A fructose transporter (GLUT 5) as a target for breast cancer therapy and imaging

By

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Abstract

Introduction: Positron Emission Tomography (PET) has revolutionized the diagnostic and imaging fields in cancer research. PET has opened new avenues in the pre-clinical study of radiotracers and radio-therapeutic compounds of which the full potentials are yet to be explored. To date ^{18}F-Fluoro-Deoxy-Glucose[1] (^{18}F-FDG) is the most widely used radiotracer for PET imaging. The success of ^{18}F-FDG is due to the existence of several trans-membrane proteins responsible for the facilitated transport of glucose. A related protein is a specific fructose selective trans-membrane transporter (GLUT 5) that has been observed to be over-expressed by some types of cancer cells suggesting that D-fructose is utilized by these cancer cells for energy production. Thus labeled D-fructose derivatives are potential candidates for selective PET imaging for cancer cells similar to ^{18}F-FDG in active cells.

Aim: The aim of this study was to investigate the effect of D-fructose on GLUT 5 positive and negative cell cultures and to evaluate the feasibility of GLUT 5 as a target for PET imaging of breast cancer.

Objectives: The following were investigated:

i. The extent of expression of GLUT 5 in three cancer cell lines: breast cancer cells (MCF-7), Baby Hamster Kidney cells (BHK) and cervical epithelial carcinoma cells (Hela).

ii. The colony formation potential of D-fructose enriched medium (glucose-free) and its effect on proliferation of the investigated cell lines.

iii. The effect of anti-GLUT 5 antibodies on the proliferation of breast cancer cells *in vitro*.

[1] Also referred to as 2-deoxy-2-fluoro-D-glucose

iv. The synthesis and characterization of a non-radioactive fluorinated D-fructose derivative (1-deoxy-fluoro-D-fructose)

Results: D-fructose was observed to mediate cell growth in MCF-7 cell lines but not in Hela and BHK cell lines. Glucose stimulated significantly greater cell proliferation than D-fructose for all 3 cell lines but more noticeably for the Hela ($p<0.001$) and BHK ($p=0.0110$) cell lines at all tested concentrations. Cell growth of MCF-7 cell lines where only D-fructose was present suggests a role for the highly expressed fructose specific transporter (GLUT 5) in the use of D-fructose for energy production and cell growth by these breast cancer cells.

No significant differences were observed in the ability of D-fructose enriched medium to induce 3D colony formation among the three cell lines studied ($p>0.05$) suggesting that D-fructose is not linked directly with aggressive carcinogenesis in these cell lines despite the observed evidence of D-fructose involvement in cell proliferation and energy consumption.

Anti-GLUT 5 antibodies did not show an inhibitory effect on MCF-7 cell proliferation at concentration up to 1 µg/ml (1:1000) despite these cells high expression of GLUT 5.

GLUT 5 is highly expressed by MCF-7 but not by Hela and BHK cell lines making it an important selective target for imaging of this type of breast cancer and a possible therapeutic target for antibody targeted therapy of breast cancer.

A chemical reaction sequence for the synthesis of 1-deoxy-fluoro-D-fructose (1-FDF) was carried out and an acceptable yield for an isotope labeling friendly reaction sequence was obtained and the product chemically characterized.

Conclusion: The D-fructose transporter GLUT 5 shows potential for possible application with PET imaging of breast cancer. Isotope labeled 1-FDF can be synthesized in good yield and should be the object of further studies such as development of an automated synthesis module for its radio-labeled derivative as well as pre-clinical animal and human studies.

Table of Contents

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Ad majorem dei Gloria!

Table of Figures

Table of Abbreviations

ADCC : Antibody Dependent Cellular Citotoxicity

ANOVA: Analysis of Variable

ATCC : American Type Culture Collection

BHK : Baby Hamster Kidney cells

$CaCl_{2:}$ Calcium chloride

CDC : Cellular Dependant Citotoxicity

cm^2 : Centimeter square

COOH-: Carbon terminal of a receptor molecule

ddH_2O : Double distilled dioinised water

DMEM : Dulbecco Modified Eagle Medium

3D : 3 Dimentional

ECL: Enhanced ChemiLuminescence

FCS : Fetal Calf Serum

1F-FDF: 1-Fluoro-deoxy-fructose

6-FDF : 6 Fluoro-deoxy-fructose

^{18} FDG : ^{18}Fluoro-deoxy-glucose

^{19}F- NMR: Fluorine 19 NMR

FT-IR : Fourier Transform Infrared scpectroscopy

g/L : Gram per liter

GLUT : Glucose Transporter

Hela : Cervical cancer cells (Henrietta Lacks)

HER2/neu: Human Epidermal Growth Receptoir 2

Ig G : Immunoglobulin G

MCF-7 : Breast cancer cells or Michigan Cancer Foundation-7

MDA-MB-231: Breast cancer cells

mM : Millimolar

µl : Microliter

ml : Milliliter

MTT : 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

ng/ml: Nanogram per milliliter

NH_2- : Amino terminal of a receptor molecule

PBS-T: Phosphate Buffer Saline- Tween 20

PET : Positron Emission Tomography

ppm: Part per million

TMs : Transmebranes

SDS : Sodium Dodecyl Sulphate

USA : United State of America

Chapter 1: Literature review

1. Breast cancer

A cancer cell is a cell that has lost its normal control mechanisms and thus has unregulated growth. Cancer can develop from any tissue within any organ. Cancers are classified by the type of cell that presumed to be the origin of the tumor. Cancer cells classification includes 5 major types:

I. Carcinomas are cancer cells originating from epithelial cells. In this group are included many of the most common cancers (breast, prostate, lung, pancreas and colon).

II. Sarcoma arise from connective tissue (bone, cartilage, fat, nerve)

III. Lymphoma and leukemia are derived from hematopoietic (blood-forming) cells.

IV. Germ cell tumors originate from pluripotent cells in either the testicle or the ovary.

V. Blastomas are derived from immature "precursor" cells or embryonic tissue.

Breast cancer (which is a sarcoma) is the most common cancer worldwide in women. It accounts for more than 22 % of all reported cases of cancers among females (Parkin et al. 2005). In the USA, 212,920 new cases of breast cancer was recorded in 2004 while 39,840 deaths (caused by breast cancer)were projected for 2010 (www.breastcancer.org).

There are several different types of breast cancer(www.breastcancer.org); these are named according to the tissue of origin of the cancer, and whether it has spread into the breast tissue:

• **Ductal**: cancer cells developed in the ducts, the tubes that carry milk to the nipple.

• **Lobular**: cancer cells began in the lobes or lobules, where milk is produced.

• **In situ**: cancer cells are completely contained within the ducts or lobes.

• **Invasive**: cancer has spread from the ducts or lobes into the surrounding breast tissue.

With these rules of classification, the following groups can be identified among breast cancer:

- **Ductal carcinoma in situ** (DCIS) is the earliest form of breast cancer. Cancer cells are in the ducts of the breast, but they haven't started to spread into the surrounding breast tissue.
- **Invasive breast cancer** is when cancer cells have spread outside the lining of the ducts or lobules into surrounding breast tissue. There are different types of invasive breast cancer:
    - *Invasive ductal breast cancer*: when cancer cells lining the duct have spread into surrounding breast tissue. It's the most common type of breast cancer (80%).
    - *Invasive lobular breast cancer*: Invasive lobular breast cancer develops from the cells that line the lobes of the breast. About 1 in 10 breast cancers (10%) are of this type.
- **Triple negative breast cancer** doesn't have receptors for estrogen, progesterone or HER2. This type of breast cancer is primarily treated with surgery followed by chemotherapy. This type of breast cancer occurs in up to 1 in 5 women (15–20%) with breast cancer and is more common in younger women.
- Some breast cancers have receptors for the protein human epidermal growth factor 2 (HER2). This protein can affect how some cancer cells grow. These **HER2 positive breast cancers** respond well to treatment with monoclonal antibodies such as trastuzumab. About 1 in 7 women (15%) with early breast cancer have HER2 positive cancer.
- Some breast cancers have receptors for the hormones estrogen and progesterone (estrogen-receptor positive) while this receptor is absent in others (estrogen-receptor negative). **Estrogen receptor positive breast cancers** (which accounts for 70% of breast cancer) respond well to hormonal treatments such as tamoxifen.
- There are some rare forms of cancer such as:

- **Inflammatory breast cancer**: an uncommon type of breast cancer. It happens when cancer cells grow along and block the tiny channels (lymph vessels) in the skin of the breast. The lymph vessels and the breast then become inflamed and swollen, which is how the condition gets its name.
- **Paget's disease of the breast** shows up as a red, scaly rash (like eczema) on the skin of the nipple. Women who have Paget's disease may have underlying DCIS or invasive breast cancer.

Breast cancer is the second leading cause of death in the USA alone with a lifetime risk (birth to death) of 1 in 8 (Jemal et al. 2005). In South Africa, breast cancer was the most common cancer reported among females (19.4%) between 1998 and 1999 with a life time risk of 1 in 26 causing up to 3000 death per year (Buccimazza, 2008; Mqoqi et al. 2004).

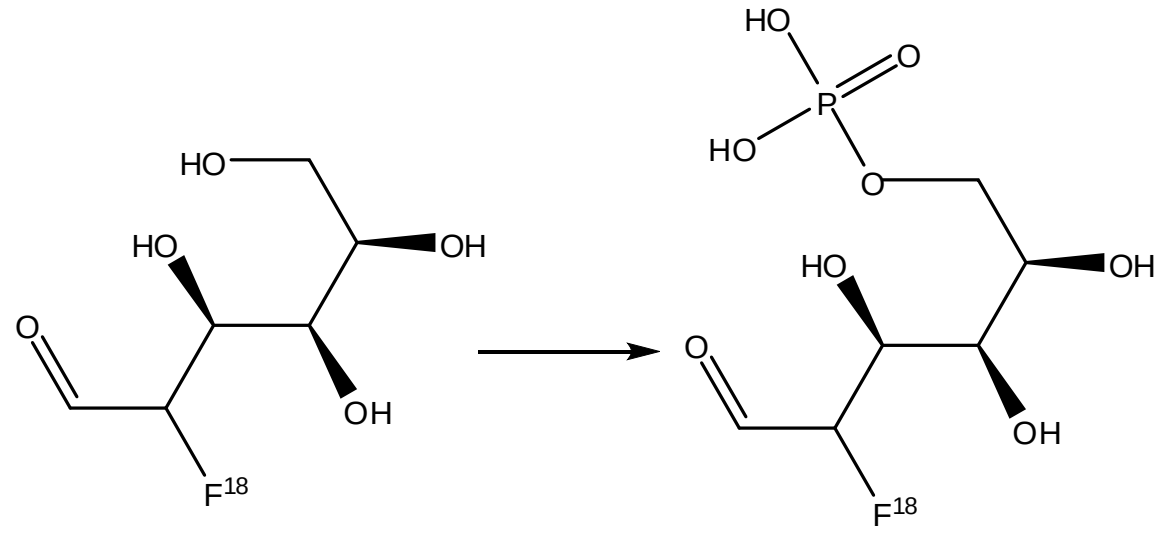
Fortunately 80% of the diagnosed cases of breast cancer can be successfully treated if the detection is in the early stages of the malignancy (Levi et al. 2007). Accurate staging through determining the extent of the cancer is an essential prerequisite of optimal management of the disease. Early detection followed by appropriate treatment is currently the most effective strategy to reduce breast cancer mortality. Among the methods currently used to detect cancer, functional imaging has proven to be a better informant of intracellular changes than modalities that rely on anatomical differences such as screening mammography. Functional imaging techniques such as Position Emission Tomography (PET) provide better indications on the stages of the progression of the malignancy. PET techniques allow better assessment of the disease as well as permit a clearer choice of the most effective treatment approach (Levi et al. 2007).

*2. Positron Emission Tomography (**PET**)*

PET has opened a new field in nuclear medicine. It is used as a valuable tool for diagnosis, disease extent and prognosis assessment, planning and monitoring treatment and detecting

recurrent cancers (Plathow and Wolfgang, 2008; Pauwels et al. 2000). ^{18}F-Fluoro-Deoxy-Glucose[2] (^{18}F-FDG), a fluorinated glucose analogue, is the most commonly used radiotracer in PET for the evaluation of patients with breast cancer (Rohren et al. 2004). ^{18}F-FDG mimics glucose and is transported into cells by the glucose facilitated transport system (GLUT 1). Once in the cell, ^{18}F-FDG is phosphorylated by hexokinase to ^{18}F-FDG-6-phosphate (Fig. 1). This phosphorylated derivative remains trapped inside the cell due to the charged phosphate group. Since ^{18}F-FDG-6-phosphate is not a substrate for aldolase (the enzyme involved in the second step of the metabolism of glucose), the glycolysis process for this molecule is blocked at this stage. All metabolically active cells are detectable by PET imaging due to the universal use of glucose as a substrate for glycolysis (Fig. 1) (Pauwels et al. 2000) making ^{18}F-FDG a non-specific marker for glucose metabolism. Unfortunately, due to the wide expression of the GLUT 1 transporter, the specificity and sensitivity of ^{18}F-FDG, particularly in the case of breast cancers, are less than desired. Little distinction can be made between well differentiated and poorly differentiated tumours leading to increased levels of false-positive diagnosis (Pauwels et al. 2000).

[2] Also referred to as 2-deoxy-2-fluoro-D-glucose



2-Deoxy-^{18} F-fluoro-D-glucose

2-Deoxy-^{18}F-fluoro-D-glucose-6-phosphate

Figure 1: Fischer Diagrams of ^{18}F-FDG metabolism; ^{18}F-FDG is metabolized by hexokinase II to ^{18}F-FDG-6-phosphate and this derivative is trapped inside the cell unable to proceed further down the glycolytic pathway.

3. Glucose Transporters

There are three known mechanisms of uptake of glucose in humans: passive diffusion, Na^+ dependent glucose transport and specific facilitated transport. Among these mechanisms the facilitated transport is the dominant pathway (Pauwels et al. 2000).

A family of transporter proteins (GLUT) mediates this facilitated transport of hexoses into cells. To date over 12 members of this family of transporters (GLUT 1-13) have been identified (Macheda et al. 2005; Shurmann, 2008; Wood and Trayhurn , 2003). It has been observed that the majority of cancers over-express one or more of these GLUT transporters, which are present at low levels in the respective tissue of origin under non-cancerous conditions. These observations make these transporters key targets for the study, diagnosis and therapy of cancer (Hanahan and Weinberg, 2011;Godoy et al. 2006; Macheda et al. 2005; Medina and Owen, 2002).

^{18}F-FDG is mainly transported into cells by GLUT 1 and GLUT 3, which are associated with normal glucose transport (Buch et al. 1998). GLUT 1 is reported to be over-expressed in most malignant tissues (Nelson et al. 1996;Younes et al. 1996). In tumor samples, GLUT 1, 2 and 5 are the three most commonly observed glucose transporters. GLUT 2 and GLUT 5 are associated with fructose transport (Godoy et al. 2006). Another fructose transporter, GLUT 7, is less commonly expressed but is reported to also transport fructose into various cancerous and normal cells (Bantle et al. 2000; Cheesman, 2008; Shurmann, 2008).

The fructose transporters are reported to be located either in the cell membrane or in the cytosol of cells (Trayner et al. 2009). GLUT 2 is thought to play a minor role in total fructose flux across the cell membrane (Trayner et al. 2009) while GLUT 5 is a high affinity fructose transporter. GLUT 5 is responsible for most of the fructose flux across the cell membrane. GLUT 5 is expressed at low levels in many tissues such as the intestine, testis, kidney, erythrocytes and lung (Douard and Ferraris, 2008; Gould and Holman, 1993; Medina and Owen, 2002). GLUT 5 is not expressed under normal conditions in breast tissue. GLUT 5 has been shown to be highly expressed *in vitro* and *in vivo* in breast cancer cells (Godoy et al, 2006; Medina and Owen, 2002; Zamora-Leon et al. 1996). This observation led to the suggestion that cancer cells may utilize fructose as an energy source during uncontrolled proliferation (Godoy et al. 2006; Medina and Owen, 2002). While there is abundant data on GLUT 5 expression in kidney cancer, breast cancer and intestinal cancer cells (Brot. 1996; Godoy et al. 2006; Zamora-Leon et al. 1996), no documented data has been reported on expression of GLUT 5 in Baby Hamster Kidney cells (BHK) while cervical epithelial carcinoma cells (Hela) are reported not to express this transporter (Godoy et al. 2006; Medina and Owen, 2002). If GLUT 5 is expressed by a limited number of healthy tissues in contrast to its common over-expression in diseased tissue, an increased detection potential is to be expected if this transporter was to be used as a target for diagnostic PET scanning. GLUT 5 therefore appears to be a potential target for improved imaging or treatment of breast cancers.

4. Antibody therapy, GLUT 5 and breast cancer

Since the development of hybridoma technology by Kohler and Milstein in 1975 (Kohler et al. 1975), it has been possible to provide monoclonal antibodies that possess high specificity and affinity for a particular target antigen. This technology has allowed the study of many antibodies targeted against specific disease markers and has proved to be a useful therapeutic regimen. Trastuzumab (Herceptin) an unconjugated anti-HER2/neu antibody is an example of the successful use of an antibody as therapeutic agent against a particular cancer known to over-express a specific surface protein (HER2/neu). Trastuzumab is widely used in breast cancer with a demonstrated reduction in relapses and prolonged disease-free and overall survival in high risk patients (Vogel et al. 2002).

The use of Anti-GLUT 1 antibodies in targeted therapy against breast cancer is under investigation. It has been observed that Anti-GLUT 1 antibodies suppress cell proliferation *in vitro* (Simon and Banerjee, 2008). No such studies have been conducted with antibodies against the fructose specific transporter GLUT 5. GLUT 5 is thought to be a better target for breast cancer therapy due to the limited expression of GLUT 5 in non-cancerous tissues in humans. Antisense oligonucleotides against GLUT 5 have been shown to have anti-proliferative effect on two breast cancer cell lines (MCF-7 and MDA-MB-231) through suppression of the GLUT 5 protein expression in these cells, which resulted from decreased fructose uptake (Chan et al. 2004).

5. D-Fructose and fluorinated fructose derivatives

Zamora-Leon and coworkers (Zamora-Leon et al. 1996) reported the over-expression of GLUT 5 in breast cancer and stated that it might have clinical implications. Heaney and Hui observed that D-fructose stimulates the growth of breast cancer cells *in vitro* (Heaney and Hui, 2007). From these findings it was inferred that cancer cells take up large amounts of available D-fructose during their metabolism to meet their increased energy need (Heaney and Hui, 2007). These observations have directed the research in this field towards the

investigation of a radiolabelled fructose derivative as imaging agent for breast cancer. A D-fructose radiolabelled imaging agent is expected to be able to detect small tumors and other more differentiated cancer subtypes much better than the commonly used radiotracer, ^{18}F-FDG. Clear and accurate images would result from an improved resolution and lower background with clear distinction from inflammation (false-positive) (Trayner et al. 2009). Various studies have been conducted on D-fructose [$^{18}F^-$] radiolabelled derivatives as potential radiotracers (Haradahira et al. 1995; Trayner, 2009). Haradahira and coworkers (Haradahira et al. 1. 1995) synthesized and tested the distribution and metabolism of 1-deoxy-1-[^{18}F] fluoro-D-fructose in rats with fibrosarcoma. The synthesis method used in Haradahira and coworkers study was based on the method described by Card and coworkers (Card and Hitz, 1984) who fluorinated a fructose molecule at carbon position 1 (C-1) (Fig. 2). This product, however, was not metabolized nor was it trapped in any of the organs analyzed possibly due to the type of cancer used (the levels of GLUT 5 expression in fibrosarcomas are yet to be determined). It was observed later by Zamora and co-workers (Zamora-Leon et al. 1. 1996) that MCF-7 cells would have been a suitable candidate because they over-express GLUT 5. Tatibouet and coworkers while studying the steric constraint around the GLUT 5 receptor with bulky fructose derivatives observed that the GLUT 5 receptor has high affinity for both the pyranose and furanose ring isoforms of D-fructose (Tatibouet et al. 2000).

Levi and coworkers (Levi et al. 2007) developed and evaluated the possible applications of two fluorescence fructose derivatives. They labeled fructose with two different fluorophores at the C-1 position, one of which accumulated specifically *in vitro* in cells expressing the GLUT 5 transporter protein.

Recently Trainer and coworkers (Trayner et al. 2009) successfully synthesized 6-deoxy-6-fluoro-D-fructose (6-FDF) which showed a dose dependent inhibition of fructose transport. A follow up study by the same group resulted in the radiopharmacological evaluation of that same molecule (Wuest et al. 2011). The radiolabelled derivative 6[^{18}F]-FDF was observed to be a substrate for human ketohexokinase and was rapidly metabolized in mice. However after

2 hours, it showed no advantage over ^{18}F-FDG. The assumption made by Trayner and coworkers (Trayner et al. 2009) of a high expression level of fructokinase in breast cancer cells compared to hexokinase levels does not correlate with literature and could explain the observed results (Levi et al. 2007).

