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ACS Chem. Biol., **Just Accepted Manuscript** • DOI: 10.1021/acschembio.0c00228 • Publication Date (Web): 15 Sep 2020

Synthetic Fluorinated L-Fucose Analogs Inhibit Proliferation of Cancer Cells and Primary Endothelial Cells

Yuanwei Dai[1†], Ruth Hartke[2†], Chao Li[1], Qiang Yang[1], Jun O. Liu[2] and Lai-Xi Wang[1,*]

1. Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, USA

2. Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

† These authors contribute equally to this work.

*To whom correspondence should be addressed. E-mail: wang518@umd.edu

ABSTRACT

Fucosylation is one of the most prevalent modifications on N- and O-glycans of glycoproteins and it plays an important role in various cellular processes and diseases. Small molecule inhibitors of fucosylation have shown promise as therapeutic agents for sickle cell disease, arthritis and cancer. We describe here the design and synthesis of a panel of fluorinated L-fucose analogs bearing fluorine atoms at the C2 and/or C6 positions of L-fucose as metabolic fucosylation inhibitors. Preliminary study of their effects on cell proliferation revealed that the 6,6-difluoro-L-fucose (**3**) and 6,6,6-trifluoro-L-fucose (**6**) showed significant inhibitory activity against proliferation of human colon cancer cells and human umbilical vein endothelial cells. In contrast, the previously reported 2-deoxy-2-fluoro-L-fucose (**1**) had no apparent effects on proliferations of all the cell lines tested. To understand the mechanism of cell proliferation inhibition by the fluorinated L-fucose analogs, we performed chemoenzymatic synthesis of the corresponding GDP-fluorinated L-fucose analogs and tested their inhibitory activities against the mammalian α1,6-fucosyltransferase (FUT8). Interestingly, the corresponding GDP derivatives of 6,6-difluoro-L-fucose (**3**) and 6,6,6-trifluoro-L-fucose (**6**), which are the stronger proliferation inhibitors, showed much weaker inhibitory activity against FUT8 than that of the 2-deoxy-2-fluoro-L-fucose (**1**). These results suggest that FUT8 is not the major target of the 6-fluorinated fucose analogs (**3** and **6**). Instead, other factors, such as the key enzymes involved in the *de novo* GDP-fucose biosynthetic pathway and/or other fucosyltransferases involved in the biosynthesis of tumor-associated glyco-epitopes are most likely the targets of the fluorinated L-fucose analogs to achieve cell proliferation inhibition. To our knowledge, this is the first comparative study of various fluorinated L-fucose analogs for suppressing the proliferation of human cancer and primary endothelial cells required for angiogenesis.

KEY WORDS: fucosylation, fluorinated L-fucose, fucosyltransferase, cancer, metabolic inhibition

INTRODUCTION

Fucosylated glycoconjugates play essential roles in numerous physiological and pathological processes including inflammation, angiogenesis, immune modulation and tumor metastasis.[1, 2] Aberrant fucosylation has been reported in many cancer types,[3] featuring the increased fucosylation of membrane receptors and the overexpression of fucosylated epitopes, such as the Lewis^b, sialyl Lewis^a, Lewis^y, sialyl Lewis^x, and Globo H carbohydrate antigens (Figure 1). Notably, these fucosylated epitopes are typical cancer-related antigens associated with tumor progression and metastasis. It has been reported that enhanced activity of certain fucosyltransferases such as the α1,6-fucosyltransferase (FUT8), which is responsible for core fucosylation, i.e., α1,6-fucosylation of N-glycans, contributes to the metastasis of melanoma[4] and non-small cell lung cancer.[5] A recent report has shown that the fucosylated carbohydrate antigen, sialyl Lewis^a (CA19-9), promotes pancreatitis and pancreatic cancer in mouse models.[6] In addition, fucosylated glycoproteins can serve as important biomarkers for cancer diagnosis and prognosis.[7] For example, the FDA-approved core fucosylated α-fetoprotein (AFP-L3) has been widely used as a biomarker for early diagnosis of hepatocellular carcinoma (HCC). [8]

