSO_4$, 25 mM $NaHCO_3$, 70 μM $CaCl_2$, pH 7.4). After the hour long incubation period, the glucose-free Krebs-Ringer solution was removed, and 300 μL of a radiotracer containing Krebs-Ringer solution was added to each well with a specific activity of 0.3 μCi mL^{-1} of ^{14}C-labeled D-fructose (Moravek Biochemicals), 3-FDF (proprietary) or 1-FDAM (proprietary). This was left to incubate within the wells for specific periods of time in a 37 °C incubator until the media was aspirated and rinsed with ice-cold Krebs-Ringer to stop further transport. 500 μL of 5 % trichloracetic acid was then added to each well lysing the cells on a rotating rocker for an hour. The cell lysate was then transferred into scintillation vials containing 4 mL of ScintiSafe™ liquid scintillation fluid for counting in a liquid scintillation counter (Beckman LS 6500 multi-purpose liquid scintillation counter). Protein quantification was performed by lysing the cells using Cellytic™ M (Sigma) and then performing a BCA protein assay (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's specifications. Inhibition studies were performed as described previously.[12,13]

Data analysis. In the inhibition experiments performed, counts per minute (CPM) were normalized to standards, background levels of substrate subtracted and then plotted against the maximum uptake of the radiolabeled tracer (i.e. [^{14}C]1-FDAM, [^{14}C]3-FDF or [^{14}C]D-fructose). For time courses, values were corrected with standards and to the protein levels present per well. IC_{50} values (concentration at which half maximum inhibition of cellular uptake of a radiotracer was observed) were determined using non-linear regression analysis in GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA), and significance was determined at $p < 0.05$ using a Student's t-test.

Author contributions

The manuscript was written with contributions from all authors. All authors have given approval to the final version of the manuscript.

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