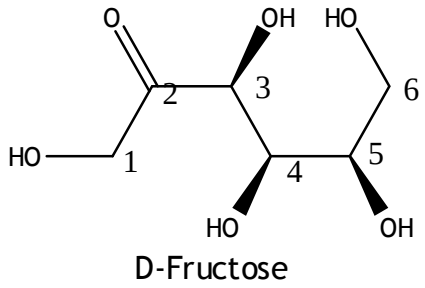
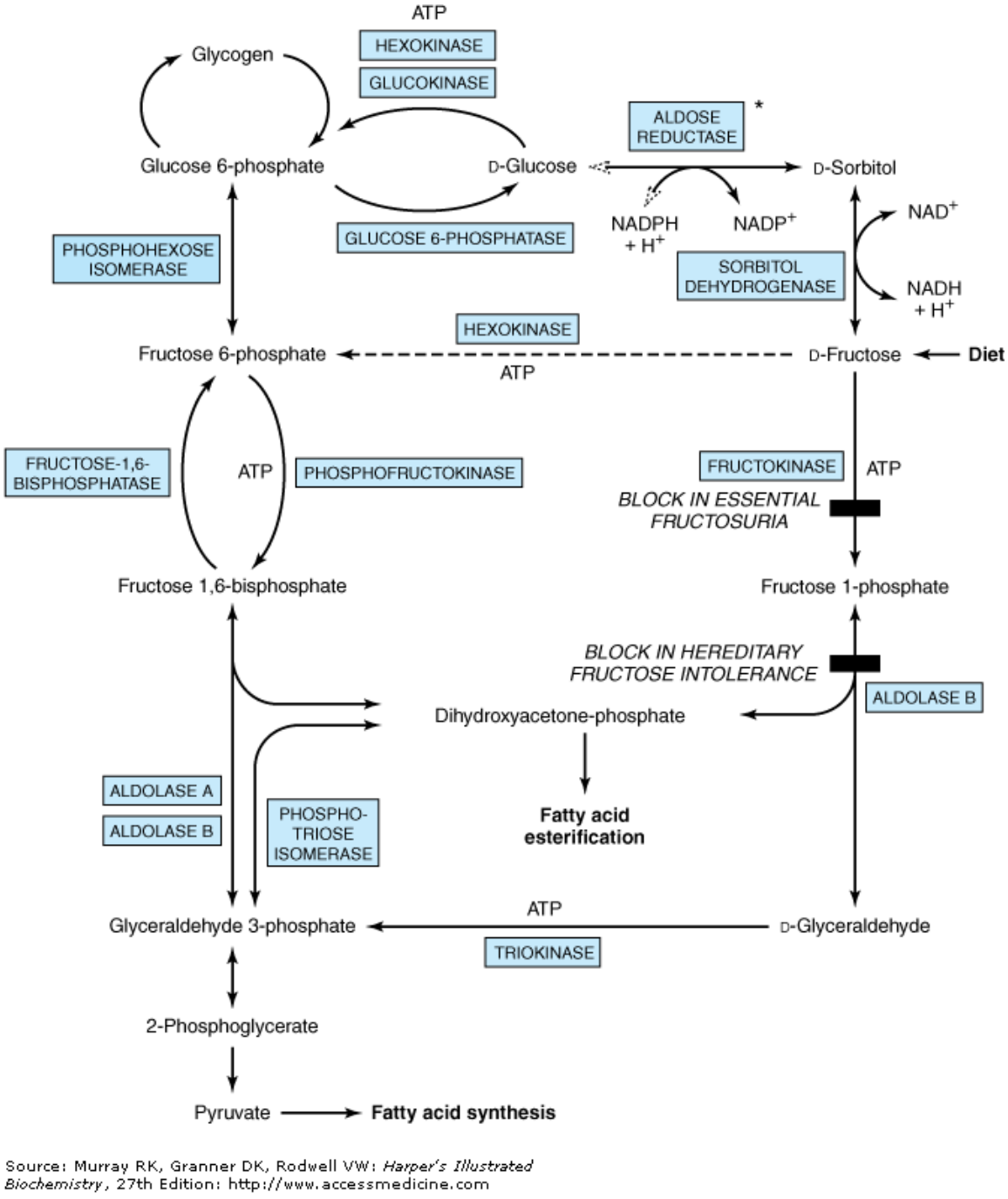


Figure 2: **General atom numbering of D-fructose;** the Arabic numbers represent carbon atoms. Note the ketone functionality at carbon 2.

It is well accepted that in some instances D-fructose can behave as a substrate for energy production by humans (Card and Hitz, 1984).

The metabolic pathway of fructose in the glycolytic phase involves two phosphorylation steps (see Fig. 3): 1) at carbon position 1 by fructokinase to yield fructose-1-phosphate or 2) at carbon position 6 by hexokinase to give fructose-6-phosphate. These phosphorylation reactions are tissue-specific as different tissues express different isoforms of the enzymes (Brown et al. 2002). Four hexokinase isoforms are known to be expressed in mammalian cells (Brown et al. 2002). Breast cells and breast cancer cells have a high level of expression of hexokinase I and II while the levels of fructokinase expression are low (Brown et al. 2002; Levi et al. 2007)

In the liver, gut and kidneys, D-fructose is primarily converted to fructose-1-phosphate by fructokinase and cleaved by aldose B to glyceraldehyde 3 phosphate and dihydroxyacetone phosphate (Haradahira et al. 1995; Heaney and Hui, 2007). In breast cancer, fructose-6-phosphate is the first derivative of enzymatic activity due to the presence of high levels of hexokinases (Brown et al. 2002; Levi et al. 2007).



Source: Murray RK, Granner DK, Rodwell VW: *Harper's Illustrated Biochemistry*, 27th Edition: http://www.accessmedicine.com

Figure 3: Overview of fructose metabolism in human; this forms part of glycolysis. Glycolysis is the major source of energy in cells that do not possess mitochondria (e.g., erythrocytes) and for cells under anaerobic conditions (e.g., contracting mycocytes).

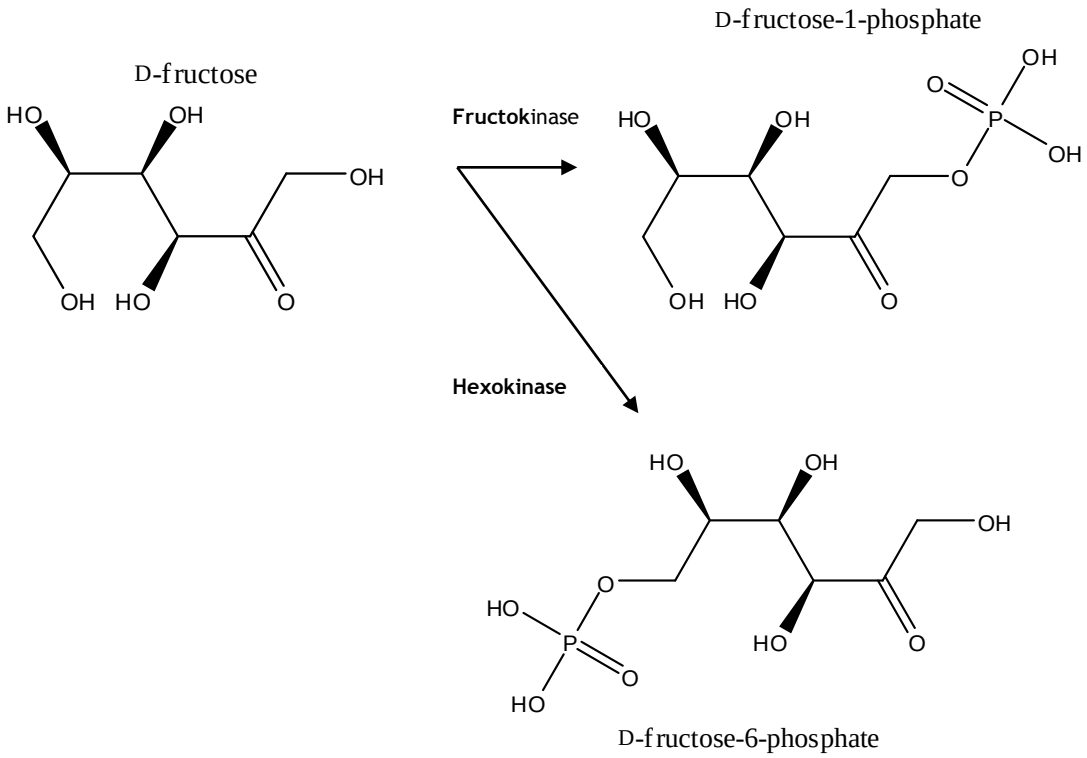


Figure 4: Scheme of D-Fructose metabolism: Upon entry into a cell D-fructose is either metabolized by fructokinase to fructose-1-phosphate or by hexokinase to fructose-6-phosphate.

It has also been demonstrated that fluoro derivatives of deoxy sugars successfully mimic a substrate with a hydroxyl group in enzyme-analogue interactions (Einsenthal, 1972). Therefore selectively labeled fluorinated fructose derivatives are predicted to interact successfully with the GLUT 5 transporters and the intracellular phosphorylating enzymes (hexokinase). ^{18}F-FDG's lack of selectivity in the diagnosis of breast cancer (Pauwels et al. 2000; Trayner et al. 2009) and the increasing evidence of a possible active role of fructose in breast cancer metabolism due to the over expression of GLUT 5 (Godoy et al. 2006; Heaney et al. 2007), are strong motivations for the development of a more specific radiotracer.

During this study, the expression of the main fructose transporter (GLUT 5) in breast cancer cells (MCF-7), Baby Hamster Kidney cells (BHK) and cervical epithelial carcinoma cells (Hela) was investigated. The three dimensional colony formation potential of D-fructose enriched growth medium (glucose-free DMEM) and its effect on cell proliferation of these cancer cell

lines were studied to assess *in vitro* cellular transformation of these cells. These results are indicative of the aggressiveness of these cancer cells. The effect of the use of anti-GLUT 5 antibodies on the proliferation of breast cancer cells *in vitro* was investigated. This work is thought to have paved the way for further investigation of the use of a radiolabelled fructose derivative as a potential agent for imaging of breast cancer.

6. Study Hypothesis

The fructose specific transporter, GLUT 5, is a potential target for imaging breast cancer cells.

7. Study aim

The aim of this study was to investigate the effect of D-fructose on GLUT 5 positive and negative cell cultures and to evaluate the feasibility of GLUT 5 as a target for PET imaging of breast cancer.

8. Study Objectives

1. To study the effect of D-fructose on the proliferation of different cancer lines *in vitro* when grown in glucose depleted medium.
2. To study the effect of anti-GLUT 5 antibodies on the proliferation of a breast cancer cell line (MCF-7) *in vitro.*
3. To study the 3-D colony formation potential of cancer cell lines cultured in a D-fructose enriched culture medium.
4. To study the expression of the specific fructose transporter (GLUT 5) in MCF7, Hela and BHK cell lines.
5. To attempt the synthesis and characterization of a non-radioactive fluorinated D-fructose derivative (1-fluoro deoxy-D-fructose or 1FDF).

Chapter 2: Cell proliferation in D-fructose enriched medium

1. Introduction

Scientists have toiled for many years to try and understand the processes behind the development of malignancies as a way to finding new avenues to prevent, or treat cancer. With this aim in mind, Otto Warburg observed that cancer cells possessed the ability to sustain high rates of anaerobic glycolysis even in the presence of oxygen (Warburg , 1956). It has since been confirmed that many tumors have a high rate of glycolysis independently of their supply of oxygen (this phenomenon is known as the "Warburg's effect" or "aerobic glycolysis"). This constitutes the basis of the use of ^{18}F-Fluoro-Deoxy-Glucose (^{18}F-FDG) for Positron Emission Tomography (PET) scans in clinical oncology.

^{18}F-FDG (a glucose analogue) is the main radiotracer used in PET for the evaluation of patients with breast cancer (Rohren et al. 2004). ^{18}F-FDG is taken into cells through a glucose facilitated-transport system (GLUT1) where it is phosphorylated by hexokinase to ^{18}F-FDG-6-phosphate (Fig. 1). This phosphorylated derivative remains trapped inside the cell due to the charged phosphate group. FDG cannot be phosphorylated at the 1 position therefore the glycolysis process for this molecule stops at this stage (Rohren et al. 2004). Theoretically all metabolically active cells will be detected by PET imaging when using FDG due to the universal use of glucose as a substrate for the energy producing glycolysis pathway (Pauwels et al. 2000).

The underlying biochemical and molecular mechanisms of the Warburg's effect are multiple. They include, among other mitochondrial malfunction (Wallace, 2005), oncogenic alterations (Dang and Semenza , 1998), as well as adaptive response to the tumor microenvironment (Gatenby and Gillies, 2004).

Glucose, the most used carbohydrate in humans, is a key molecule in energy production in humans and an essential element in glycolysis. Glucose absorption into cells is mediated by

specific glucose transporters. The majority of cancers over-express one or more of the GLUT transporters, which are not normally expressed at high levels in the respective tissue of origin under non-cancerous conditions, thus making these transporters a possible target in the study, diagnosis and therapy of cancer (Medina and Owen, 2002).

Since GLUT 1 is not always over expressed in malignant tissues (Nelson et al. 1996;Younes et al. 1996), how could the increase glycolysis observed by Otto Warburg that is characteristic of most malignancy, be explained?

D-fructose has been proposed as a possible source of the energy needed by cancer cells in the Warburg effect (Heaney et Hui, 2007). GLUT 2 and GLUT 5, two isoforms in the GLUT transporters, are associated with fructose transport (Trayner et al. 2009; Zamora-Leon et al. 1996). It is well recognized that GLUT 5 transporters have a high affinity for D-fructose in both its pyranose and furanose ring forms (Tatibouet et al. 2000). Fructose could be a major substrate for energy production by selected cells in humans. It is estimated that the baseline D-fructose concentration in human plasma is around 2-5 mM (Heaney et Hui, 2007; Macdonald et al. 1978). D-fructose is primarily converted to D-fructose-1-phosphate by fructokinase and cleaved by aldose B to glyceraldehyde phosphate and dihydroxyacetone phosphate in organs such as liver, gut and kidneys (Fig 3.) (Haradahira et al. 1995; Heaney and Hui, 2007).

2. Aim

To assess cell proliferation in glucose free media (DMEM) enriched with different concentrations of D-fructose or glucose 5mM (0.930g/L), 10 mM (1.86g/L), 15 mM (2.79g/L) and 25 mM (4.650g/L) and incubated over a period of time (24 -168 hours) on two cancer cell lines (Hela and MCF-7) and one non-cancerous cell line: Baby Hamster Kidney (BHK) .

3. Material and methods

3.1. Cells

- MCF-7 (ATCC catalogue number: HTB-22)
- Hela (ATCC catalogue number: 229CL-2. 1)
- BHK (ATCC catalogue number: 238-1 CCL10C-13)

3.2. Media

Glucose free DMEM (PAA Laboratories, Austria) supplemented with 10% FCS was used for culturing all four cell lines. Glucose of analytical grade was used. D-fructose and glucose at different concentrations (5, 10, 15 and 25 mM) were added to aliquots of the medium. DMEM (Glucose= 5.56 mM or 1 g/L) supplemented with 10% FCS and used as positive control.

3.3. Cells harvesting

Growth medium was poured off from the 75 cm^2 culture flask containing the selected cancer cells (80% confluence). Trypsin/EDTA (5 ml) was added to the flask and incubated at 37°C in a CO_2 incubator for 20 minutes. After the cells had detached, the contents of the culture flask was transferred into a 15 ml centrifuge tube and the tube was filled with fresh medium (Glucose free DMEM). This tube was centrifuged at 200 g for 10 minutes. The supernatant was removed and cells re-suspended in 1 ml DMEM (Glucose free). The cell concentration in the suspension was determined using a hemocytometer and adjusted to the desired cell concentration.

3.4. Proliferation assay

Each of the 3 selected cell lines was incubated in media with different concentrations of D-fructose (5 mM, 10 mM, 15 mM and 25 mM) or different glucose concentrations (5 mM, 10 mM, 15 mM and 25 mM) for 24, 48, 72 and 168 hours at 37°C in a CO_2 incubator. Aliquots of 100 µl

of a cell suspension containing 5 x 10^4 cells per ml were added into each well of a 96 well plate (in the case of 168 hours incubation 1.25 x 10^4 cells were used) followed by 100 µl of the specific medium.

Triplicate wells were used for each treatment. All experiments were repeated at least 3 times.

3.5. Viability assay

After the respective incubation time, 20 µl of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma-Aldrich, Steinheim, Germany) (5 mg/ml in PBS) were added to each well of the 96 wells plate. The plate was incubated for 4 hours at 37ºC in a CO_2 incubator. The plate was then centrifuged at 800 g for 10 minutes. The supernatant was removed and 150 µl of PBS was added to each well to wash off any excess reagent and media. The plate was centrifuged again at 800 g for 10 minutes and the PBS was removed. After a brief drying period, 100 µl of dimethyl sulfoxide (DMSO) was added to each well on the plate and the plate was shaken gently for 60 minutes. The plate was analyzed spectrophotometrically at 570 nm with the reference wavelength at 630 nm on ELX 800 universal microplate reader (Biotek Instruments.inc).

3.6. Statistical analysis

GraphPad version 5 was used for statistical analysis. The statistical tools were descriptive and analytical using t-tests or analysis of variance (ANOVA). Testing was conducted at the $p \leq 0.05$ level of significance.

4. Results and discussion

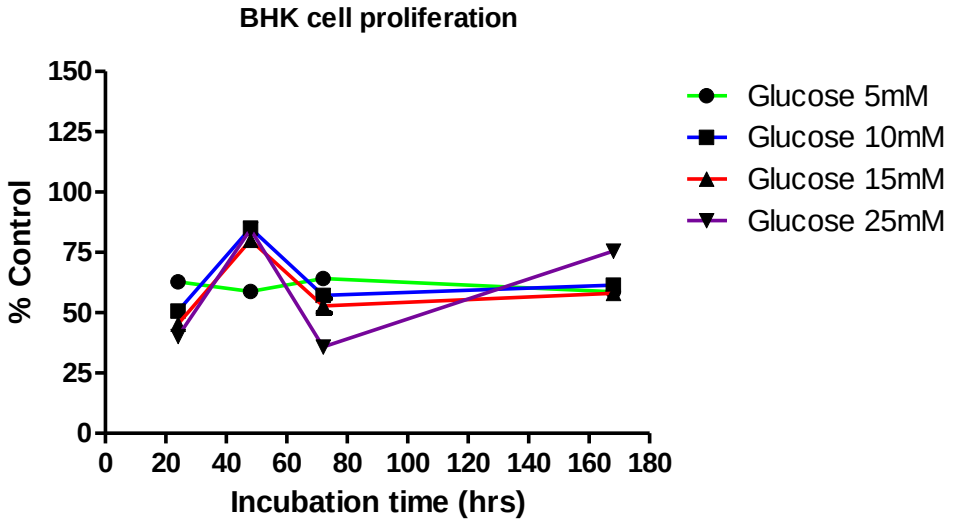
Substituting the glucose with D-fructose in the cell culture medium did not enable normal cell proliferation for Hela and BHK cells however a significant but suppressed cell proliferation was observed in MCF-7 cells after the carbohydrate substitution.

Glucose allowed significantly higher proliferation than D-fructose for all tested cell lines, at all incubation periods and all carbohydrate concentrations (Fig.5, 6 and 7).

D-fructose substitution allowed cell proliferation in MCF-7 breast cancer cells but glucose showed a significantly higher proliferation than fructose at all incubation periods and carbohydrate concentrations (Fig. 7).

It is thought that D-fructose might be taken up by both BHK and Hela cells leading to minimal cell proliferation (Fig.5 and 6). No significant difference was observed between 24, 48, 72 and 168 hrs in D-fructose cell proliferation rates among these two cell lines while glucose was observed to have greater proliferation rates at all tested glucose concentrations(Fig.5 and 6).

The MCF-7 cell proliferation in the presence of D-fructose as the only carbohydrate source as observed in the present study suggests the potential use of fructose in these cells for proliferation. This is supported in the literature by the widely observed presence of the fructose specific receptor GLUT 5 in breast cancer tissues (Godoy et al. 2006; Medina and Owen, 2002; Zamora-Leon et al. 1996). Chan and co-workers who observed that cell proliferation in human breast cancer cells (MCF-7 and MDA-MB-231) was inhibited by antisense oligonucleotides against GLUT 5 (Chan et al. 2004).