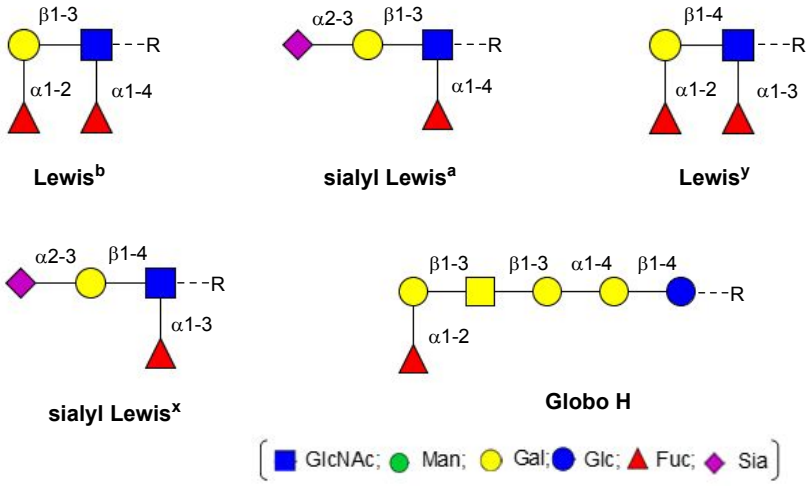


Figure 1. Selected examples of fucosylated glycans as cancer-associated antigens

The addition of L-fucose to glycans and glycoproteins is catalyzed by various fucosyltransferases (FUTs) using GDP-β-L-fucose as a donor substrate. [1, 9] Two distinct pathways, the *de novo* pathway and the salvage pathway, are involved in the synthesis of GDP-L-fucose in the cell. Fucosyltransferase inhibitors can down-regulate the activity of FUTs and have shown potentials as therapeutic agents for cancers. [10-12] Several GDP-L-fucose analogs have been reported as inhibitors of fucosyltransferases. [3, 10] However, application of those inhibitors in biological systems was hampered by the negatively charged nature of the sugar nucleotide moieties, which have low cell permeability. On the other hand, several small-molecule fucose analogs, including fluorinated L-fucose analogs, have been developed as inhibitors of fucosylation.[13-17] For example, 2-deoxy-2-fluoro-L-fucose has shown potential therapeutic efficacy in animal models for treatment of sickle cell disease[18] and arthritis[19]. Paulson and co-workers[13] have reported an elegant study using peracetylated 2-deoxy-2-fluoro-L-fucose as a global metabolic inhibitor of fucosyltransferases for remodeling cell-surface glycans. 2-Deoxy-2-fluoro-L-fucose, 6,6,6-trifluoro-L-fucose (fucostatin I), and their per-O-acetylated derivatives were also applied as an inhibitor for core-fucosylation to generate non-fucosylated monoclonal antibodies with enhanced antibody-dependent cellular cytotoxicity (ADCC) against cancer.[14, 17]

Despite wide applications of the 2-deoxy-2-fluoro-L-fucose as a global fucosylation inhibitor, it does not appear to have significant effects on the proliferation of cancer cells in cell-based assays, even present at very high concentrations. [13, 20, 21] Indeed, while 2-deoxy-2-fluoro-L-fucose was shown to retard tumor growth in prophylactic tumor xenograft models, relatively large dosage was required.[14, 21] On the other hand, 6,6,6-trifluoro-L-fucose (fucostatin I) and its per-O-acetylated derivative have been shown to be a potent inhibitor for core-fucosylation, clearly by primarily targeting the GDP-mannose 4,6-dehydratase (GMD) in the *de novo* GDP-fucose biosynthetic pathway,[17] but its effects on cancer cells have not been described. Herein, we describe the synthesis of an array of fluorinated L-fucose analogs bearing fluorine

atoms at C2 and/or C6 positions of L-fucose, and a study of their effects on cell proliferation in cancer and angiogenetic cells. Our data revealed that the previously reported 2-deoxy-2-fluoro-L-fucose and 6-fluoro-L-fucose had only marginally inhibitory activity against proliferation of several cancer cells, while 6,6-difluoro-L-fucose and 6,6,6-trifluoro-L-fucose analogs showed significant inhibitory activity against proliferation of human colon cancer and angiogenetic cells. To understand the putative mechanism of cell proliferation inhibition by the fluorinated fucose analogs, we also synthesized the corresponding fluorinated GDP-L-fucose analogs and compared their inhibitory activities against the mammalian α1,6-fucosyltransferase (FUT8). Interestingly, the corresponding GDP derivatives of the 6,6-difluoro-L-fucose and 6,6,6-trifluoro-L-fucose analogs, which are much more efficient inhibitors of cell proliferation than the 2-deoxy-2-fluoro-L-fucose, showed much less activity in inhibiting FUT8, implying that the 6,6-difluoro-L-fucose and 6,6,6-trifluoro-L-fucose analogs may achieve their inhibition of cell proliferation by targeting other components along the fucosylation pathway, concurrent with the inhibition of FUT8.

RESULTS AND DISCUSSION

Design. Mammalian cell fucosylation, the attachment of an α-linked L-fucose to the oligosaccharides of glycoproteins and glycolipids, is catalyzed by a respective fucosyltransferase using GDP-L-fucose as the donor substrate. GDP-L-fucose can be generated in cells through two biosynthetic pathways: the salvage pathway, in which free L-fucose is converted to GDP-fucose via the catalysis of fucokinase and fucose pyrophosphorylase, and the *de novo* pathway where GDP-fucose is generated from GDP-mannose via the catalysis of GDP-mannose 4,6-dehydratase (GMD) and GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (FX).[1] Several fluorinated L-fucose analogs, including the 2-deoxy-2-fluoro-L-fucose, the 6-fluoro-L-fucose, and the 6,6,6-trifluoro-L-fucose have been used as global metabolic inhibitors of fucosylation in cells.[13, 14, 17] A prerequisite is that the metabolic inhibitors

must get into the cells and are converted to the corresponding fluorinated GDP-L-fucose analogs to exert their inhibitory activities on fucosylation (Figure 2). In the present study, we sought to test a panel of C2/C6 fluorinated L-fucose analogs (compounds **1-6**, Figure 2) for their effects on proliferation of various cancer cells. So far, no systematic comparative study of fluorinated L-fucose analogs on cancer cell proliferation has been conducted.

We decided to use the peracetate of the fluorinated L-fucose analogs as a "pro-drug" for the present cellular studies, because peracetylated sugar analogs usually have better cell permeability than the corresponding free sugars and have been frequently used for cellular metabolic incorporation studies.[20, 22-26]. It should be noted that the enhancing effects of acetylation on metabolic incorporation and inhibition of monosaccharide precursors may depend on the sugars and the cellular systems. A recent comparative study has demonstrated that acetylation of 2-deoxy-2-fluoro-L-fucose and 5-alkynyl-L-fucose had only moderately enhanced inhibitory effect on the core fucosylation of IgG antibodies produced in CHO cells.[26] When the peracetylated derivatives get into cells, the O-acetyl groups can be readily removed by the cellular non-specific esterases to provide the free fluorinated L-fucose analogs, which are then converted to the corresponding fluorinated GDP-L-fucose analogs by fucokinase and fucose pyrophosphorylase, *via* the salvage biosynthetic pathway.[1] The resulting fluorinated GDP-L-fucose analogs could exert inhibition of fucosylation by a mechanism of dual action. On the one hand, the fluorinated GDP-L-fucose could act as a competitive inhibitor of fucosyltransferases. Ample experimental data have demonstrated that the presence of a fluorine atom proximal to the anomeric center in the substrate provides a strong destabilizing effect on the oxocarbenium ion transition state being developed during glycoside hydrolysis and/or glycosyl transfer.[27-29] As demonstrated in the pioneering work from Withers and co-workers, 2-deoxy-2-fluoro-glycosides can act as a class of mechanism-based inhibitors of retaining glycosidases, which proceed to form a covalently linked