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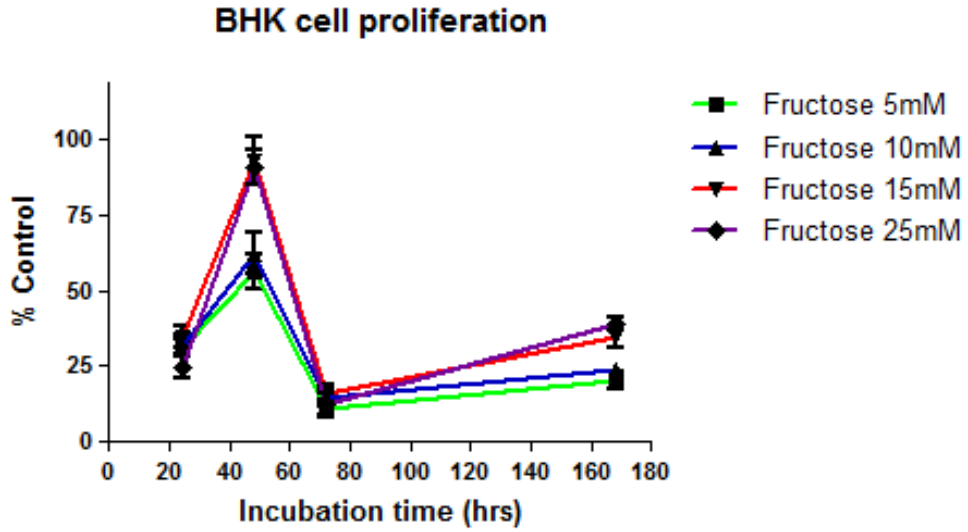
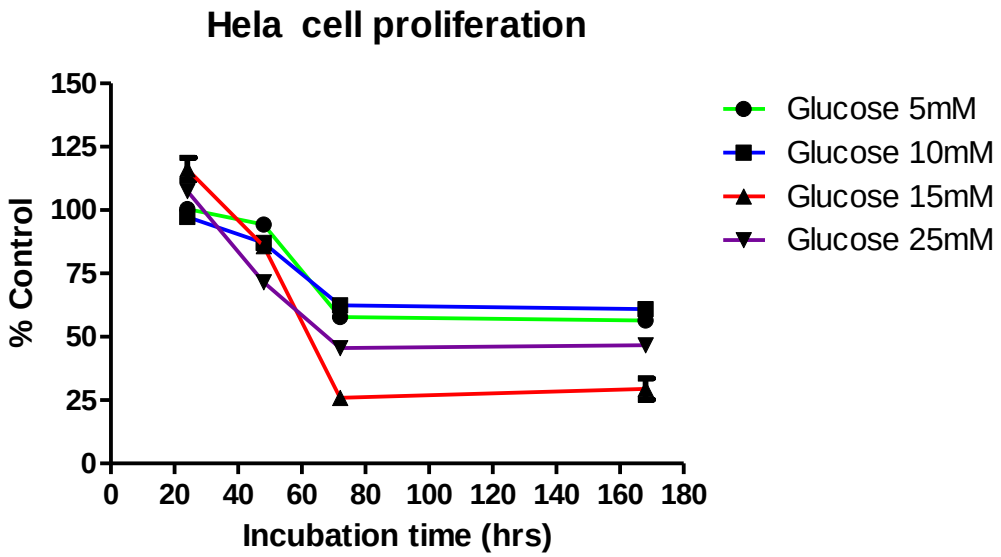


B.

Figure 5: Effect of fructose as source of carbohydrate on BHK cells proliferation. (Glucose free DMEM supplemented with 10% FCS and a hexose as indicated in the legend). ANOVA shows significant difference between the glucose (A) and fructose (B) enriched DMEM ($p<0.001$). No significant difference could be observed between the different fructose concentrations.



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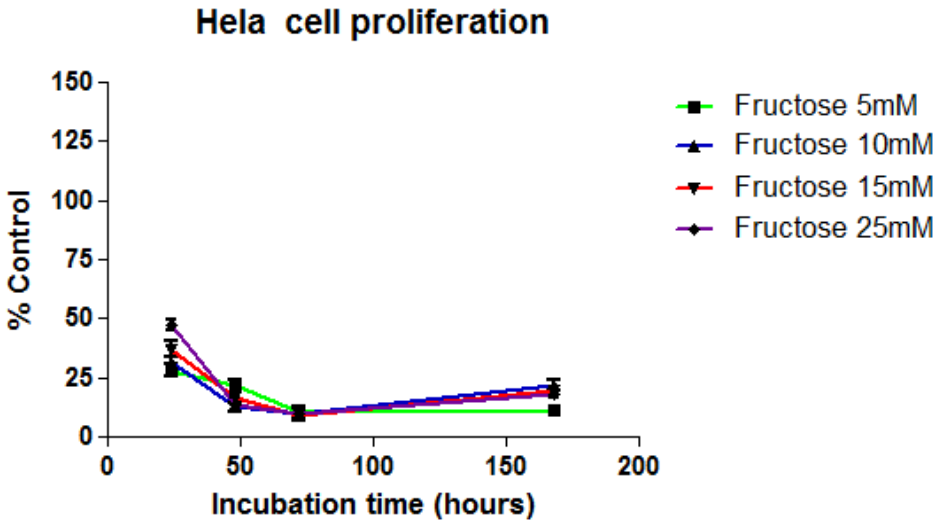
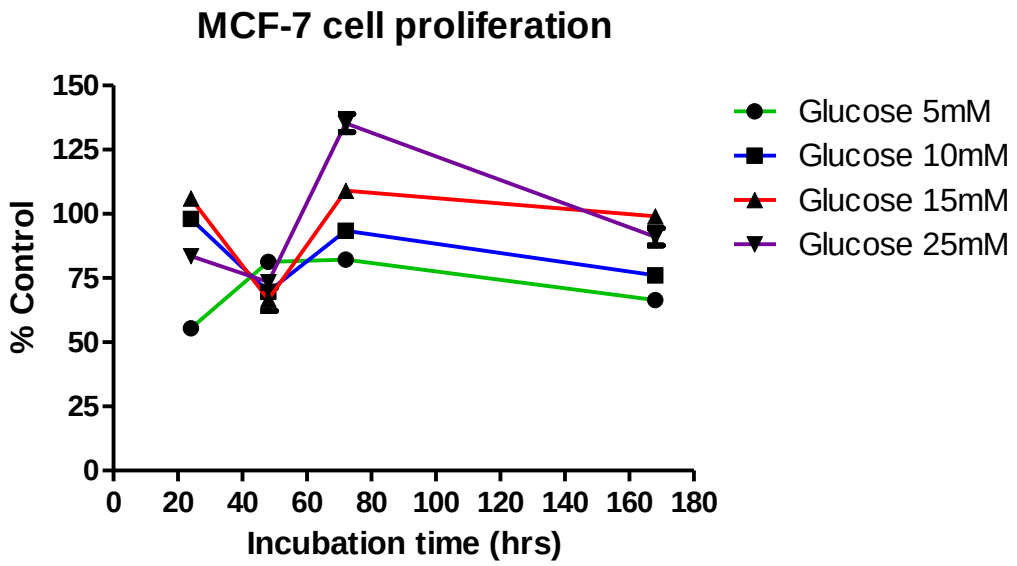


B.

Figure 6: Effect of fructose as source of carbohydrate on Hela cells proliferation. (Glucose free DMEM supplemented with 10% FCS and a hexose as indicated in the legend). ANOVA shows significant difference between the glucose (A) and fructose (B) enriched DMEM ($p<0.001$). No significant difference could be observed between the different fructose concentrations.



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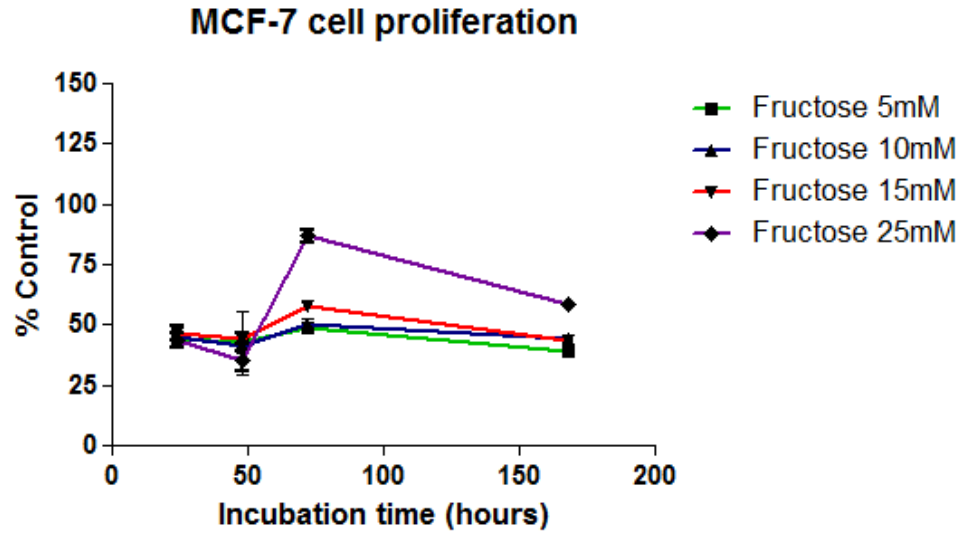


B.

Figure 7: Effect of different concentrations of D-fructose on MCF-7 cells proliferation in Glucose free DMEM supplemented with 10% FCS and either glucose or fructose as the source of carbohydrate. ANOVA- shows significant difference between the glucose (A) and fructose (B) based media ($p<0.05$). The medium with high fructose levels (25mM) is comparable to the low glucose level (5mM) media in its ability to allow cell proliferation. There is significant difference ($p=0.0002$) in the ability of D-fructose to allow cell proliferation among the 4 fructose concentrations in MCF-7 cell lines (ANOVA).

The results observed in this study align with the hypothesis that D-fructose is associated with glycolysis in breast cancer cells and therefore contributes to growth and cell proliferation.

It has been hypothesized that high carbohydrate intake is a risk factor for breast cancer, possibly mediated by elevated levels of free insulin, estrogen and insulin-like growth factor-I (Heaney and Hui, 2007). This hypothesis is supported by population-based case-control studies. In a Mexican population characterized by relatively high fat and high carbohydrate intakes, carbohydrate intake was positively associated with breast cancer risk. The strongest associations were observed for sucrose and fructose (Romieu et al. 2004) .

In animal studies, chronic administration of a 60% fructose diet to normal rats led to both hyperinsulinemia and *in vivo* insulin resistance. The fructose induced insulin resistance was mainly due to a diminished ability of insulin to suppress hepatic glucose output, and not due to decreased insulin-stimulated glucose uptake by muscle (Tobey et al. 1982). In another study fructose feeding to lean and obese Zucker rats, led to increased kidney fat, liver fats, and retroperitoneal adipose tissue weights, emphasizing the hyperlipidemic effect of fructose (Koh et al. 1985).

In humans, epidemiological studies support an association between fructose intake specifically, and cancer risk. Food-frequency questionnaires, documenting carbohydrate intake, glycemic load, in addition to sucrose, and fructose intake, collected from a cohort of women in the USA (n = 88,802) participating in the Nurse' Health Study, revealed a 53% increased risk of pancreatic cancer development in women who had a high glycemic intake, and particularly in the cohort who reported a high fructose-intake (57% increased risk) (Michaud et al. 2002).

A direct link between the presence of the glucose transporter GLUT1 and the use of glucose as a source of energy by the cells can be suggested (Fig.5, 6 and 7). There is abundant data reported on the presence of glucose transporter GLUT1 in all 3 cell lines used in the present study (Godoy et al. 2006; Gould and Holman, 1993; Medina and Owen, 2002). These observations correlate with the high cell proliferation (relative to those under fructose enriched media) in all cell lines studied in the presence of glucose as energy source (Fig.5, 6

and 7). Although the observed growth of Hela and BHK cells was not as expected (lower than the control), it still was significantly higher under glucose enriched medium than the growth observed under fructose enriched environment(Fig.6 and Fig.7). This can be attributed to many reasons For BHK (kidney cells) and Hela (cervical epithelial carcinoma) cells not much is documented on the expression of the two fructose transporters, GLUT2 and GLUT 5. The lack of cell proliferation of these two cell lines in the presence of a range of DMEM (glucose free) enriched with different D-fructose concentrations suggests only minimal uptake of D-fructose by its specific transporters in these two cell lines.

To explain these results, it has been suggested that tumors seeking extra energy sources may resort to using fructose in addition to glucose due to the constraint cause by the two rate limiting enzymes in glycolysis: glucokinase and phosphofructokinase-1. In the Caco2 colon cancer cell line, GLUT 5 is expressed endogenously in those cells that are fully differentiated and exhibit low rates of glucose consumptions (Mahraoui et al. 1994). This may be an attempt to obtain additional sources of sugars and thus increase proliferation as observed in MCF-7 cells (Fig.7). The observed increased expression of GLUT 5 could indicate additional use of fructose by cancer cells although the link between fructose and cell growth is still to be explained. The high rate of glycolysis observed in the “Warburg effect” could thus be explained by the presence of increased expression of the fructose specific transporter GLUT 5.

5. Conclusion

Glucose and fructose are the most abundant carbohydrate in humans. They are known to take part at different level of energy production in human cells. Cancer cells are reported to demonstrate an increase rate of energy consumption which correlates with their high and fast proliferation abilities. This property is thought to be directly linked to the presence of glucose transporters in relatively high levels which mediate the easy transport and use of these carbohydrates by cancer cells.

The MCF-7 cell lines (breast cancer cells) were observed to show proliferation in the presence of only D-fructose as the source of carbohydrate but for Hela and BHK cell lines this effect was not seen. Glucose stimulates proliferation in MCF-7, Hela and BHK cells significantly greater than D-fructose at all tested concentrations.

The observed cell proliferation in only the MCF-7 cell line in the presence of D-fructose suggests a possible role of the fructose specific transporter GLUT 5 in the use of D-fructose in energy production and cell growth by these breast cancer cells.

MCF-7 cells (a type of breast cancer cells) use D-fructose for cell proliferation.

Chapter 3: Effects of anti-GLUT 5 antibodies on the proliferation of breast cancer (MCF-7) *in vitro*

1. Introduction

In the past decades cancer therapy has witnessed a dramatic shift from the traditional chemotherapeutic approaches. Targeted therapies have joined traditional cytotoxic chemotherapy as components of treatment for many malignancies such as breast, colorectal, lung, leukemia, multiple myeloma and pancreatic cancers (Gerber et al. 2008). Targeted therapy blocks the proliferation of cancer cells by interfering with specific receptors required for tumor development and growth (Gerber et al. 2008). Many of these receptors are mutated or over expressed in tumors which make them available targets.

One such application of targeted therapy is the used of monoclonal antibodies. Over three decades since the development of hybridoma technology, it has been possible to provide monoclonal antibodies with high specificity and affinity for a particular target antigen (Kohler and Milstein, 1975). Monoclonal antibodies have become one of the most rapidly expanding classes of pharmaceuticals for treating a wide variety of human diseases including cancer.

Trastuzumab (Herceptin) an anti-HER2/neu antibody is widely used in therapy against breast cancer (Vogel et al., 2002). Similar approaches have been suggested for GLUT transporters due to their observed role in cancer proliferation (Simon and Banerjee, 2008).

Monoclonal antibodies typically use a combination of mechanisms in directing cytotoxic effects to a tumor cell. Most interact with components of the immune system through antibody-dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC), and many alter signal transduction within the tumor cell or act to eliminate a critical cell-surface antigen. ADCC occurs when antibodies bind antigens on tumor cells and the

antibody Fc domains are engaged by Fc receptors (FcR) on immune effector cells (Steplewski et al. 1983). ADCC is viewed as a mechanism to directly induce a variable degree of immediate tumor destruction that leads to antigen presentation and the induction of tumor-directed T-cell responses. CDC is another cell killing mechanism that can be directed by antibodies. In this cascade the formation of antigen-antibody complexes results in the assembly of a membrane attack complex which creates pores in the cell membrane that facilitate free passage of water and solutes into and out of the cell resulting in cell death.

2. Aim:

To determine the effect of anti-GLUT 5 antibodies on the proliferation of breast cancer cells (MCF-7) *in vitro*

3. Materials and methods

3. 1. Cells

- MCF-7 (ATCC catalogue number: HTB-22)

3.2. Media

Glucose free DMEM (PAA Laboratories, Austria), supplemented with 10% FCS was used. D-fructose used was of analytical grade. D-fructose at two different concentrations either 5 mM (0.930 g/L) or 25 mM (4.650 g/L) was added to the medium. DMEM Glucose free medium was used as positive control (enriched with either 5 or 25 mM fructose).

3.3. MCF-7 harvesting

Trypsin/EDTA (5 ml) was added to the 75 cm^2 culture flask containing the MCF-7 cells at approximately 80% confluence. The flask was incubated at 37°C in a CO_2 incubator for 20 minutes until the cells had detached. The content was transferred to a 15 ml sterile

centrifuge tube and fresh medium (Glucose free DMEM) was added. This tube was centrifuged at 200 g for 10 minutes. The supernatant was removed and cells resuspended in 1 ml DMEM (Glucose free). The concentration of cells in the suspension was determined using a hemocytometer and adjusted to the desired cell concentration with the same medium.

3.4. Proliferation study

One hundred microliters of a cell suspension containing 5 x 10^4 cells per ml was incubated for 72 hours at 37°C in a CO_2 incubator with one of three dilutions of the anti-human GLUT 5 (Rabbit anti-human polyclonal IgG ;1mg/ml-US Biological, Massachusetts, USA) antibodies solutions (1 µg/ml or 200 ng/ml or 100 ng/ml) prepared from the stock solution (1.0 mg/ml). The cells were incubated in DMEM media enriched with either 5 mM (0.930g/L) or 25 mM (4.650g/L) D-fructose. These were compared to an equivalent anti-body free cells as controls.

Triplicate wells were used for each treatment. All experiments were repeated at least 3 times.

3.5. Viability assay

The viability of cells was assessed using the MTT assay (Mossman, 1983). After the incubation period, 20 µl of MTT (5 mg/ml in PBS) were added to each well. The plate was incubated for 4 hours at 37ºC in a CO_2 incubator then centrifuged at 500 g for 10 minutes. The supernatant was removed and 150 µl of PBS was added to each well to wash away any excess of reagents and medium. The plate was centrifuged again at 500 g for 10 minutes and the PBS was removed. The individual pellets were treated with 100 µl of DMSO and the plate was shaken gently for 60 minutes to dissolve the colored formazan then read on a microplate reader at 570 nm with the reference wavelength at 630 nm.

4. Results and Discussion

Anti-GLUT 5 antibodies at the following 2 concentrations 1:5000 (200 ng/ml) and 1:10 000 (100 ng/ml) did not significantly inhibit MCF-7 cell line proliferation (Fig.8) at either of the tested D-fructose concentrations (low 5mM and high 25mM). At 1 µg/ml the anti-GLUT 5 antibodies seem to exhibit an inhibitory effect on MCF-7 cell proliferation. This however was not significantly significant.

Strategies to use Anti-GLUT1 antibodies in targeted therapy against breast cancer are under investigation (Simon and Banerjee, 2008). It has been observed that Anti-GLUT1 antibodies suppress cell proliferation *in vitro* (Simon and Banerjee, 2008). No such studies had been conducted with antibodies against the fructose specific transporter GLUT 5 although GLUT 5 would by virtue of its more selective distribution be a better target for breast cancer therapy (Chan et al. 2004) particularly since it has been established that antisense oligonucleotides against GLUT 5 have anti-proliferative effect on two breast cancer cell lines (MCF-7 and MDA-MB-231) through suppression of the GLUT 5 transporter expression in these cells. In the present study, it was expected that the inhibitory effect of the anti-GLUT 5 antibodies would be concentration dependent both for the antibody and the level of D-fructose in the medium. The higher concentration of antibody did show an inhibitory effect but this was not the case for lower concentration of the antibody.

Using a 1 way ANOVA statistical analysis test no significant difference could be observed among the different concentrations of anti-GLUT 5 antibodies groups across D-fructose concentrations ($p=0.096$). At both 1:5000 (200ng/mL) and 1:10 000 (100ng/mL) concentrations of the GLUT 5 anti-antibody a normal growth pattern was observed for MCF-7 cell line but this was not significantly different from that of the control (DMEM glucose free media enriched with either 5 or 25 mM).

These observed results could mainly be attributed to higher dilutions of the antibodies. It was expected that if anti-GLUT 5 antibodies were able to inhibit proliferation of MCF-7, then a

decrease in cell proliferation would have been observed, ideally showing a dose dependent effect. A clear (even though not significant p=0.0990 using an unpaired student *t*-test) decrease in cell proliferation was observed at the 1:1000 (1 µg/ml) concentration at all tested D-fructose concentrations.

There a D-fructose dependent mediated cell proliferation was observed. MCF-7 cells seem to be less susceptible to the inhibitory effect of anti-GLUT 5 antibodies in the presence of higher D-fructose concentration. At 1000ng/mL the anti-GLUT 5 antibodies demonstrated a 30% inhibition compared to the control at both D-fructose concentrations tested (Fig.8).This hints to the effectiveness of the antibodies in reducing/preventing cell proliferation possibly through blockage of GLUT 5 transporters at this concentration.