fluorosugar-enzyme intermediate that cannot proceed further or only react slowly to form product, due to the destabilizing effect of the proximal fluorine atom on the oxocarbonium ion transition state.[27, 30-32] Mammalian α-fucosyltransferases are inverting glycosyltransferases that do not form a covalently linked enzyme-GDP-fucose intermediate, but the fluorinated GDP-L-fucose, which mimics the natural GDP-L-fucose substrate, can be recognized by the enzyme and form a fluorinated GDP-L-fucose/enzyme complex at the catalytic center, which might not proceed further for fucosyl transfer or might have only a slow turnover, due to the destabilizing effect of the proximal fluorine atom(s) on the oxocarbenium ion transition state. In fact, synthetic GDP-2-deoxy-2-fluoro-L-fucose and GDP-6-fluoro-L-fucose have been shown to be competitive inhibitors against several fucosyltransferases including FUT3, FUT5, FUT6, and FUT7, with a Ki of low μM.[33] On the other hand, the slow turnover of the fluorinated GDP-L-fucose-enzyme complex results in the accumulation of the fluorinated GDP-L-fucose analog, which can exert feedback inhibition of fucosylation by targeting the GDP-mannose 4,6-dehydratase (GMD), a key enzyme in the *de novo* GDP-fucose biosynthetic pathway, through allosteric binding.[33, 34] GDP-L-fucose feedback inhibition is an important mechanism to regulate cellular fucosylation.[34] Allen and co-workers have solved the crystal structure of GDP-mannose 4,6-dehydratase (GMD) complexed with synthetic GDP-6,6,6-trifluoro-L-fucose.[17] The structural study has revealed that the trifluoromethyl group is completely buried in a small pocket adjacent to the sugar moiety, and extensive Van der Waals interactions are observed between the trifluoromethyl group and several amino acid residues in GMD. Thus, the replacement of the methyl group in the natural substrate GDP-L-fucose with the trifluoromethyl group leads to increased Van der Waals contacts, which may explain the increased potency of the precursor 6,6,6-trifluoro-L-fucose in reducing core-fucosylation in cells over the 2-deoxy-2-fluoro- or 6-fluoro-L-fucose.[17] Many studies have demonstrated that, due to the strong electronegativity and the size similarity of fluorine to hydroxyl group or hydrogen, the introduction of

fluorine into a molecule at a selective site can influence the membrane permeability, protein and enzyme recognition, metabolic pathways, and pharmacokinetic properties of the molecule.[28, 29] In general, C–F bond can undergo multipolar interactions with protein's backbone amide bonds, side-chain amide residues, and/or the positively charged guanidinium in the binding pocket, resulting in an enhancement of protein-ligand interactions.[35, 36] For example, it has been shown that introduction of a tetrafluorinated methylene group dramatically strengthens the interactions between a fluorinated UDP-galactofuranose analog and UDP-galactopyranose mutase.[37] Thus, it will be particularly interesting to examine and compare how the 2-deoxy-2-fluoro-, 6-fluoro-, 2-deoxy-2,2-difluoro-, 2-deoxy-2,6-difluoro-, 6,6-difluoro- and 6,6,6-trifluoro-L-fucose analogs (compounds **1-6**, Figure 2) affect the global fucosylation and cell proliferation of cancer cells. In addition, we also sought to synthesize the corresponding fluorinated GDP-L-fucose analogs and test their inhibitory activity against fucosyltransferases such as FUT8, which may provide insights into the mechanism by which the fluorinated L-fucose analogs exert their effects.

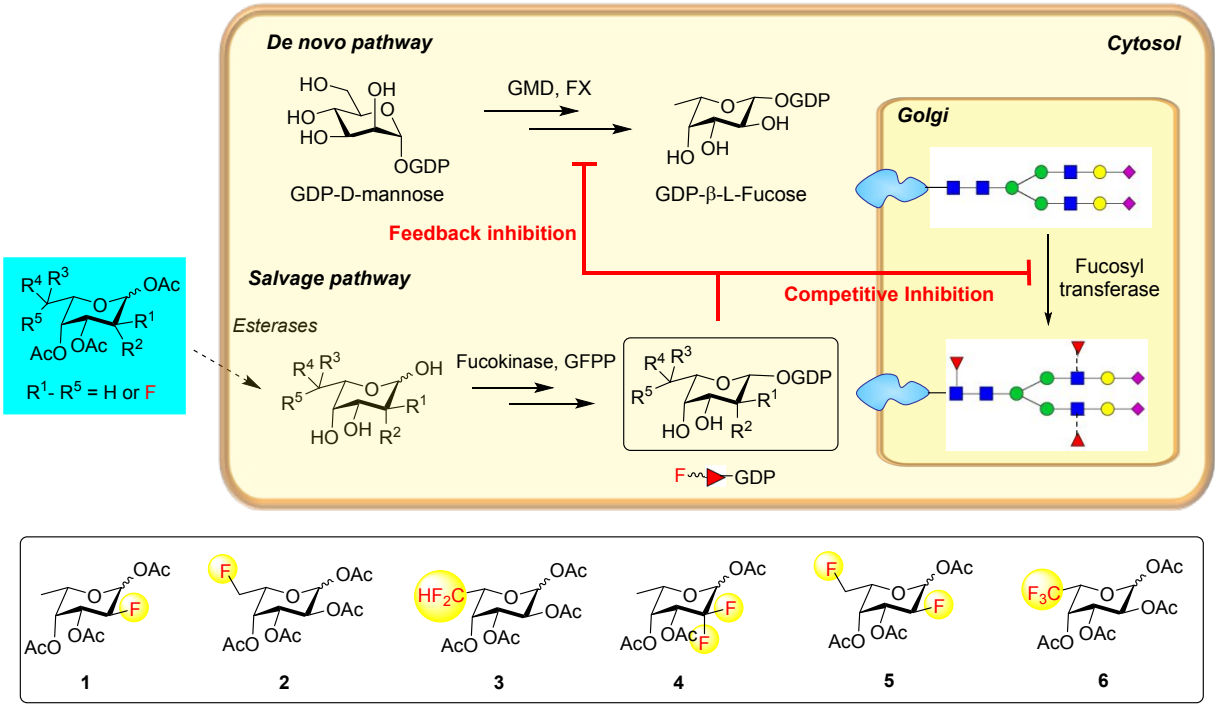


Figure 2. Fluorinated L-Fucose Analogs as Metabolic Inhibitors of Fucosylation. Peracetylated analogs of L-fucose (**1-6**) are up-taken by cells and converted into the corresponding GDP-fucose analogs through the salvage pathways. The fluorinated GDP-fucose analogs may inhibit fucosylation by two mechanisms: 1) The fluorinated GDP-fucose analogs can act as a competitive inhibitor of various fucosyltransferases. The accumulated fluorinated GDP-fucose analogs act as a feedback inhibitor to shut down the *de novo* GDP-fucose biosynthesis.

Synthesis of the Fluorinated L-Fucose Analogs. Among the 6 fluorinated fucose analogs designed to be examined and compared, **1**, **2**, **3**, and **6** are known compounds, while **4** and **5** are new fluorinated L-fucose analogs. The peracetylated 2-deoxy-2-fluoro-L-fucose (**1**) was synthesized by following the previously reported procedure.[38] The peracetylated 6-fluoro-L-fucose (**2**) was prepared starting from 1,2;3,4-di-O-isopropylidene-L-galactose, as described by Wong and co-workers.[33]

Peracetylated 6,6-difluoro-L-fucose (**3**) has been also synthesized previously. [39] We developed a modified synthesis of **3**, which gave an improved overall yield (Scheme 1, panel A). Oxidation of **7** with Dess-Martin periodinane gave the aldehyde (**8**) in 92% yield. Deoxyfluorination was achieved by treatment of **8** with diethylaminosulfur trifluoride (DAST) [40] to afford **9** in 77% yield. Importantly, slow addition of DAST to the solution of **8** in dichloromethane with ice-bath cooling was essential to give a good yield without the generation of the vinyl fluoride by-product. Notably, the long-range coupling of fluorine and proton was observed in the proton NMR spectrum of **9** between F(6) and H(3) ($^5J_{F6,\,H3}$ = 1.3 Hz) and between F(6) and H(l) ($^5J_{F6,\,H1}$ = 1.8 Hz). The removal of isopropylidene groups was achieved by refluxing **9** with 80% acetic acid to give the tetraol, which was acetylated with acetic anhydride in pyridine to give the 6,6-difluorofucose derivative (**3**) in 91% in two steps. Thus, the synthesis of 6,6-difluoro-L-fucose peracetate (**3**) was achieved in 4 steps with a 64% overall yield from the known compound **7**. Interestingly, replacement of the methyl group in fucose with the difluoromethyl group