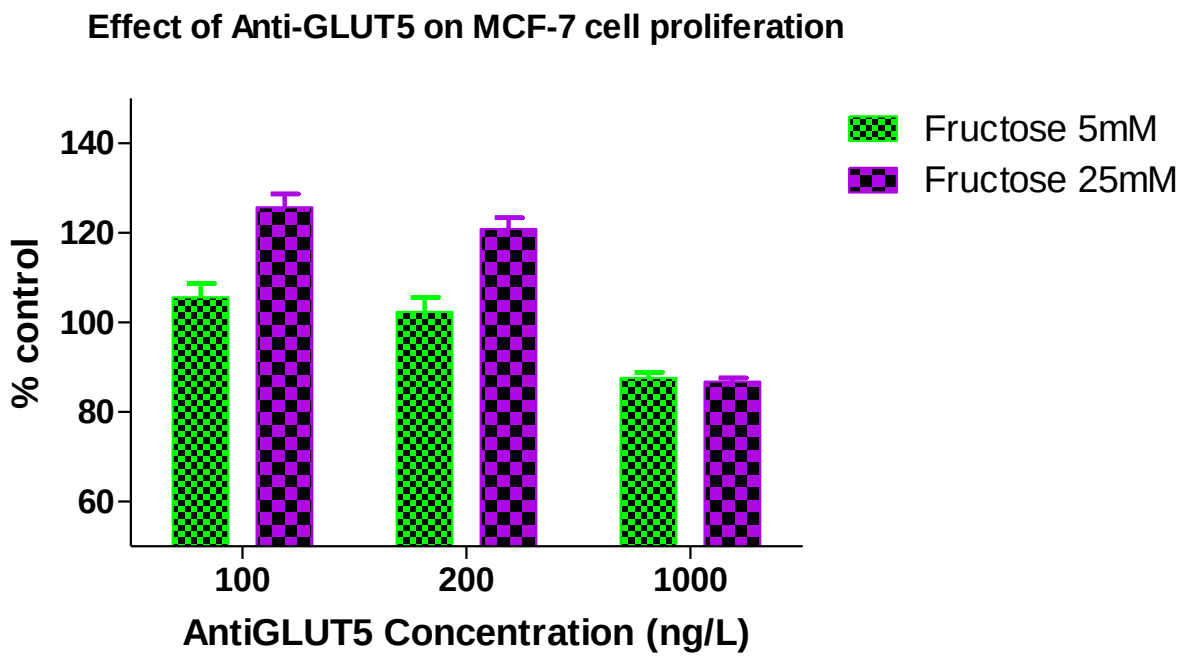


Figure 8: MCF-7 proliferation (72hours) incubation with different concentration of anti-GLUT 5 antibodies in glucose-free DMEM enriched medium (5mM and 25mM D-fructose). Anti-GLUT 5 antibodies showed no significant inhibition effect on MCF-7 cell lines (p=0.096 one way ANOVA). No significant difference could be observed between the different concentrations of D-Fructose groups (p=0.5754 unpaired *t*-test).

It is possible that by blocking GLUT 5 transporters there is a decrease in D-fructose available within the cell and therefore a decrease in energy production. This is in support of the results observed where incubation with glucose free but D-fructose enriched media resulted in modest MCF-7 proliferation.

In an *in vivo* scenario the inhibition of cell proliferation would be hypothesized to be mediated by either ADCC or CDC. This will result from the effect of ADCC or CDC on the GLUT 5 transporters as a consequence of the binding of the antibody on the transporter resulting in the destruction of the cell membrane and/or the inhibition of the transporters functionality and/or attack on the cell by phagocytic cells of the immune system. The destruction of the cell membrane would subsequently be followed by cell death while the destruction of the GLUT 5 transporter would result either in a decrease of the influx of fructose thus limiting cell proliferation due "cell starvation" caused by scarcity of energy source or in cell death caused by a failure in keeping the integrity of cell membrane due to the CDC action.

Apart from an increase in anti-GLUT 5 concentrations, the results of the present study could be improved through combination of anti-GLUT 5 antibodies with cytotoxic drugs. This technique of combination therapy is widely and successfully used in clinical oncology since it increases the response rate and duration of individual drugs. The underlying principle of combination therapy is a known synergistic effect of the drugs used mostly mediated through different targeted receptors. For example Herceptin (anti-HER2/neu antibody) has shown not only a synergistic antitumor activity when in use with cisplatin and carboplatin but also an additive benefit when used in conjunction with doxorubicin, cyclophosphamide, methotrexate, taxol or celecoxib (Baselga et al. 1998; Mann et al. 2001; Pegram et al. 1999; Pietras et al. 1999; Pietras et al. 1998; Pietras et al. 1994).

A possible combination of the anti-GLUT 5 antibodies with one cytotoxic drug could give better inhibition of breast cancer (the use of a cytotoxic pro-drug would give still better

results through a major reduction in possible adverse side effects). This technique would have the advantages provided by blocking GLUT 5 transporter in breast cancer cells but also those afforded by the cytotoxic drug. The cytotoxic drug may even be used at lower dosage in the event of synergistic effects and increase selectivity mediated by the specificity of GLUT 5 antibodies to this transporter which is over-expressed in several malignant tissues.

5. Conclusion

Monoclonal antibodies provide the ability to target specifically a known receptor or transporter on a particular cell. In the case of breast cancer cells, one such target receptor is the specific fructose transporter GLUT 5. Anti-GLUT 5 antibodies can be used as "magic bullets" to target this transporter.

Anti-GLUT 5 antibodies showed minor inhibitory effect on MCF-7 cell proliferation at concentrations equal to 1 µg/ml. Higher concentrations (>1 µg/ml) of the antibodies as well as possible combination with cytotoxic drug/prodrugs could be investigated for their inhibitory effect on MCF-7 cell proliferation.

There was no significant difference observed among the 2 D-fructose concentrations (5mM and 25mM) suggesting that D-fructose has little added-on role to play in the effect of anti-GLUT 5 antibodies when it comes to reducing or inhibiting cell proliferation in MCF-7 cell lines.

It is difficult to determine whether MCF-7 cells do rely solely on the GLUT 5 transporter as the major source of carbohydrate delivery into the cell in the presence of fructose as the only source of carbohydrate. It is known that GLUT 2 (and reportedly GLUT 7) can also be used as an alternative transporter of fructose into the cell although this transporter is rarely used for this function (GLUT 2 serves mainly as a glucose transporter).

Anti-GLUT 5 demonstrate inhibitory effect on breast cancer cells (MCF_7 cell line) at concentration above 1 µg/ml.

Chapter 4: The role of D-fructose enriched culture medium on the 3D colony formation potential of selected cell cultures

1. Introduction

In vitro cellular transformation detection assays are semi-quantitative and measure the morphological transformation of cell colonies induced by chemicals. This transformation is linked to certain cellular changes such as loss of contact inhibition and anchorage independence. The process by which these changes occur is closely associated with the process of *in vivo* carcinogenesis and thus linked to intracellular mechanisms of energy consumption. Among the techniques used to assess *in vitro* cellular transformation is the 3D colony formation in soft agar gel which is indicative of the aggressiveness of the tumor growth (Neugut et al. 1979; Tatsuyoshi et al. 1986; Liu et al.2010). In the presence of a molecule with anchorage dependent inducing abilities (such as forskolin and many other carcinogenic molecules), the number of colonies formed by the treated cells is expected to be proportional to the concentration of the molecule to which the cells are exposed. These molecules promote invasiveness. The same principle would apply in cases of a aggressive forms of cancer cell line. The number of colonies formed under the 3D soft agar colony formation essay will be proportional to the aggressiveness of the specific cell line.

2. Aim:

The aim of this study was to investigate the effect of D-fructose ability to cause *in vitro* 3D colony formation on GLUT 5 expressing cells in a soft agar matrix.

3. Materials and methods

3.1. Cells

- MCF-7 (ATCC catalogue number: HTB-22)

- Hela (ATCC catalogue number: 229CL-2.1)
- BHK (ATCC catalogue number: 238-1 CCL10C-13)

3.2. Media

- Glucose (ANALAR-BDH, London, UK) supplemented cell culture medium (DMEM)
- D-fructose (Merck, Johannesburg, South Africa) supplemented cell culture medium (DMEM)

3.3. Soft agar assay for colony formation

The colony formation ability of a D-fructose enriched medium was assessed using the method previously described by Roberts and co-workers with slight modification (Roberts et al. 1981).

Preparation of 1% nutrient agar: 500 mg nutrient agar (Whitehead Scientific, Johannesburg, South Africa) was weighed and added to 50 ml sterile deionised H_2O (ddH_2O). This solution was autoclaved for 20 min at 121°C, mixed well, placed in a water bath at 45°C and allowed to solidify while kept sterile.

Preparation of 0.7% nutrient agar: 14 ml of 1% nutrient agar was mixed with 6 ml of sterile ddH_2O to give a final agar concentration of 0.7%. This solution was placed in a water bath at 45°C.

Preparation of 2 x DMEM: DMEM medium was prepared from the powder using half of the required volume of water (for both the glucose and fructose based media) and sterile heat inactivated fetal calf serum was added to yield a final concentration of 10% FCS.

Bottom layer: One millilitre of 1% nutrient agar was mixed with 1 ml 2 x DMEM with 10% FCS to give a final agar concentration of 0.5% nutrient agar. A total volume of 0.5 ml of this 0.5% nutrient agar solution was added to each well (24 well plates) and allowed to solidify at room temperature.

Top layer: To 1 ml of 0.7% nutrient agar was added 800 µl of 2 x DMEM with 10% FCS and 200 µl of a cell suspension containing 1250 cells per ml. One half a milliliter of the diluted 0.7% agar containing the suspended cells was added onto the solidified bottom layer (final concentration of the top nutrient agar layer being 0.35%) and cultured in an incubator at 37°C, 5% CO_2 for 2–3 weeks.

3.4. Colony counting

Cells were grown in either glucose or D-fructose based medium at the 2 different concentrations: 5 mM (0.930 g/L) and 25 mM (4.650 g/L). Each well was stained with 500 µl of 0.5% aqueous crystal violet for 3 hours. The number of cell colonies per well larger than 0.2 mm diameter on the plate were counted under a microscope (Olympus IX70, Japan) at a magnification of 100x.

DMEM (5.56mM-1 g/L glucose supplemented with 10% FCS) used routinely in the laboratory was used as positive control while a glucose/D-fructose free DMEM supplemented with 10% FCS will was used as negative control.

Triplicates wells were used for each treatment. All experiments were repeated at least 3 times.

Results and discussion

BHK cell line had the highest number of colonies for all the tested media but particularly in the highest concentration (25 mM= 4.560 g/L) of glucose containing medium.

The Hela cell line showed the least number of colonies formed. MCF-7 cell lines have some colonies growth range between these two cell lines. These results go hand in hand with those observed in the proliferation study (Chapter 2). There was no significant difference between the three cells lines across the tested concentrations (Fig.9).

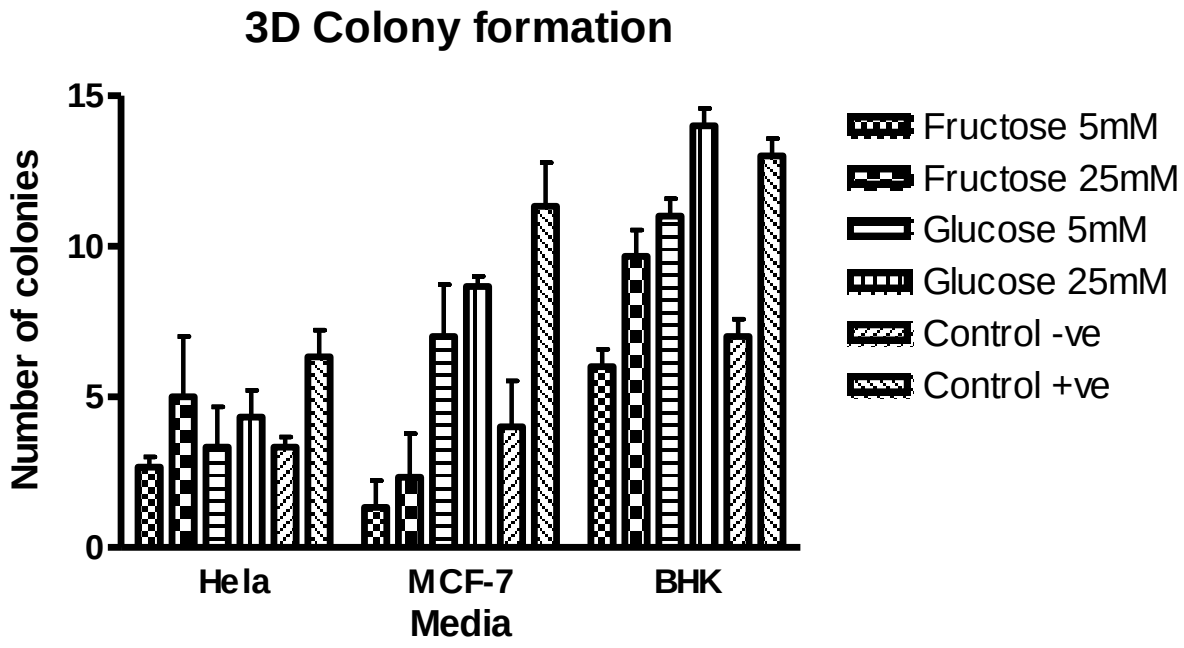


Figure 9: 3 D colony formation study. Number of colonies formed after 21 days incubation period on 0.35% nutrient agar layer in 10% FCS. No significant difference could be observed among the 3 cells lines: MCF-7, Hela and BHK (1 way-ANOVA: $p>0.05$).

Heaney and Hui, (2007) reported that D-fructose is directly associated with breast cancer proliferation particularly in MCF-7 cell line *in vitro* which is supported by results seen in the

present study. It is further known that tumor environment causes specific adaptations of cellular metabolism that increase the uptake of metabolic substrates (Gatenby and Gillies, 2008). Based on the proliferation study results in different concentrations of glucose or fructose it would have been expected that a greater degree of colony formation be seen for the MCF-7 cell line compared to the BHK cell lines. BHK cells are a rapidly growing primary culture of new born hamster kidney tissue, with fibroblastic cells and widely used as viral host in studies of oncogenic transformations and of cell physiology. BHK cells cultures showed minimal degree of proliferation when exposed to D-fructose enriched medium. The present results did not correlate with predictions that these cell lines (BHK) would show minimal colony formation especially in the presence of D-fructose. The colony formation pattern of Hela cell line observed in this study was consistent with results observed under cell proliferation in the different carbohydrate supplemented media. One possible reason why the results observed for MCF-7 and BHK cell lines fell outside of the predictions is that the incubation time during the proliferation study was a maximum of 7 days while in the present study the incubation time was a minimum of 14 days. An incubation period of more than 7 days may allow enough time for the BHK cell lines to adapt to their new environment (D-fructose enriched DMEM medium) thus explaining the increased colony formation ability observed when their ability to thrive in such environment is considered. The level of expression of the GLUT 5 transporter in each of the studied cell lines could be another explanation. There is abundant data on the expression of GLUT 5 in MCF-7 cell lines but nothing is known about the presence of this transporter in BHK cell lines (Medina and Owen, 2002). Only MCF-7 expresses GLUT 5 transporter in high enough levels to be detected. Yet it should be noted that GLUT 5 has been observed to self-induced in the presence of D-fructose in some cancer cell lines (Heaney et al. 2007). If these two observations are considered together, a possible induction of the GLUT 5 transporter in BHK cells could result when these cells are exposed to a D-fructose enriched medium for extended periods. This would need to be investigated further but falls outside the scope of this study.

The difference in the cell lines ability to form colonies could also be attributed to their intrinsic properties. The colony formation test is based on morphological transformation linked to certain changes such as loss of contact inhibition and anchorage independence. The experiment was to assess if D-fructose could mediate these changes in cells that would otherwise not express GLUT 5. Grover et al. (1998) have demonstrated that invasiveness of breast cancer cells increase the expression of GLUT 1 but decrease the expression of GLUT 2 and GLUT 5 (Groveret al. 1998). Colony formation potential of a particular cell lines has been reported to be associated with cancer aggressiveness (Aapro et al. 1987). From the results reported here, it appears that D-fructose does not promote colony formation in any of the three cell lines studies and thus cannot be linked with aggressive forms of these cancers.

4. Conclusion

No significant differences in 3D colony formation were observed among the three cell lines used in the study ($p>0.05$) when exposed to glucose or D-fructose supplemented media. These observations suggest that the cell lines ability to form colonies could not be attributed to the enrichment of the media with D-fructose.

Anchorage dependent growth is an important property of aggressive cancer species. This is primarily essayed on soft agar by assessing the 3D colony formation potential of these cancer species. The number of colonies formed by any species is directly proportional to its anchorage dependent growth and thus its aggressiveness. Since increased D-fructose cannot be directly associated with colony formation, it thus cannot be directly linked to aggressive forms of carcinogenesis despite the observed evidence of its involvement in cell proliferation and energy consumption as seen in the cell proliferation assays performed in the early part of this study.

It is unclear whether D-fructose would favor cancer aggressiveness and metastasis in the presence of carcinogenic molecules. Such a study should be undertaken at a future stage to determine the full impact of D-fructose action in cancer.

Chapter 5: Expression of fructose transporters, GLUT 5, in MCF-7, Hela and BHK cell lines.

1. Introduction

1.1. Glucose transport in cells

Virtually all mammalians cells use glucose as the primary source of energy production. The hydrophilicity of glucose, doesn't allow it to penetrate the lipid bilayer. Specific carriers are required to facilitate its entry into cells despite a concentration gradient (Joost and Thorens, 2001). A facilitated transport of hexoses into all cells types, through a family of glucose transport proteins (GLUT1-13) mediates this entry of glucose into the cells. (Joost and Thorens, 2001; Macheda et al; 2005; Shurmann, 2008; Wood and Trayhurn, 2003). These GLUT isoforms have been widely described and their expression has demonstrated to be cell-specific with kinetics and substrate specificity.

1.2. Localization and structure of GLUTs

The GLUTs are intrinsic trans-membrane proteins with different tissue specificity and responses to regulatory stimulus.

The facilitative sugar transporters are predicted from genetic sequence data to have 12 membrane-spanning regions with both the amino (NH_{2}-) and carboxyl (COOH-) terminal ends intracellularly located (Gould and Holman, 1993; Joost and Thorens, 2001; Wood and Trayhurn, 2003). Sequence comparison of these transporters resulted in the identification of "sugar transporter signatures" which are conserved glycine and tryptophan residues regarded to be essential for facilitative transport of sugars (Joost and Thorens, 2001). The genes belong to the solute carrier 2A family (SLC2A).

These transporters can be divided into three main families or classes (see Fig. 10) (Joost and Thorens, 2001). Based on the amino acid sequence of the GLUTs, a model with 12 hydrophobic α-helical domains has been proposed predicting the orientation of facilitative GLUTs in the cell membrane. There is an intracellular loop between transmembrane (TMs) domains 6 and 7 while the amino and carbon tails are located in the cytoplasm (Macheda et al. 2005):

- Class I: GLUT 1, 2, 3 and 4. These are thoroughly characterized. They are mainly distinguished by their tissue distribution, their affinity to glucose and their hormonal regulation. A glutamine in helix 5 and the STSIF-motif in the extracellular loop 7 are the residues that appear to be characteristic of this class (Joost and Thorens, 2001). They have a large extra cellular loop between TMs 1 and 2 that contains an N-linked glycosylation site (Macheda et al.2005).
- Class II: GLUT 5, 7, 9 and 11 are characterized by the lack of the tryptophan following the conserved GPXXXP motif in helix 10 (Joost and Thorens, 2001). They also have a large extracellular loop between TMs 1 and 2 that contains an N-linked glycosylation site (Macheda et al. 2005).
- Class III: GLUT 6, 8, 10, 12 and H^+/*myo*-inositol transporter (HMIT1) are characterized by a shorter extracellular loop 1 that lacks a glycosylation site and by the presence of such a site on a large predicted extracellular loop and potential site for glycosylation between TMs 9 and 10 (Joost and Thorens, 2001; Macheda et al. 2005). They do not have a large extracellular loop with a N-linked glycosylation site between TMs 1 and 2 as for Class I and II.

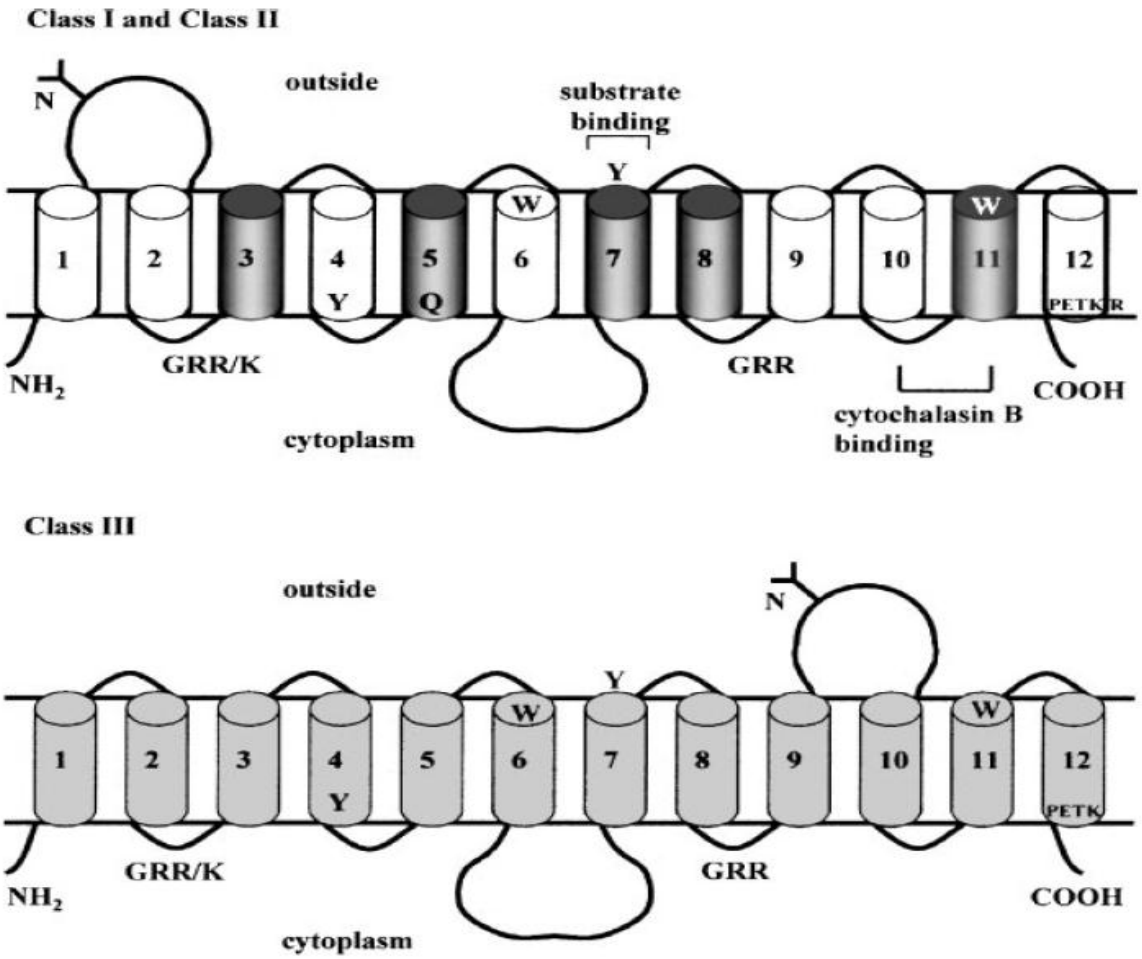


Figure 10: Model of the orientation of Glucose transporter protein in the cell membrane. Class III has a large predicted extracellular loop and potential site for glycosylation (N) between TMs 9 and 10 (The TMS are numbered 1-12).They do not have the extracellular loop and N-linked glycosylation site between TMs 1and 2 as for class I and II. The conserved sequences are shown (GRR/K and GRR). Both the NH_2 and COOH tails are within the cytoplasm (Macheda et al. 2005).

The nomenclature, location, hexose transported and references for the GLUT transporters described to date is summarized in Table 1.

Table 1. Tissue expression of GLUT transporters

(Gould and Holman, 1993; Joost HG and Thorens, 2001; Medina and Owen, 2002; Wood and Trayhurn, 2003)

Protein	Alias	Gene name	Tissue of expression	Substrate	Reference
GLUT 1		SLC2A1	All tissues	Glucose Galactose	(Mueckler et al. 1985) (Gould and Holman , 1993)
GLUT 2		SLC2A2	Liver, retina and pancreatic islets cells	Glucose Fructose	(Fukomoto et al. 1988) (Watanabe et al. 1999) (Zamora-Leon et al.1996)
GLUT 3		SLC2A3	Brain	Glucose Galactose	(Kayano et al.1988)
GLUT 4		SLC2A4	Muscle, fat, heart	Glucose	(Fukomoto et al.1988)
GLUT 5		SLC2A5	Intestine, testis, kidney, erythrocytes	Fructose	(Concha et al.1997; Kayano et al.1990)

GLUT 6	GLUT 9	SLC2A6	Spleen, Leukocytes, brain	Glucose	(Doege et al. 2000a)
GLUT 7		SLC2A7	Liver, small intestine, colon	Glucose Fructose	(Joost and Thorens, 2001) (Cheesman, 2008)
GLUT 8	GLUTX1	SLC2A8	Testis, brain, blastocyst	Glucose	(Doege et al.2000b) (Carayannopolous et al.2000) (Linsinki et al.2001)
GLUT 9	GLUTX	SLC2A9	Liver, Kidney	n.d	(Phay et al.2000)
GLUT 10		SLC2A10	Liver, pancreas	Glucose	(McVie-Wylie et al.2001) (Dawson et al. 2001)
GLUT 11	GLUT10	SLC2A11	Heart, muscle	Glucose	(Doege et al.2001) (Wood and Trayhurn, 2003)

				Fructose	
GLUT 12	GLUT 8	SLC2A12	Heart ,prostrate, muscle, intestine	n.d	(Roger et al. 2002)
Myosinositol transporter (HMIT)	GLUT 13	SLC2A13	Brain	H^+-*myo*-inositol	(Joost and Thorens, 2001) (Uldry et al. 2001)

1.3. Glucose transporters and cancer

Any living cell needs energy in increasing amounts to be able to divide and grow. Malignant cells have a higher metabolism and increased requirement for energy than cells from the surrounding tissue. In the 1950s Otto Warburg observed that a characteristic feature of ischemic conditions is the production of large amounts of lactic acid from glycolysis due to the reduced oxygen levels (Warburg,1956). This was later to be known as the Warburg theory. The increase in the amount of lactic acid present with reduced oxygen results from an increase of the rate of transport of glucose (Birnbaum et al.1987; Pedersen,1978). Thus the demand for energy is satisfied by an increased sugar intake which is accomplished through the increase in glucose transporter expression.

It was believed for some time that only GLUT 1 was over-expressed in most types of cancer cells until Nelson and Younes (Nelson et al. 1996; Younes et al. 1996) observed that GLUT 1 over expression in malignant tissues was not characteristic of all cancers. Today it is widely accepted that the majority of cancers over-express one or more classes of the GLUT family members, which are present in the respective tissue of origin under non-cancerous conditions, thus making these transporters an important target in the study, diagnosis and therapy of cancer (Medina and Owen, 2002).

In tumor samples, GLUT 1, 2 and 5 have been observed to be the three most commonly observed glucose transporters, where GLUT 2 and GLUT5 are associated with fructose transport (Godoy et al. 2006). Another less commonly expressed fructose transporter GLUT 7, has also been recently reported to transport fructose into cells (Cheesman, 2008; Shurmann, 2008). These fructose transporters are found either on the surface membrane or in the cytosol of cells (Trayner et al. 2009). The ability to transport D-fructose into the cell varies for each receptor. GLUT 2 is reported to have a minor role in total fructose flux across the cell membrane about 12 % in MCF-7 and 30 % in MDA-MB-231 (Trayner et al.

2009). GLUT 5 is a high affinity fructose transporter (GLUT 5 is responsible for a major part of the fructose flux for both MDA-MB-231 and MCF-7 cells). GLUT 5 is expressed at low levels in the intestine, testis, kidney, erythrocytes and lung (Douard and Ferraris, 2008; Gould and Holman, 1993; Medina and Owen, 2002). GLUT5 is not expressed by normal breast cells while it is reported to be highly expressed *in vitro* and *in vivo* in breast cancer cells (Godoy et al. 2006; Medina and Owen, 2002; Zamora-Leon et al. 1996). The observation of a high over expression of GLUT5 in cancerous breast cells lead to the assumption that breast cancers utilize fructose as an energy source during uncontrolled proliferation (Godoy et al. 2006; Medina and Owen, 2002). While there is abundant data on GLUT 5 expression in kidney, breast and intestinal cancer cells (Brot et al. 1996; Godoy et al. 2006; Zamora-Leon et al. 1996), no documented data on expression of GLUT 5 in cervical epithelial carcinoma cells (Hela) could be found. Due to its selective expression in breast cancer cells and not in normal breast tissues GLUT 5 would appear to be a potential target for imaging or treating breast cancers.

2. *Aim*

To confirm the presence of the fructose specific transporter, GLUT 5, in selected cancer cell lines.

3. *Materials and Methods*

3.1. Cells

- MCF-7 (ATCC catalogue number: HTB-22)
- Hela (ATCC catalogue number: 229CL-2.1)
- BHK (ATCC catalogue number: 238-1 CCL10C-13)

3.2. Cell cultures

After cells reach 80% confluence in a 75 cm^2 culture flask, they were gently removed by scraping, suspended in medium, centrifuged at 700 *g* and the pellet stored at -70°C. The cells were lysed in 10 ml HES buffer (20 mM HEPES, 5 mM sodium azide, 250 mM sucrose) plus protease inhibitors (HESpi; 10 µl of enzyme inhibitor cocktail-(Sigma Aldrich, Steinheim, Germany). Cells were homogenized with 10 strokes of a Teflon Dounce homogenizer on ice. The cell debris were removed by low speed centrifugation at 12000 g for 15 minutes followed by the isolation of membrane bound proteins with high speed centrifugation (100 000 *g* for 60 minutes at 4ºC) in HESpi buffer. Protein concentrations determined using the Bradford assay (Bradford,1978). Extracted proteins were aliquoted in 1 ml vials and kept frozen at -70 ºC until ready to be used.

3.3. Sodium Dodecyl Sulphate (SDS) polyacrylamide electrophoresis of GLUT5 proteins

SDS polyacrylamide electrophoresis (SDS-PAGE) were carried out using a Hoeffer Mighty Small II Slab Gel electrophoresis system (SE 250-Hoeffer scientific instrument, San Francisco) based on the SDS-discontinuous buffer system method developed by Laemmli (Laemmli UK. 1970). Proteins samples were separated on a 12% SDS running gel with 12 % T and 1.4 % C in 1.5 M Tris/HCl, 0.1% SDS pH 8.8 and a 5 % SDS stacking gel in 0.5 M Tris/HCl, 0.1% SDS pH 6.8. Samples were prepared by 1:1 dilution with 2 x Laemmli sample buffer, 0.125 M Tris/HCl pH 6.8 containing 12.5% SDS, 12% glycerol, 3% ß-mercaptoethanol and 0.001 % bromophenol blue. Samples were boiled for 5 minutes and centrifuged at 10 000 g for 10 minutes. Protein samples were separated at 30 mA per gel with Tris glycine 0.192 M, pH 8.3 reservoir buffer containing 0.1% SDS at 25ºC until the tracking dye had advanced 85% down the gel.

GLUT proteins were quantified as follows: 30 µg of total protein were loaded per lane and resolved on the 12% SDS-polyacrylamide gel electrophoresis. Gels were fixed and stained

with 0.05% Coomassie blue R-250 solution prepared in 50% methanol and 10% acetic acid and destained in 50% methanol, 10% acetic acid in water (Bester MJ. 1996).

3.4. Protein transfer

The Gels were transblotted to HyBond C membrane (Biovision Research Products, California, USA) using a LKB Bromma 2117 Multiphor II electrophoresis unit. A transfer buffer system was used with two anode buffers (Anode buffer 1: 0.3 M Tris, pH 10.4 and Anode buffer 2: 25 mM Tris, pH 10.4) and one cathode buffer (25mM Tris, 40 mM glycine and 40 mM 6-amino-n-hexanoic acid, pH 9.4) all containing 10% methanol (Bester,1996). The gels were equilibrated for 5 minutes in the cathodic buffer. The Hybond C membrane was cut to size taking care not to touch the membrane with ungloved hands, equilibrated as per the manufacturer's instructions by wetting the membrane in deionized water for 10 minutes then equilibrating it in the second anode buffer for 10 minutes. Proteins were transferred for 60 minutes at 2.5 mA/cm^2. The membrane was either stained for protein with Coomassie blue to confirm successful transfer or used for immunodetection of the GLUT 5/GLUT 1 transporters.

3.5. Immunodetection of GLUT 5

Protein immunodetection was conducted as per the method described by Grover and co-workers (Grover et al. 1998). Membranes with transferred protein bands were blocked by incubation in 3 % Bovin Serum Albumin (BSA) in phosphate buffered saline containing 0.1% Tween-20 (PBS-T; pH 7.4) for 60 minutes then rinsed twice in PBS-T for 5 minutes before being incubated with diluted primary antibody (polyclonal rabbit) anti-human GLUT 1 (0.2 mg/ml- Genetex Inc, California, USA) 1:1000 or anti-human GLUT 5 (1.00 mg/ml- US Biological, Massachusetts, USA) 1:1000 for over 60 minutes at room temperature with agitation, washed twice with PBS-T for 5 minutes, incubated at room temperature for 60 minutes with horseradish peroxidase-linked secondary antibody, anti-rabbit IgG (1.00 mg/ml-Rockland, Pennsylvania, USA) (1:1000) then washed twice at room temperature for

5 minutes with PBS-T. Membranes were visualized by enhanced chemiluminescence (ChemiFast Chemiluminescence Substrate-Syngene/Cambridge) on a BIORAD precision ChemiDoc™ XRS+ System at 120 seconds exposure time.

4. Results and Discussion

GLUT 5 is expressed by MCF-7 but was not observed to be expressed by Hela cell lines. BHK cell lines demonstrated a limited expression of the transporter compared to MCF-7 cell line (Fig.12).

Coumassie blue staining of the gel showed that there were proteins present in our region of interest between in 40 and 80 kDa (Fig.11). GLUT transporters are located within this region. The approximate masses are respectively 55kDa for GLUT 1 and 66 kDa for GLUT 5 (Godoy et al.2006).

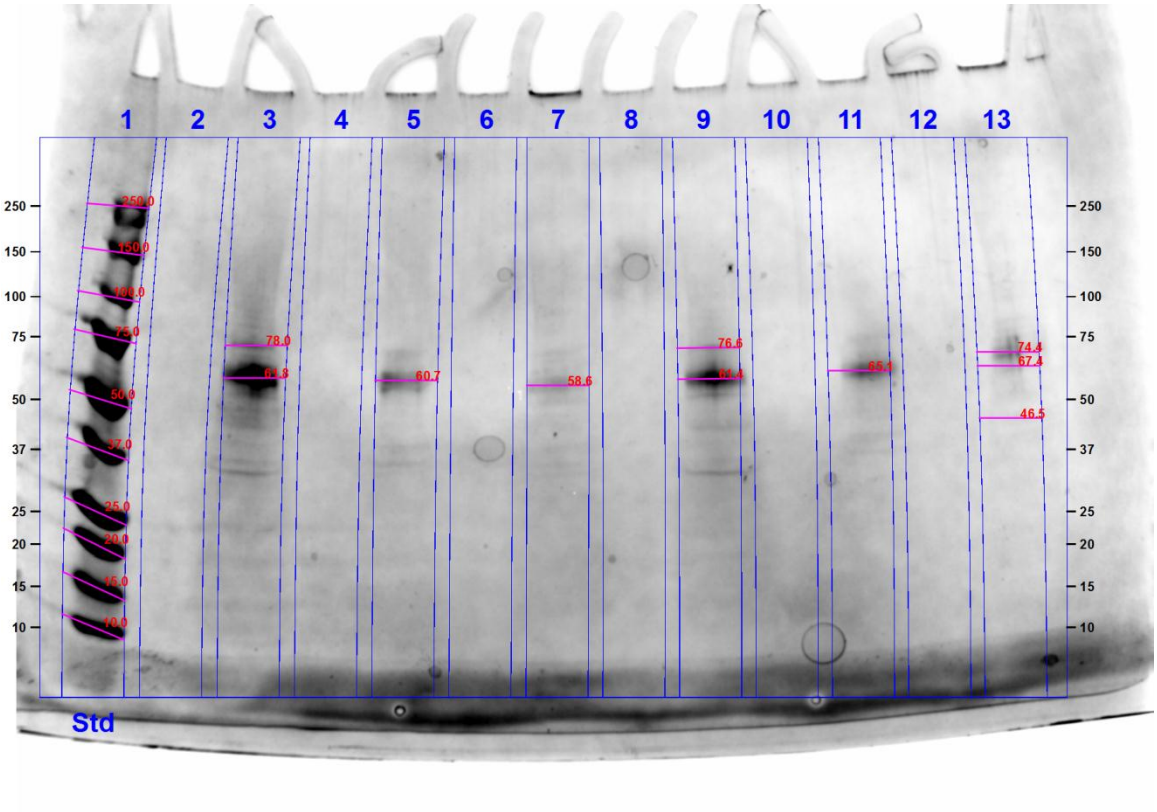


Figure 11: SDS-PAGE of protein extract of the different cell lines stained with Coomassie Brilliant blue; **3** & **9:** Hela cell line; **5** & **11:** MCF-7 cell line and **7** & **13:** BHK cell line. Proteins are represented by a dark spot; GLUTs transporters are known to have molecular weights between 40-80kDa which was chosen as region of interest. Images were acquired with a BIORAD Precision Plus Gel Doc™ EZ at 0.400 seconds exposure time (with a Bio Rad precision plus standard in lane **1**)

The high expression of GLUT 5 by MCF-7 cell lines is widely recorded in literature (Godoy et al. 2006; Medina and Owen, 2002; Zamora-Leon et al. 1996). Godoy and coworkers observed that after GLUT 1, the two most widely expressed transporters in tumors samples they analyzed were GLUT 2 (31%) and GLUT 5 (27%). These same authors found that in all normal breast cells they studied only GLUT 5 was expressed while GLUT 2 and GLUT 5 were expressed in 90% of breast ductal carcinomas samples mainly in the cytoplasmic area of. (Godoy et al.2006). While there was no available data on GLUT 5 expression in BHK cell lines. The Hela cell line is reported not to express this particular fructose transporter (Godoy et al.2006). The results from the study align with the data available in the literature. It has been observed that the over-expression of GLUT 5 is characteristic of breast cancer cells, yet this receptor is not expressed at the same high levels in normal breast cells (Godoy et al.2006;Zamora-Leon et al. 1996).

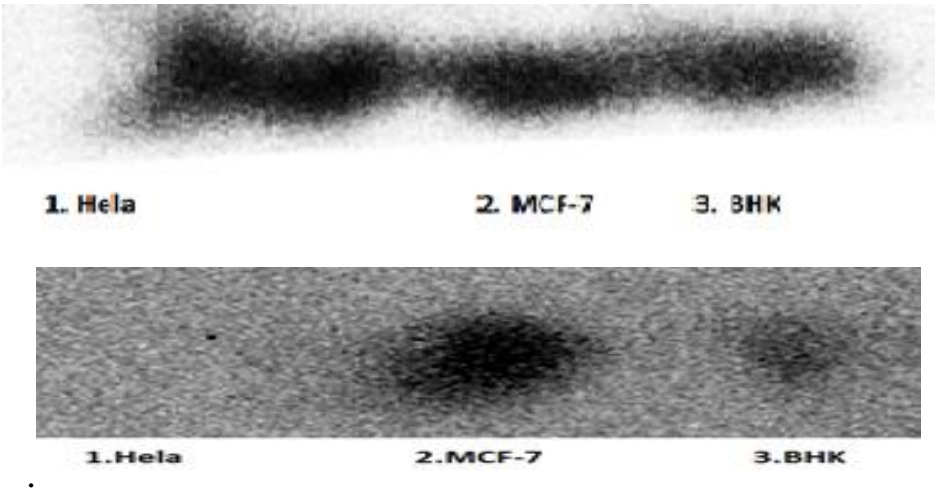


Figure 12: Western Blots of protein extract of the different cell lines visualized with Enhanced Chemiluminescence. **A:** GLUT 1 on 1.Hela, 2. MCF-7 and 3.BHK cell lines. **B:** Glut 5 on 1.Hela, 2. MCF-7 and 3.BHK cell lines. Proteins detected are represented by a dark spot; GLUTs transporters are known to be of molecular weight between 40-80kDa (55 kDa here). Images were acquired with Enhanced Chemiluminescence on a BIORAD Precision ChemiDoc™ XRS+ System at 120 seconds exposure time.

It is interesting to note that RT-PCR and sequencing experiments have shown the presence of GLUT 5 mRNA in cancerous breast cells. Neoplastic transformation of breast epithelial

cells leads to over-expression of high affinity fructose transporter GLUT 5 allowing the enhanced uptake of fructose (Godoy et al. 2006).

Recent works have recognized the role of the adjustment of energy metabolism following deregulation of cell proliferation in cancer in order to fuel cell growth (Hanahan and Weinberg, 2011). It has been suggested that the increase energy demand on cancer cells results in fructose entering the glycolytic pathway and being used as a substrate for energy generation in cancerous cells. Lactic acid generation through fructolysis may not be regulated by the same mechanism that control glycolysis thus providing the neoplastic cells with a metabolic advantage (Zamora-Leon et al. 1996). Zamora and coworkers demonstrated using functional kinetics that breast cancer cells lines such as MCF-7 and MDAB468 use glucose and fructose in vitro. Godoy and coworkers found that ZR-75 breast cancer cells take up fructose. These cell lines express GLUT 5. The expression of the facilitative transporter coupled to the results to the functional analysis studies indicate that these cancer cells have the capacity to take up fructose in vitro.

It has been recorded that GLUT 5 is expressed at low levels in kidneys, erythrocytes and lung (Gould and Holman, 1993; Medina and Owen, 2002; Godoy et al.2006; Douard and Ferraris, 2008). This may be an explanation of the low level expression of this transporter in BHK cell lines which are kidney cells. This phenomenon becomes clearer with the understanding of the physiological role of kidney cells in any organism. The main function of the kidney is the excretion of waste products such as urea, uric acid and creatinine. In the course of this activity, the kidney regulates homeostasis through reabsorption of some key electrolytes and molecules such as glucose as dictated by the needs of the organism.

It is mainly in the kidneys proximal tubule that the sodium dependent glucose transporters are more active in reabsorption of glucose. GLUTS 5 are also expressed in these cells (Godoy et al. 2006). These transporters are thought to be responsible for fructose reabsorption by the kidneys back into the blood stream (Thorens, 1993). It has been

observed that fructose was reabsorbed actively by the renal tubular cells (Gammeltoft and Kjerulf-Jensen, 1943). Since kidney cells express GLUT 5, it would be expected to find an overexpression of GLUT 5 in malignant kidney cells. Godoy and coworkers found no expression of GLUT 5 in renal cell carcinomas while there was a marked reduction in GLUT 1 expression in these cells (Godoy et al. 2006).

Transport across the cell membrane is the first rate-limiting step for sugar metabolism in cells. One of the cellular alterations in normal cells infected with sarcoma viruses is an increased rate of uptake of glucose or other sugars compared to non-transformed cells. This corresponds to an increased expression of GLUTs and is typical of cancer cells (Macheda et al, 2005). Thus it is clear that up regulation of GLUT 5 expression occurs in the transformation process of MCF-7 and that the increase GLUT 5 levels and transport activity contributes to tumor growth. It is still to be determined whether the increased GLUT 5 expression in MCF-7 represents a cause or an effect of the malignant transformation process.

5. Conclusion

GLUT 5 was shown to be expressed by MCF-7 but not by Hela cell lines. BHK cell lines seem to display a limited expression of the GLUT 5 transporter. MCF-7 cells are well known and characterized estrogen receptor positive breast cancer cells. It is thus clear that MCF-7 use fructose in some form or other for cell growth and proliferation. GLUT 5 was observed to be expressed in this cell line more than in the other cell lines tested and thus should be considered as an important potential target for the development of imaging agents for breast cancer. The selective expression of this receptor would play a critical role in the success of a potential imaging agents designed to selectively bind to GLUT 5.

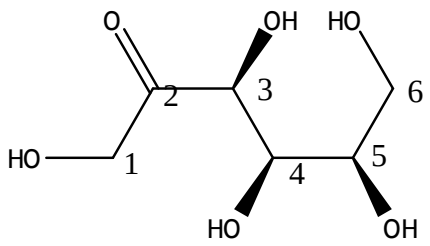
The results in this study will need to be correlated with *in situ* and *in vivo* laboratory experiments to confirm the importance of this particular fructose receptor. The exact role of this receptor in human cases of breast cancer as well as its importance is still to be elucidated. Future studies should focus on these points to maximize the chances of success for the possible radiotracer to be designed based on the receptor.

Chapter 6: Synthesis and characterization of 1-deoxy-D-Fructose

1. Introduction

For decades fluorinated carbohydrates have been widely utilized in biochemical investigations. The wide success of ^{18}F-fluorinated molecules such as ^{18}F-FDG has led to a growing interest in PET imaging resulting in many studies being conducted on the use of fluorinated sugars as radiotracers with various degree of success.

D-Fructose, a simple reducing sugar found in many foods is the second most abundant simple sugar in nature. D-fructose is one of the three most important blood sugars along with glucose and galactose. A fluorinated D-fructose derivative has been proposed as potential agent for PET imaging (Heaney and Hui, 2007). D- Fructose ($C_6H_{12}O6$) is a levo-rotatory monosaccharide and an isomer of glucose.

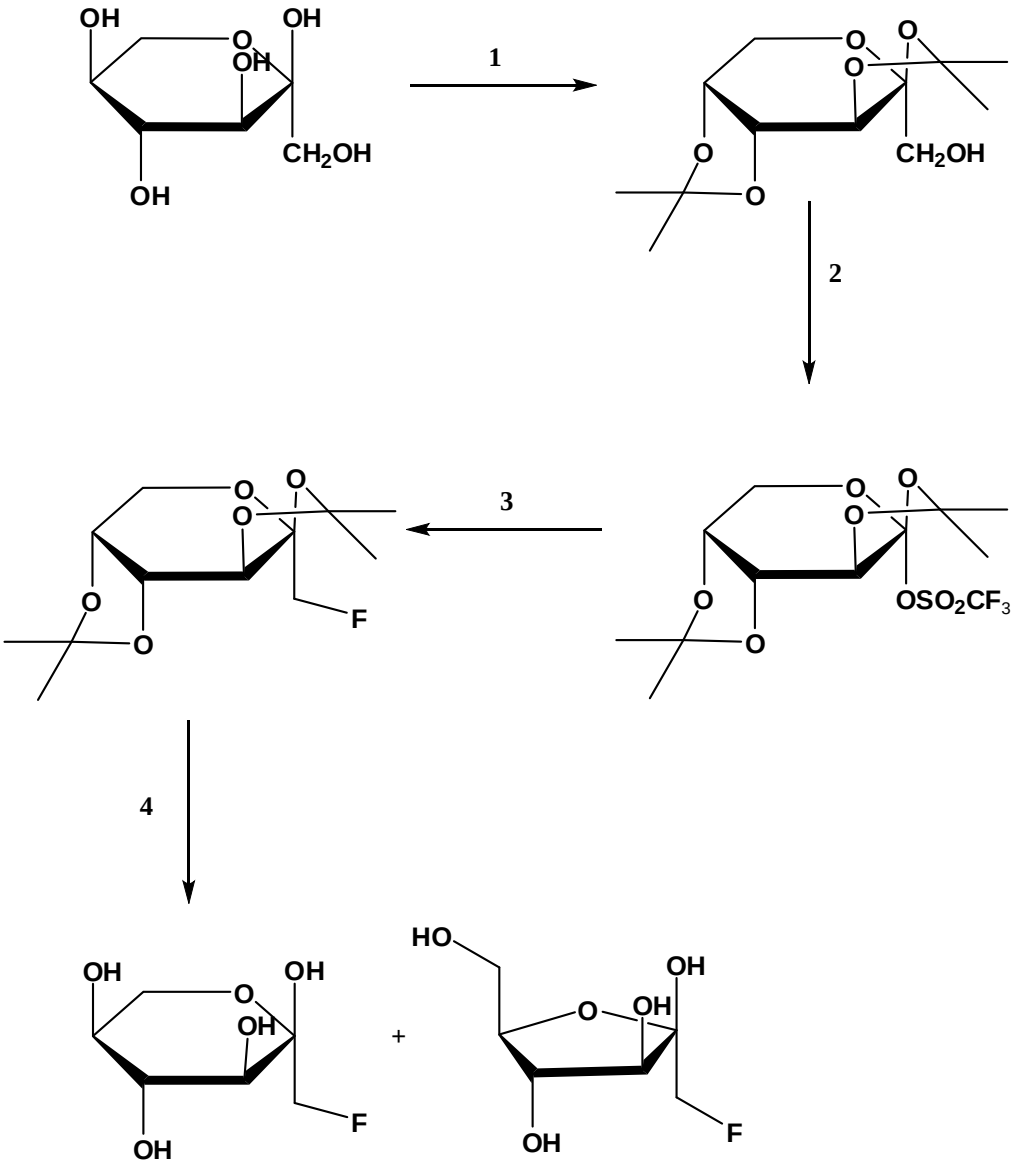


D-Fructose

Figure 13: Line drawing and atom numbering of D-fructose; Carbon atoms are represented by the Arabic numbers.

D-fructose is generally found in its furanose form (5-member ring). Tatibouet and coworkers (Tatibouet et al. 2000) found that both D-fructofuranoside and D-fructopyranoside forms are well tolerated by the GLUT 5 transporter. Synthesis of fluorinated sugars is both tedious and time consuming because of the prerequisite protection and deprotection steps. For D-fructose the C1 and C6 are the only two positions for which a replacement of the hydroxyl group by a halogen does not result in a disruption of the GLUT 5 transporter interaction (Tatibouet et al. 2000). However Trayner and coworkers observed that D-fructose fluorinated at position C6 resulted in the inhibition of GLUT 5 (Trayner et al. 2009). Fluorination at C1 remained thus the most likely option to have great potential as a radiodiagnostic compound (see Fig. 13). Therefore 1-deoxy-1-fluoro-D-fructose was the molecule of choice to be synthesized. The first reaction step in the production of 1-deoxy-1fluoro-D-fructose is a blocking or protection step where a ketal formation reaction is conducted to prepare a diisopropylidine product. Ketalation (isopropylidenation) is the most widely used reaction for the initial step of carbohydrates derivatization and many methods are known to accomplish this reaction. These isopropylidine groups acts as easily removable protecting groups for the hydoxyl groups that must not be altered during the reaction sequence (Fig . 14).

The formation of ketals by reacting D-fructose and acetone was one of the first condensation reactions to be described (Meunier, 1888) and is still the most widely used reaction for the initial step of carbohydrate derivatization. Nowadays the ketal protecting group is widely used with success. This is due to features such as: cheap reagents, the ease of protection with corresponding high yields, the inertness of the protection group to reagents used for structural modification and the ease of deprotection with corresponding high product yield (Hanessian, 1996). This route was chosen in this study for these reasons.



1-Deoxy-1-[F]-D-Fructose

Figure 14: The general reaction scheme followed during the production of 1-deoxy-1-[F]-D-Fructose.

Ketalation of D-Fructose happens under "acidic conditions" and is a direct condensation of the carbonyl derivative. An illustration of such a reaction mechanism is presented in Fig.15.

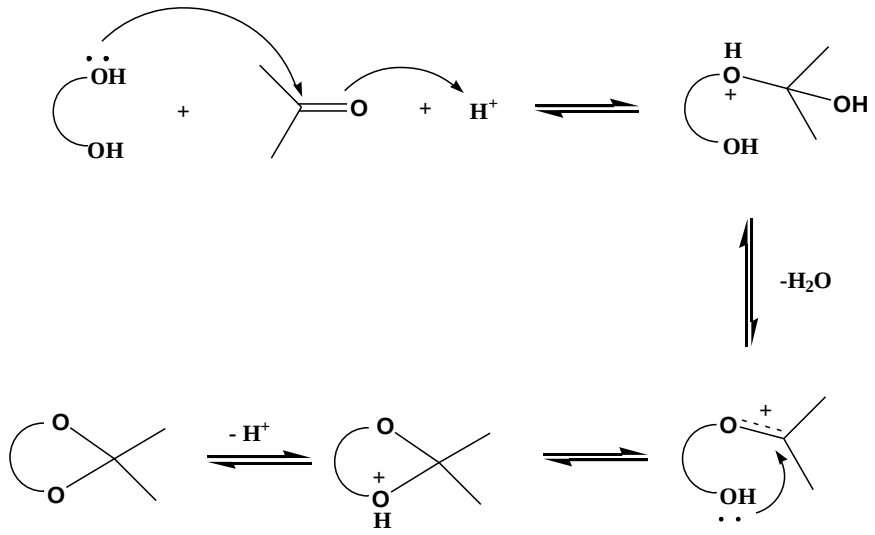


Figure 15: An illustration of a reaction mechanism during direct condensation of a carbonyl derivative with a vicinal diol group to form a ketal.

2. *Experimental*

Unless otherwise stated, the individual procedures were carried our using standard Schlenk line techniques. All solvent used were dried using standard procedures and all reagents were purchased from Sigma Aldrich. Dichloromethane was predried with $CaCl_2$ then distilled under reflux from CaH_2. D-Fructose was dried under vacuum over phosphorous pentoxide. Thin Layer chromatography (TLC) was performed on the Merck Kieselgel 60 F_{245} plates and visualized in an iodine chamber or with 5% anisaldehyde in sulfuric acid.

NMR spectroscopic data was acquired on a Bruker 300 MHz spectrometer. ^{1}H chemical shifts are reported relative to TMS using $CDCl_3$ as reference (7.24 ppm). ^{19}F-NMR spectra were obtained with trichlorofluoromethane as external standard.

FT IR spectra were obtained using KBr pellet on a Bruker Tensor 27 and reported in (cm^{-1}).

2.1. Synthesis of 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose *(F101)*

2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose, with no water scavenger.

The preparation was based on a method previously described (Brady, 1970). A cooled solution (0◦c) of sulphuric acid (3.4 ml) in dried acetone (100 ml) was added to a round bottom flask containing powdered D-Fructose (3.6 g, 20 mM). The reaction mixture was stirred at room temperature for 2 hours and cooled. An aqueous solution of sodium hydroxide (11 g in 70 ml) water was slowly added to the reaction mixture and left stirring for 5 min. The solution was freed of acetone by evaporation and the remaining slurry extracted with dichloromethane (3 x 50 ml). The dichloromethane was removed under *vacuo* to yield the product as a white precipitate. This product was dissolved and recrystalised in petroleum ether to yield slight yellow neddles (2.53 g); m.p. 89˚C (ether-pentane); lit.m .p. 97 ˚ C; 70% yield (See Fig .16 for the ^{1}H-NMR spectra of the product).

2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose, using $CuSO_4$ as a water scavenger.

The preparation was based on a described procedure with slight modification (Hanessian, 1996). Finely powdered dried D-fructose (10 g, 55.5 mmol) was added to an anhydrous acetone solution (100 ml) containing anhydrous $CuSO_4$ (9 g, 56.9 mmol). A solution of 5 ml anhydrous acetone containing 1.1 ml of H_2SO_4 was slowly added to the reaction mixture and left to stir for 24 hours. A swivel frit was packed with a neutralizing agent, CaOH (10 g), and the $CuSO_4$ filtered off as the reaction mixture passed over the CaOH layer and the frit itself. The acetone was removed in *vacuo* to yield a dark precipitate. The yellow

product was extracted with MeOH:Pentane (1:1) and the solvent removed in *vacuo* (See Fig.17 for the ^{1}H-NMR spectra of the product). 2:3, 4:5-Di-O-isopropylidene-ß-D-fructopyranose was obtained as colorless needles (7.48 g): m.p. 94 ° C (ether-pentane); lit.m .p. 97◦C; yield 75%.

2.2. Preparation of 2,3:4,5-di-O-isopropylidene-1-O-(trifluoromethanesulfonyl)-ß-D-fructopyranose (F102)

The procedure was based on a method previously reported (Card and Hitz,1984). To a solution of 2,6-di-tert-butyl-4-methylpyridine (1.13 g, 5.50 mmol) in dry dichloromethane (20 ml), was added drop wise, under stirring, redistilled trifluoromethanesulfonic anhydride (1.44 g, 5.10 mmol), which resulted in the formation of a precipitate. To this stirred mixture was added drop wise a solution of 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose (F101) (0.663 g, 2.55 mmol) in dichloromethane (10 ml). The resulting slurry was stirred, and the reaction was monitored at 5 min intervals by t.l.c. (1:1 hexane-ethyl acetate). After 30 min when all of F101 had reacted, ice-cold water (30 mL) was added, and the mixture was extracted with dichloromethane (8 x 20 mL). The combined organic extracts were dried ($MgSO_4$), and the solvent was evaporated at 40◦C *in vacuo*. Column chromatography of the crude product (silica gel, 8:2 hexane-ethyl acetate) and evaporation of the solvent from the appropriate fractions gave F102 (0.761 g) as clear, yellow syrup; recrystalization in dichloromethane afforded clear/purple crystals; m.p. 89◦C; lit.m .p. 85-95◦C; yield 53%.

2.3. Preparation of 1-Deoxy-1-Fluoro-D-Fructose (F104)

A slight modification was performed to a previously described method (Haradahira etal. 1995). 2,3:4,5-di-O-isopropylidene-1-[(trifluoromethylsulfonyl)oxy]-D-fructopyranose (1.83 g-4.67mM) was reacted with tetrabutylammonium fluoride (20 ml - 1M solution in dichloromethane) at room temperature for 1 hour and subsequent purification by silica gel column chromatography with n-Hexane:Ethyl acetate (8:1) gave 1-deoxy-1-fluoro-

2,3:4,5-di-O-isopropylidene-D-fructopyranose (F103) as a yellow syrup. Recrystallization in dichloromethane gave clear white crystals. Deprotection of the obtained intermediate was accomplished by treatment with 1.5 mL of 90 % (v/v) trifluoroacetic acid (CF_3COOH) at room temperature for 1 hour. The acid was removed under reduced pressure and the residue chromatographed on silica gel with chloroform:methanol (15:1) to give pure 1-deoxy-1-fluoro-D-fructose (F104; 1.02g) as yellow syrup (which eventually crystallized as sharp needles). m.p. 90 ° C; lit: not determined; 55.7 % yield.

3. Results and Discussion

1-deoxy-1-fluoro-D-fructose was successfully synthesized. TLC, IR and NMR data correspond to those reported in the literature (Haradahira et al. 1995; Lopez and Gruenwedel, 1991). These are summarized in Fig.16 & 17 and 19-26 as well as in Table 2 and 3.

2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose (F101)

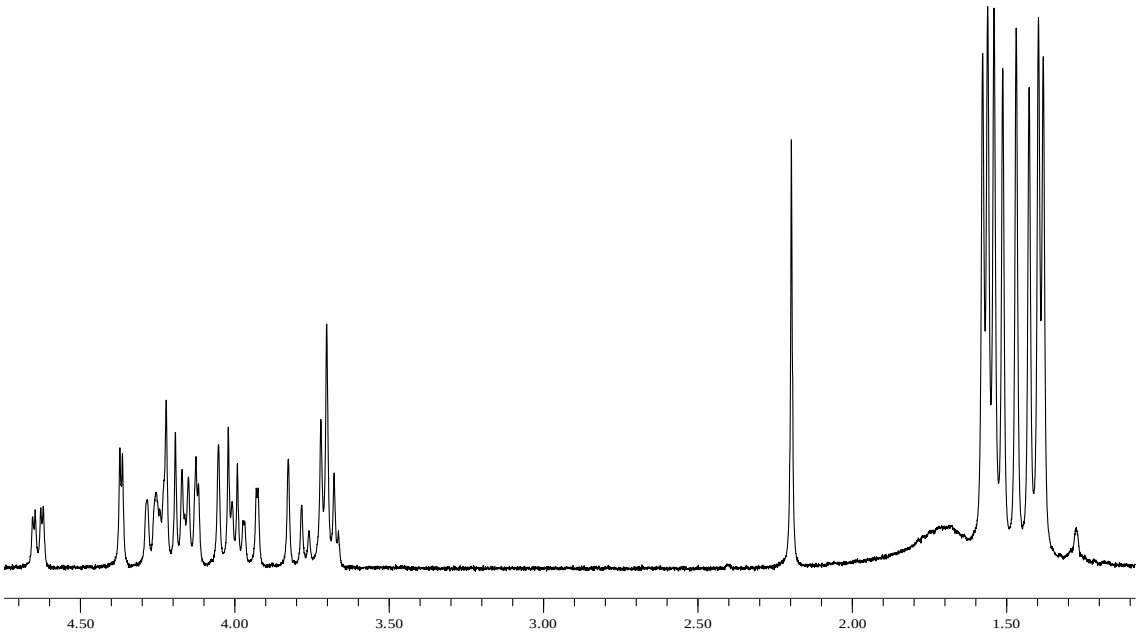


Figure 16:1H-NMR spectra displaying the impure product obtained from D-Fructose ketalation in the absence of a water scavenger (1.6-1.30ppm)

Preparation of pure 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose is a very important step in the synthesis of 1-deoxy-1-fluoro-D-fructose. The synthesis and purification of 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose was found to be more problematic than initially expected. A reason for this was that the synthetic procedure initially used was based on a procedure described by Brady (1970). To synthesize pure 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose using acidic acetonation according to the method 1,2:4,5-di-O-isopropylidene-ß-D-fructopyranose is thought to be formed first. This

compound then isomerizes to 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose depending on the concentration of the acid. Careful selection of the reaction conditions permits the synthesis of either isomer in satisfactory yields. Acid concentrations above 0.5% and shorter reaction time are said to favor the formation of a mixture solution of the diketals where 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose is predominant. It is also important to note that for each mole of reagent, 2 moles of water are produced per mole of di-blocked fructose produced in the reaction. These water molecules should be removed continuously due to the fact that the hydrolysis of the acetal reaction is reversible (Fig.16). This reaction can however be conducted without azeotropic distillation or a desiccant, but the use of either would lead to various by-products and hydrolysis of the formed ketal groups. This is clearly noticeable on the ^{1}H-NMR spectra (Fig.16) as eight strong signals are present in the methyl region of the spectra.

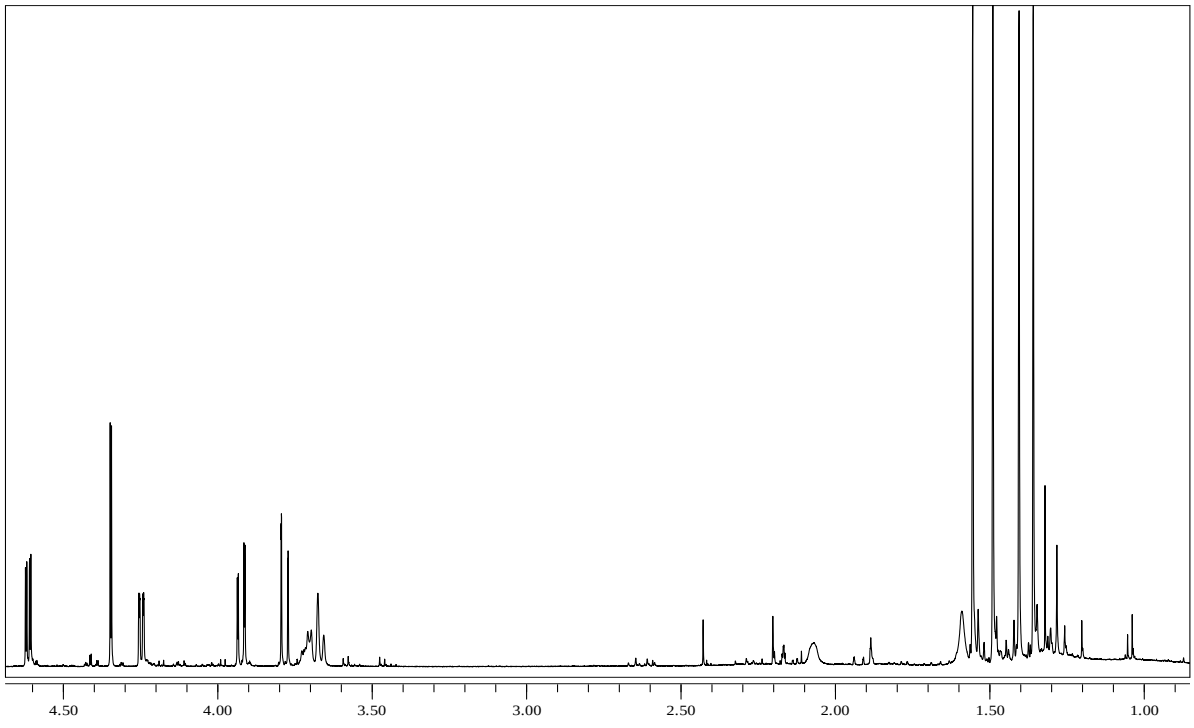


Figure 17: 1H-NMR spectra displaying the pure product obtained from D-Fructose ketalation when a water scavenger is added to the reaction mixture(1.6-1.30ppm).

A synthetic approach based on a procedure by Hanessian was therefore attempted to solve this problem (Hanessian, 1996). 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose was prepared using a method that included anhydrous copper sulfate as a desiccant to absorb the water molecules produced during the reaction. This procedure yielded pure 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose as noted on the ^{1}H-NMR spectra (Fig. 17) with only four strong signals in the methyl region of the spectra.

After numerous attempted synthesis and purification methods pure 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose was finally produced. This was achieved by conducting the reaction under standard Schlenk line conditions (dry conditions) and by adding a desiccant to the reaction mixture. The desiccant removes the formed water molecules and limits the formation of by-products and ketal hydrolysis.

Once pure 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose was synthesized in the desired purity and yield the next step in the production of 1-deoxy-1-[F]-D-fructose could be attempted.

2,3:4,5-di-O-isopropylidene-1-O-(trifluoromethanesulfonyl-oxy)-ß-D-fructopyranose (F102)

Various sulfonic esters including the trifluoromethyl sulphonic ester (Triflate) are useful groups of carbohydrates derivatives in synthetic organic chemistry due to the kinds of reactions which they can undergo (Tipson, 1953). An *ester of a sulfonic acid* ($R'SO_3H$) usually undergoes *alkyl-oxygen* fission resulting in the transitory formation of a carbonium cation. This kind of behavior towards nucleophilic reagents is the basis for many transformations which can be brought about with sugar derivatives through reaction of their sulfonic esters.

The activation for nucleophilic displacement at position C-1 of 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose by preparing a triflate derivative is a sensitive process. Tertiary nitrogenous bases such as pyridine, quinoline and diethylamine have been used in the formation of sulfonic esters of alcohol. This is conducted in the presence of extraneous, dry inert or non-polar solvents such as dichloromethane.

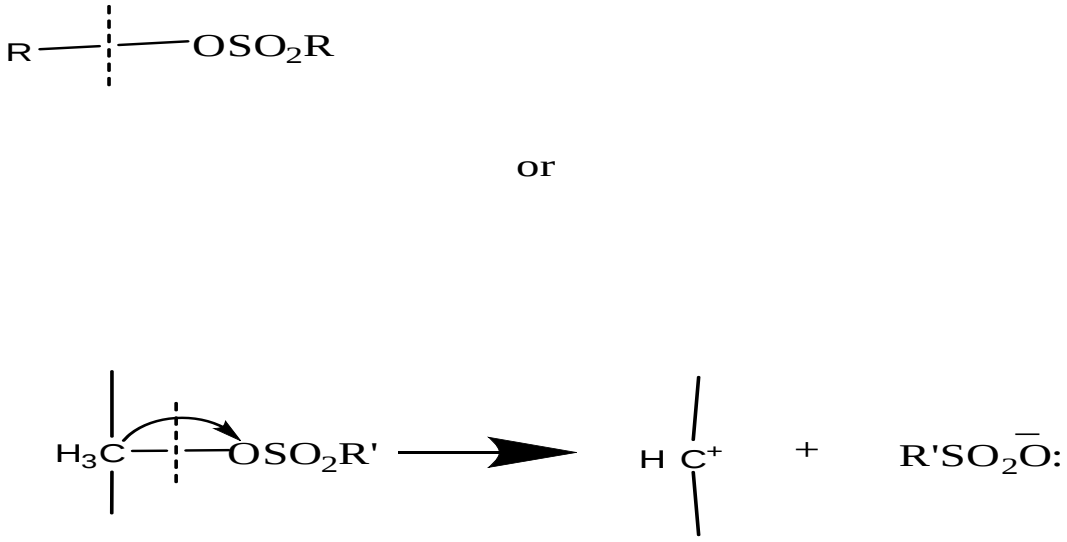


Figure 18: Alkyl-oxygen reaction of an ester of a sulfonic acid ($R'SO_3H$)

Unfortunately in the presence of pyridine (an organic base as initially used in this experiment) at least four types of side reaction (usually undesired) can occur (Tipson, 1953);

- Ether formation: This mainly occurs under drastic or prolonged conditions and results in intermolecular formation of inner cyclic ether (dehydration).
- Formation of pyridinium quaternary salts: Mainly account for poor yields and is most likely to occur when the carbohydrate to be sulfonylated has a free group at

carbon atom 1 or if the group is already substituted by a halogen atom which although unlikely could have happened during the experiments.

- Alkyl-chloride formation or "Chlorination": This is the most important type of side reaction as far as carbohydrates are concerned. The use of low temperatures during the reaction can help prevent this.
- Double bond formation: This arises from the interaction of an active hydrogen atom with a vicinal sulfonyloxy group particularly on heating a tertiary nitrogenous base.

Sterically hindered base such as 2,6-di-tert-butyl-4-methylpyridine as a non-nucleophilic proton acceptor has been successfully used to circumvent base-adduct formation during sulfonation (Lopez and Gruenwedel, 1991). In addition, cooling retards the reaction sufficiently that a greater portion of the primary hydroxyl groups are targeted by the sulphonating agent while drop wise addition of the base has been shown as a successful technique to eliminate the formation of pyridinium salts (Tipson, 1953). Taking all these points into consideration in the design of the synthesis procedure, allowed successful synthesis of 2,3:4,5-di-O-isopropylidene-1-O-(trifluoromethanesulfonyl-oxy)-ß-D-fructopyranose (F102) as seen in Table 3, Fig.22 and Fig.23.

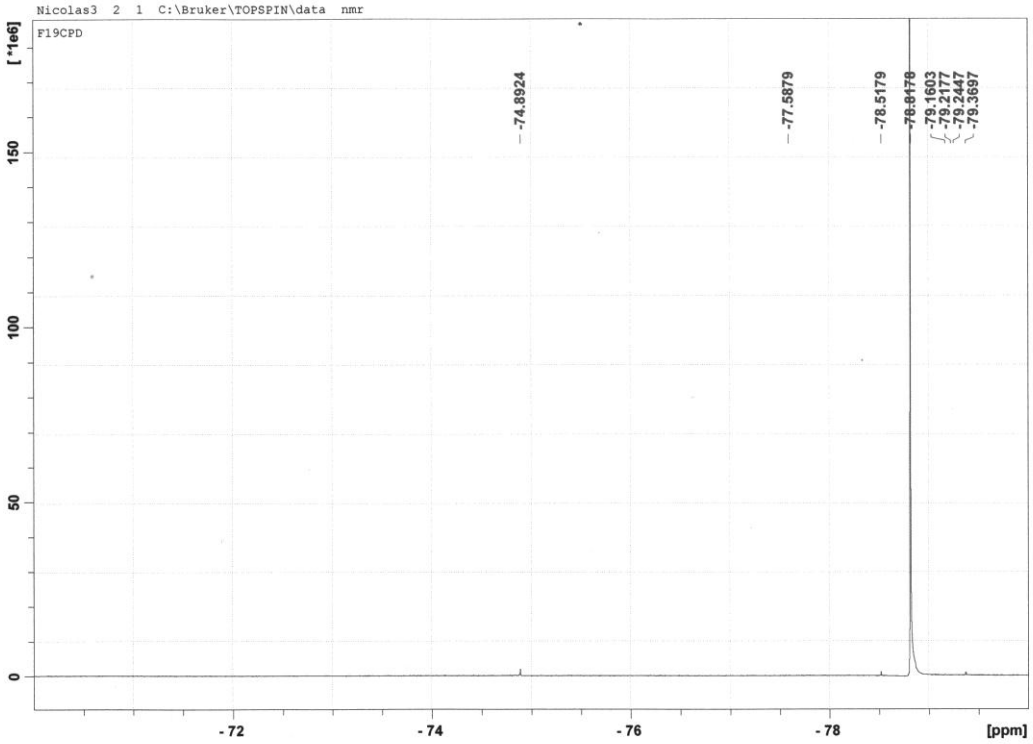


Figure 19: ^{19} F NMR spectra of 1-deoxy-1-fluoro-2,3:4,5-di-O-isopropylidene-D-fructopyranose (F103)

1-deoxy-fluoro-2,3:4,5-di-O-isopropylidene-D-fructopyranose (F103) and 1-deoxy-1-Fluoro-D-Fructose (F104)

It is accepted that deoxy and deoxy fluoro sugars can be substituted for natural monosaccharides in a variety of reactions. Their success is based on the fact that covalent fluorine might prove a better hydroxyl analogue than hydrogen in enzyme-analogues interactions suggested by the similarity in electro-negativity and size of the fluoro- and hydroxyl groups. It has been demonstrated that covalently bound fluorine, like hydroxyl group can participate in hydrogen bonding (Einsenthal, 1972). Such sugar analogues have attracted interest as potential agents for PET imaging. This increased importance of

fluorinated sugars has made it imperative to investigate new routes of synthesis of this class of compounds. Fluorination of carbohydrates is a complex and complicated procedure. Two routes: nucleophilic or electrophilic displacement reactions, are used to this purpose. Electrophilic fluorination refers to the addition of fluorine atoms across a double bond producing a difluoro derivative of the parent compound (Yu, 2006). Nucleophilic substitution is a chemical reaction involving the addition of a nucleophilic molecule (highly negatively charged molecule) into a molecule with a suitable leaving group (Yu, 2006). Nucleophilic displacement reactions are preferred to electrophilic reactions due to the short-comings in controlling the reaction conditions and reactivity of free fluorine in an electrophilic displacement (Schlyer, 2004). The main reaction in a nucleophilic fluorination displacement reaction is the S_N2 reaction or bimolecular nucleophilic substitution (or backside attack). In an S_N2 a lone pair form a nucleophile attacks an electron deficient electrophonic center (most often at an aliphatic sp^3 carbon center) and binds to it, expelling another group called a leaving group. Thus the incoming fluorine containing group replaces the leaving group in one step. As a result, the nucleophilic molecule forms a covalent bond with the parent molecule and displaces the leaving group. The stereo-configuration of the parent molecule is also changed. This reaction is slow, rate limiting and dependent on the solvent in which the reaction is carried out, the steric hindrance of the parent molecule, the basicity of the leaving group as well as the size of the nucleophile. The choice of the leaving group depends on the yield, stability of precursors, ease of subsequent separation of the fluorinated product from the precursors and the formation of potential side products (Schlyer, 2004). Trifluoromethane esters (triflates) are frequently used due to their reactive ability and excellent yield in nucleophilic reactions. These groups have considerably increased the synthetic usefulness of the displacement reactions involving sulfonates esters in carbohydrates systems. Other leaving groups such as cyclic sulfates, mesylates and

tosylates tend to give lower yield due to the harsh and destructive reactions conditions required (Haradahira et al. 1995).

Many nucleophilic and electrophilic fluorinating agents have been used with varying degrees of success. The choice of the fluorinating agents is based on whether the reaction is aimed at being nucleophilic or electrophilic, the desired reaction procedure conditions, formation of by-products as well as the subsequent purification of the final product. Hydrogen fluoride, diethylaminosulfur trifluoride (DAST), metal fluorides (such as silver fluoride, zinc fluoride, trifluoromethyl zinc bromide) and tetrabutylammonium fluoride (TBAF) have been used for nucleophilic fluorination of sugars (Yokoyama, 2000). In this study TBAF was used due to its known effect in previous studies (Haradahira et al. 1995).The resulting product (1FDF) was obtained with relatively good yield (55%) suggesting successful fluorination and deprotection reactions.

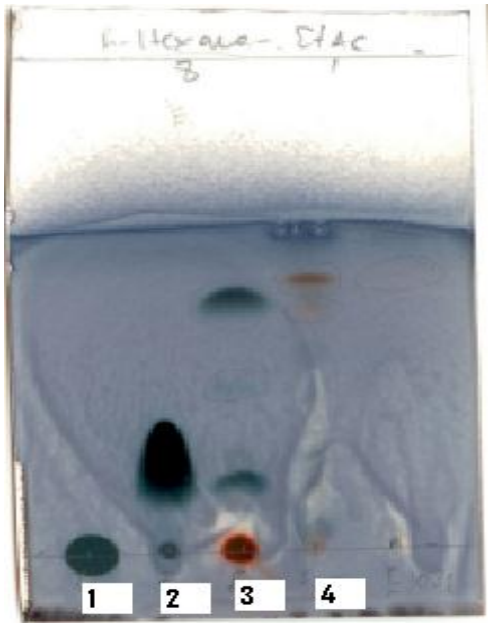


Figure 20: TLC of D-fructose derivatives; Mobile Phase: Hexane: Ethyl Acetate (8:1); visualizing agent: 5% Anilsadehyde in Sulfuric Acid ; 1) D-fructose Rf 0.0 ;2)2,3:4,5-di-O-isopropylidene-D-fructopyranose (F101) Rf 0.4 ; 3) 1-deoxy-fluoro-2,3:4,5-di-O-isopropylidene-D-fructopyranose(F103) Rf 0.9 and 4) 1-deoxy-1-Fluoro-D-fructose (F104) Rf 0.97.

Table 2. TLC characterization of steps in the synthesis of 1-deoxy-1-[F] Fluoro-D-fructose

Step in the synthesis	Mobile phase	Identification (Rf)	Note (5% Anilsadehyde in Sulfuric Acid)
D- Fructose	n-Hexane: EtAc (8:1)	0.0	Dark/blue spot
Step1 (F101)	n-Hexane: EtAc (8:1)	0.52	Dark/blue spot
Step2 (F102)	n-Hexane: EtAc (8:1)	0.97	Yellow/bleached
		0.0	Purple
		0.3-0.4	Dark/blue
	Chloroform: Methanol (15:1)	0.1	Yellow/bleached
Step 3			
After fluorination (F103)	n-Hexane: EtAc (8:1)	0.90	Dark/blue
		0.55	Dark/blue
	Chloroform: Methanol (15:1)	0.28	Yellow/bleached
After deprotection (F104)	n-Hexane: EtAc (8:1)	0.97	Grey/Orange
	Chloroform: Methanol (15:1)	0.55	Yellow/bleached

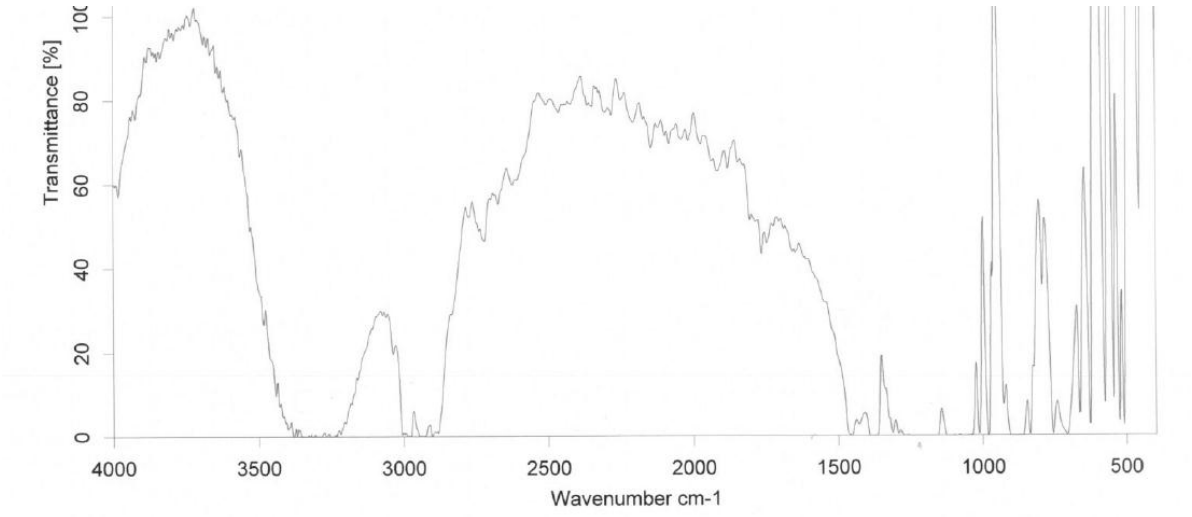


Figure 21: IR spectra of 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose (F101)

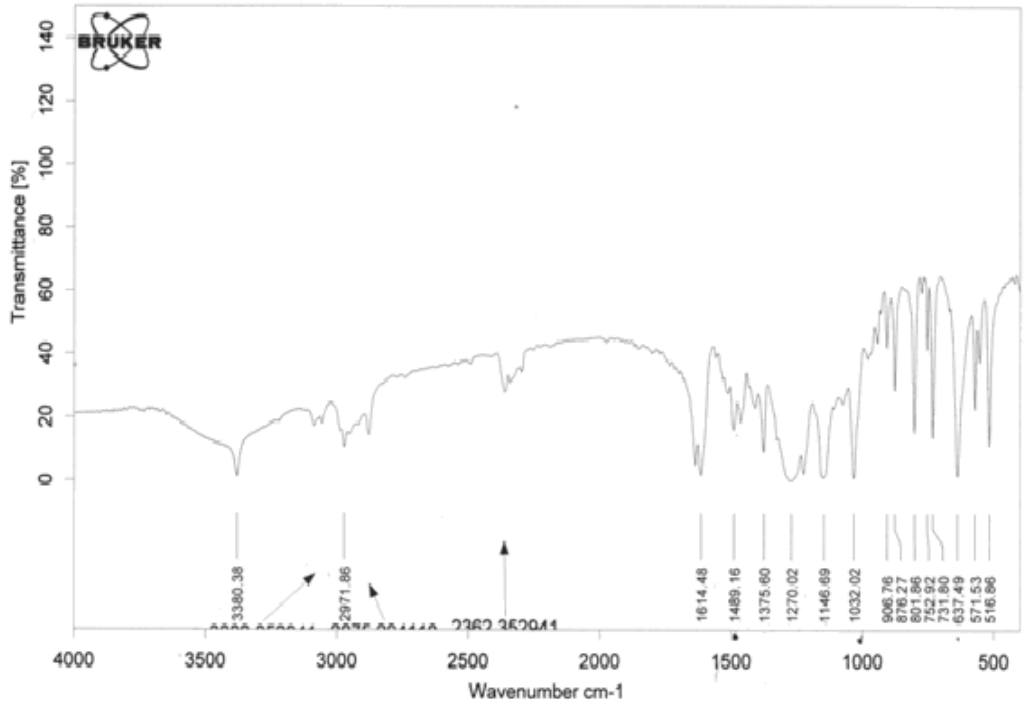


Figure 22: IR spectra of 2,3:4,5-di-O-isopropylidene-1-[(trifluoromethylsulfonyl)oxy]-D-fructopyranose(F102);

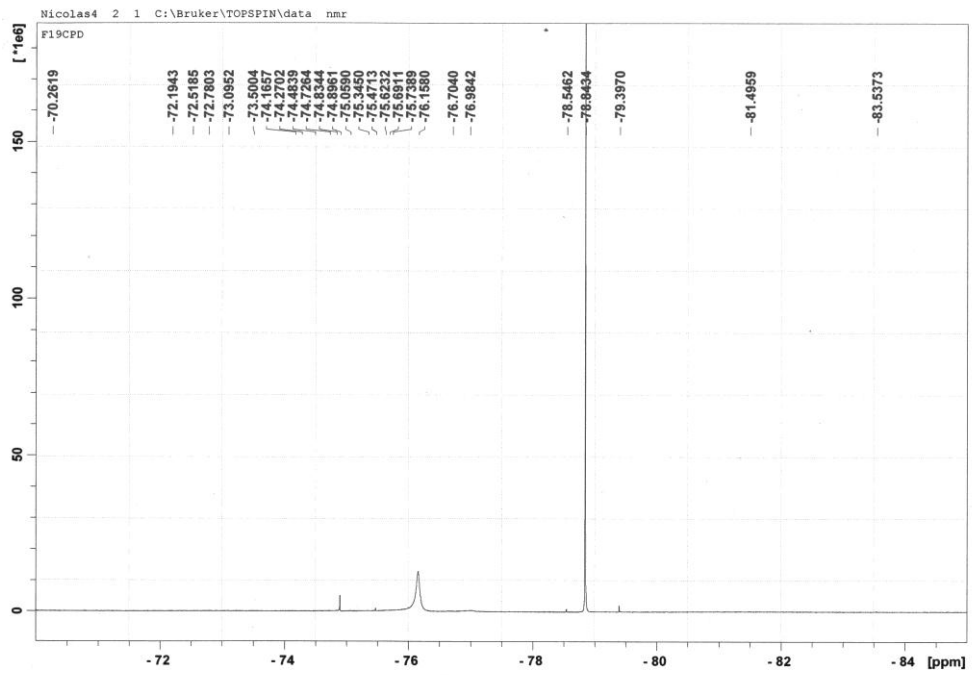


Figure 23: ^{19}F NMR spectra of the 2,3:4,5-di-O-isopropylidene-1-[(trifluoromethylsulfonyl)oxy]-D-fructopyranose(F102).

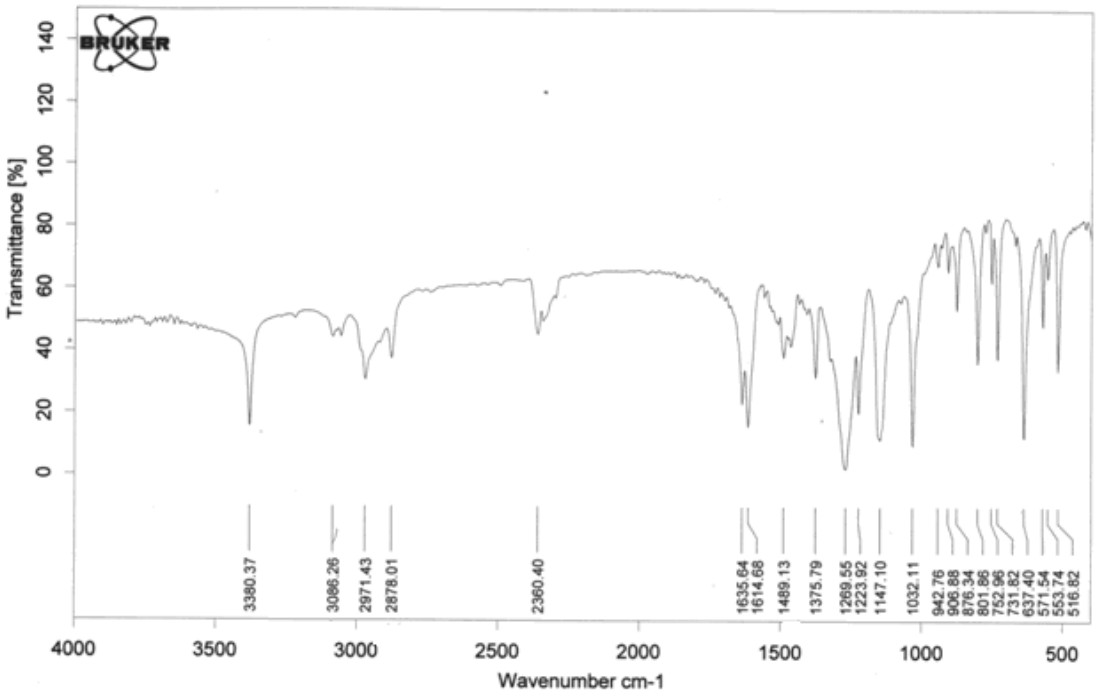


Figure 24: IR spectra of 1-deoxy-fluoro-2,3:4,5-di-O-isopropylidene-D-fructopyranose(F103).

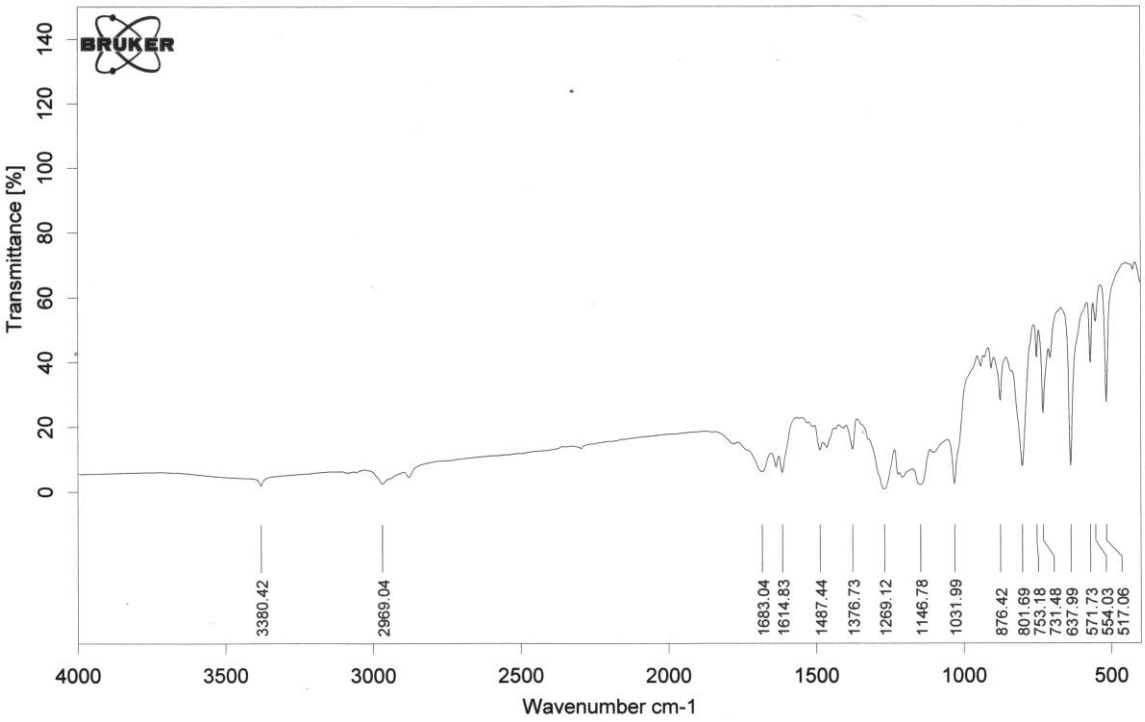


Figure 25: IR spectra of the pure 1-deoxy-1-Fluoro-D-fructose (F104).

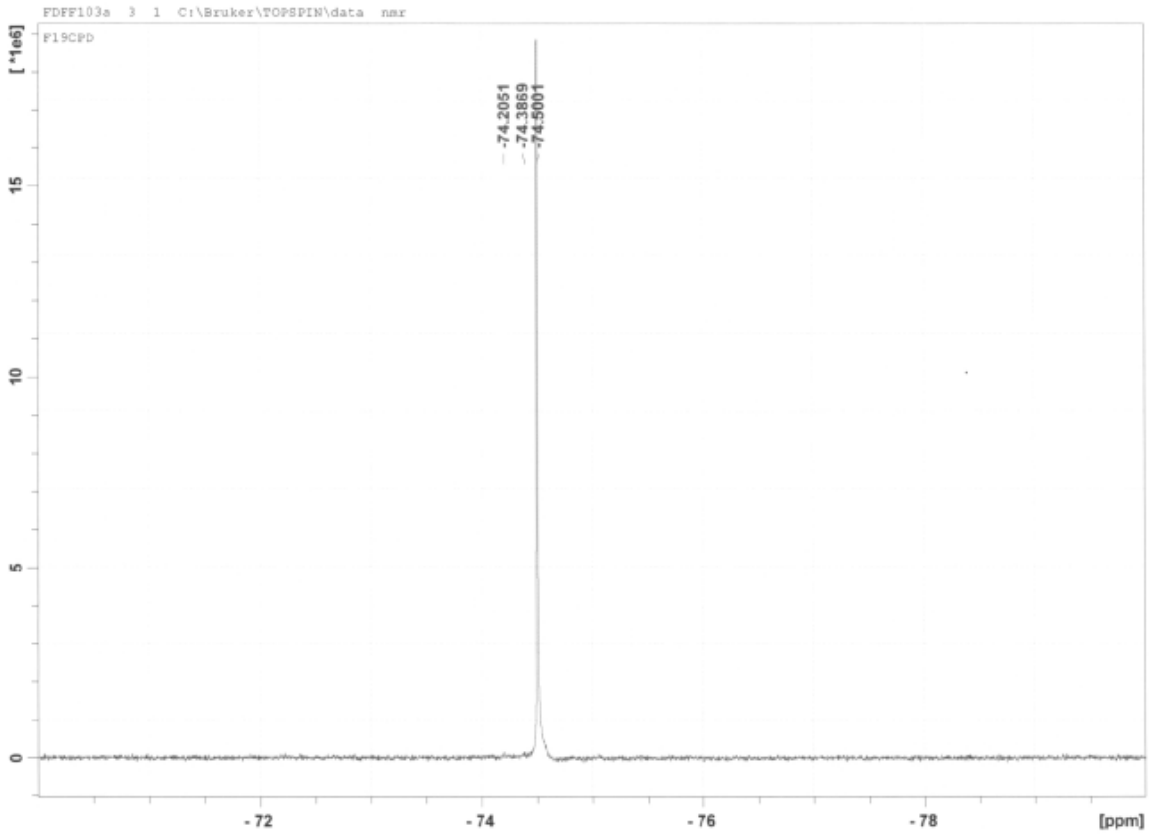


Figure 26: 19 F NMR spectra of the pure 1-deoxy-1-Fluoro-D-fructose (F104).

Table 3. IR characterization of steps in the synthesis of 1-deoxy-1-[F] Fluoro-D-fructose

Step in Synthesis	IR peaks(cm^{-1})	NMR peaks (δppm)	Cited in Literature
2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose(F101)	3325(OH) 3000-2800 (CH) 1379 (CMe_2)	Nd	(Lopez and Gruenwedel, 1991)
2,3:4,5-di-O-isopropylidene-1-[(trifluoromethylsulfonyl)oxy]-D-fructopyranose(F102)	No (OH) 3000-2800(CH) 1381 (CMe_2) 1418, 1246,1208, 1145,1071,980,609 All (OSO_2CF3:CF,SO_2)	Nd	(Card and Hitz,1984) (Lopez and Gruenwedel,1991)
1-deoxy-1-fluoro-2,3:4,5-di-O-isopropylidene-D-fructopyranose(F103)	No (OH) 3000-2800(CH) 1381 (CMe_2) 1418, 1246,1208, 1145,1071,980,609 All	^{1}H-NMR(500MHz,DMe) 1.43(dddd,$^3J_{H-F}$ =78.0Hz,J_{H-H} =41.5,28.15,16.35Hz);260 (dd, $^3J_{H-H}$ =40.6Hz,J_{H-H}=19.9Hz);3.15(ddd, $^2J_{H-H}$ =6.55, $^2J_{H-H}$ =3.3, $^2J_{H-H}$ =43.15Hz),5.32(ddd, $^2J_{H-F}$ =24.2Hz, $^2J_{H-H}$ =4.75Hz,2.15Hz) ^{19}F NMR (370 MHz) -78.84ppm	(Card and Hitz, 1984;Haradahira et al.1995)

Table 4. IR characterization of steps in the synthesis of 1-deoxy-1-[F] Fluoro-D-fructose (continued)

1-deoxy-1-fluoro-D-fructose (F104)	3380(OH) 3000-2800 (CH) No 1381 (CMe_2) 1418, 1246,1208, 1145,1071,980,609 All	^{1}H-NMR(500MHz,DMe) 1.43(dddd,$^{3}J_{H-F}$ =78.0Hz,J_{H-H} =41.5,28.15,16.35Hz);260 (dd, $^{3}J_{H-H}$ =40.6Hz, J_{H-H} =19.9Hz);3.15(ddd,$^{2}J_{H-H}$ =6.55, $^{2}J_{H-H}$ =3.3, $^{2}J_{H-H}$ =43.15Hz),4.29(dd,$^{1}J_{o-H}$=3.25),5.32(ddd, $^{2}J_{H-F}$ =24.2Hz, $^{2}J_{H-H}$ =4.75Hz,2.15Hz) ^{19}F NMR (370 MHz) -74.34ppm(dd)	(Card and Hitz, 1984;Haradahira et al.1995)

4. Conclusion

1-deoxy-1-fluoro-D-fructose has been successfully synthesized. Despite literature recording instances where relatively pure 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose was obtained without the use of a water scavenger, the use of a water scavenger in the synthesis of 2,3:4,5-di-O-isopropylidene-ß-D-fructopyranose proved to have a very important effect on the purity and yield (increased by about 30%) of the final product. A sterically hindered base (2,6-di-tert-butyl-4-methylpyridine) was successfully used as a non-nucleophilic proton acceptor to circumvent base-adduct formation during sulfonation that would have been caused by pyridine. It also helped to efficiently eliminate pyridine residue in the final product resulting in improved yield in the synthesis of the triflate derivative : 2,3:4,5-di-O-isopropylidene-1-[(trifluoromethylsulfonyl)oxy]-D-fructopyranose. TBAF was used with relatively good success as a nucleophilic agent. Other nucleophilic fluorinating agents could be studied for their effect on yield and purity of the final product. This fell out of the scope of the present work. The obtained product of each steps in the synthesis procedure compared well with the published results indicating that the few changed introduced to the procedures were successful in giving products of higher purity in relatively good yield. Future work should consider other precursors and possible fluorination using other nucleophilic agents.

Chapter 7: General Discussion and conclusion

Cancer is the uncontrolled growth of abnormal cells anywhere in a body. In cancer growth, functional changes such as altered metabolism, proliferation, invasiveness and metastatic potential that produce a chaotic but complex situation have been detailed (Hanahan et al.2011; Gatenby and Gilles, 2008; Hanahan et al. 2000). Such information gathered through a systematic analysis of growth mechanism in cancer cells can be used in a beneficial manner in the fight against cancer, either to help treat the disease or to provide better means of diagnosis. This ultimately helps to effectively deal with the disorder. This work aimed at contributing to this objective through the study of GLUT 5 transporter.

The proliferation study using glucose free medium demonstrated that only the MCF-7 cell line can grow in D-fructose supplemented media. Hela and BHK cell lines did not show the same ability to proliferate in fructose enriched glucose free media. It was observed that proliferation in the presence of glucose in MCF-7, Hela and BHK cells was significantly greater than when using D-fructose at any of concentrations tested, the MCF-7 cell line had better ability to proliferate in media containing only D-fructose. This finding suggests some role played by the fructose specific transporter, GLUT 5. Potentially breast cancer cells, in the present case, MCF-7 cells, can use D-fructose in energy production and cell growth. MCF-7 cells are thus of great interest for any future study of the application of D-fructose to the imaging of cancer.

The ability of fructose to facilitate cell growth in the absence of glucose was further confirmed by the 3D colony formation study. Determining the potential of fructose to cause anchorage-dependent cell growth as assayed by the 3D soft agar assay is very important. The question it answered was whether once in a fructose rich medium in the

absence of glucose, cells were prone to induced anchorage dependent growth in cells that otherwise would not form colonies on their own (as mimicked by the controls). No major changes to the cells ability to form colonies could be attributed to the enrichment of the media with D-fructose. No significant differences were observed among the three cell lines studied (Hela, MCF-7 and BHK) in their ability to cause anchorage-dependent cell growth at the 3 concentrations of fructose used. These observations suggest that D-fructose cannot directly be associated with colony formation; it thus cannot directly be linked to aggressive forms of carcinogenesis. This is despite the observed evidence of its involvement in cell proliferation and energy consumption as seen in the cell proliferation assays.

Antibodies are renowned for their ability to specifically bind to a predetermined target. This property has been extensively used in medicine to deliver specific drugs or to counter the ability of a molecule to bind to its receptor. In either case the intended effect is the result to a certain extent of the binding of the antibody to its target. Anti-GLUT 5 antibodies were used to potentially bind to the GLUT 5 transporter with the aim of blocking it, thus preventing or reducing fructose uptake into the cell. The expected result was a decrease in cell proliferation and potentially cell death. The addition of anti-GLUT 5 antibody to the growing cells *in vitro* demonstrated that anti-GLUT 5 antibodies have no inhibitory effect on MCF-7 cell proliferation at either of the two tested concentrations of antibodies (0.1 and 0.5 µg/mL). A small decrease in cell proliferations was observed at the 1 µg/mL concentration of antibody but this did not afford any statistical significance. Concentrations above 1µ g/mL could be investigated in future and may potentially result in better inhibition of cell proliferation. This however may not be of any major clinical relevance due to the high cost that such a treatment would necessitate in humans. In future, possible combination of anti-GLUT 5 antibodies with a well-known cytotoxic drug could be investigated for their potential synergistic cytotoxic effect on MCF-7 cells.

Cancer cells are thought to have an increase metabolic rate compared to non-malignant cells. GLUT 5 was observed to be highly expressed by MCF-7. In the case of MCF-7, an over-expression of GLUT 5 is indicative of its ability to make use of fructose as an alternative source of energy with better ability than non-malignant breast cells. Not only did MCF-7 grow better under fructose enriched medium but they did so under conditions where BHK and Hela cells were not growing at all! This observed expression of GLUT 5 in breast cancer cells makes this transporter an important target for imaging of breast cancer and a possible therapeutic target for antibody targeted therapy of breast cancer.

For future studies, different types of breast cancer cell lines (i.e. MDA-MB-468, MDA-MB-231) as well as normal breast cells (e.g. MCF10A) should be included in the study to effectively evaluate GLUT5 expression, the effect of anti-GLUT5 antibodies and the influence of glucose/fructose regiment on energy metabolism of these cell lines.

2,3:4,5-di-O-isopropylidene-β-D-fructopyranose was synthesized with the use of a water scavenger in the synthesis of 2,3:4,5-di-O-isopropylidene-β-D-fructopyranose in good purity and high yield. The water scavenger increased the yield while decreasing the level of contaminants.

The use of 2,6-di-tert-butyl-4-methylpyridine (which is a sterically hindered base) was successful in efficiently eliminating pyridine reaction products in the final fructose product resulting in improved yield for the synthesis of the triflate derivative: 2,3:4,5-di-O-isopropylidene-1-[(trifluoromethylsulfonyl)oxy]-D-fructopyranose. For any organic synthesis it is imperative to obtain products of the best possible quality at the end of each step during the synthesis before moving on to the next.

The obtained product of each of these steps in the synthesis procedure compared well with the published results indicating that these changes introduced to the procedures were successful in giving products of higher purity in relatively good yield. The synthesis

of the non-radiolabelled fructose derivative paves the way to that of a radiolabelled product to be used as imaging agent with a single step reaction and a deblocking step which are both achieved sequentially in a single pot reaction scheme without the requirement for a cleanup step between these two reactions.

Many radiotracers are used for radiolabelling in nuclear medicine. For PET imaging only 4 possible radioisotopes can be used for the labeling of imaging tracers: carbon 11 (half-life=20 min), nitrogen 13 (half-life=10 min), oxygen 15 (half-life=2 min) and fluorine 18 (half-life=110 min). Fluorine 18 is preferred due to its relatively long half-life (110 min) making it the PET radiolabel of choice. The synthesis of 1-deoxy-fluoro-D-fructose in high yield using a method that is conducive to radiolabelling with ^{18}F was a very important step in this process. The way forward would require the synthesis and study of the fluorine [18] derivative of 1-deoxy-fluoro-D-Fructose in both primary cell cultures and in small animals to establish what the background activity of the 1-deoxy-fluoro [18]-D-fructose imaging abilities, organ specificity, metabolism and kinetics *in vivo*.

The use of radiolabelled-fluorinated D-fructose derivatives as PET theranostic agent for breast cancer has tremendous clinical application. But a recent immunohistochemistry study (only available after the completion of the present study) has observed that GLUT 5 is not over-expressed in most breast cancer tissues (Gowrishankar et al. 2011). This study however found that fructose was "a marker that distinguishes breast cancer cells from normal cells" (Gowrishankar et al. 2011). A comprehensive study evaluating the expression of GLUT 5 in patient tissue samples is needed before the synthesis of a radiolabelled fructose derivative is undertaken.

To be effective and successful an imaging agent should selectively target one specific receptor among the many complex and interrelated pathways involved in the growth of cancer. For 1-deoxy-fluoro-D-fructose, there is still the need to determine exactly what intracellular metabolic pathways will be used and to what extent. The real power of non-

invasive molecular imaging methods in clinical decision-making processes will be seen when multiple biologic processes of the tumor in a given patient are specifically and selectively targeted using a set of tracers that will give enough information about the pathway being targeted. In the case of breast cancer, a possible approach is to target GLUT 5, using a radioactive fluorine D-fructose derivative suggesting once more that PET with $^{18}F^-$ has many more alleys yet to be fully explored.

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