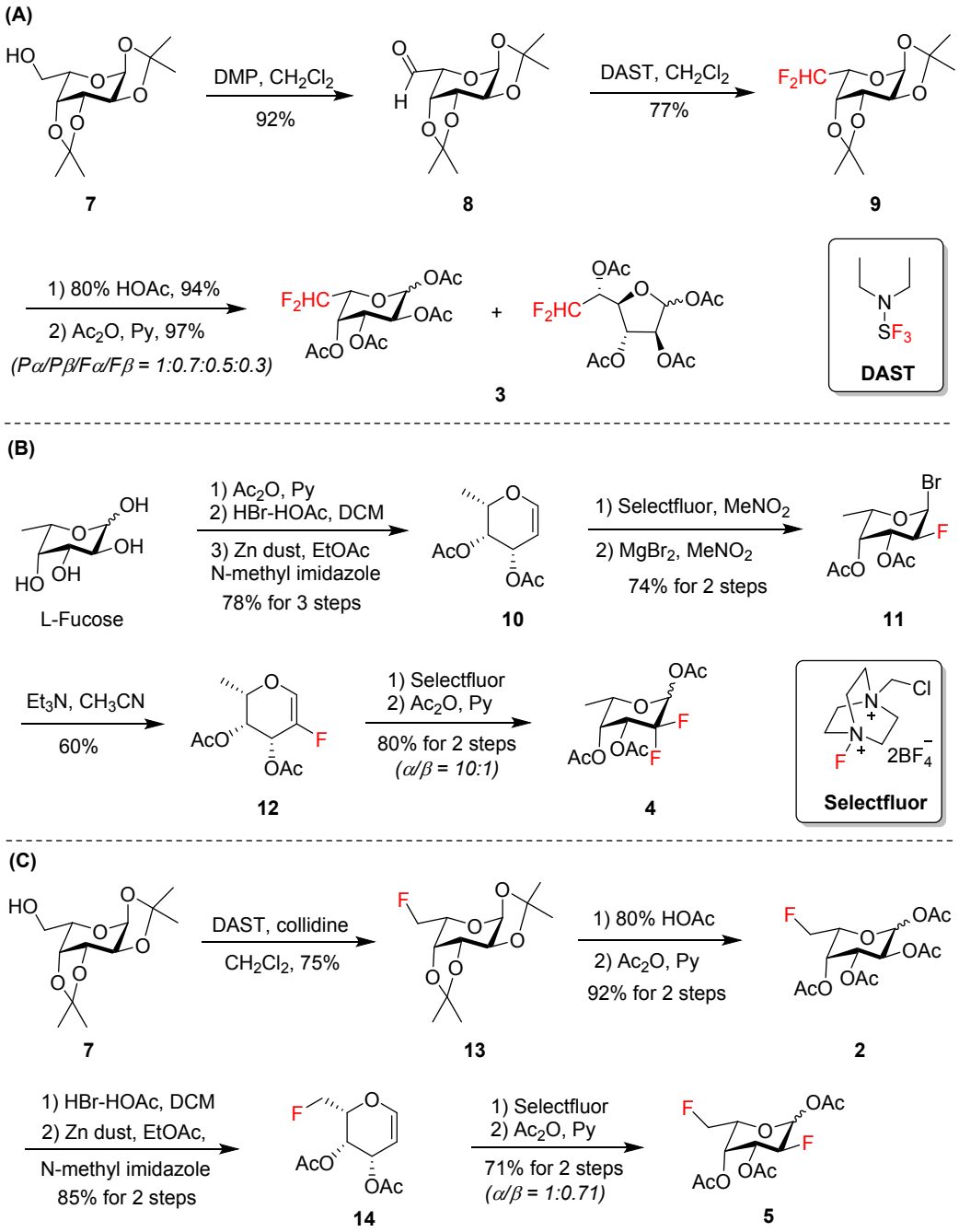
increased the formation of the furanose isomer. NMR analysis indicated that compound **3** was a mixture of α,β-isomers of pyranose and furanose, which was in good agreement with the reported data. [39]

Next, we turned our attention to the synthesis of 2-deoxy-2,2-difluoro-L-fucose derivative (**4**) from L-fucose as outlined in Scheme 1, panel B. Sequential electrophilic fluorination strategy was applied to efficiently introduce the two fluorine atoms at C2 in the process. Acetylation of L-fucose, treatment with hydrogen bromide in acetic acid, and subsequent zinc-promoted reductive elimination afforded the diacetyl-L-fucal (**10**) in 78% yield over three steps. Compound **10** was treated with Selectfluor[38] in dry nitromethane, followed by adding $MgBr_2$ as a nucleophile to furnish 2-deoxy-2-fluoro-L-fucosyl bromide (**11**). [41] Elimination of the anomeric bromide with triethylamine in acetonitrile afforded the desired vinyl fluoride (**12**) in 60% yield. Subsequent electrophilic fluorination of **12** by using Selectfluor, followed by acetylation of anomeric position provided the peracetylated 2,2-difluorofucose (**4**) in 80% yield over 2 steps. Interestingly, compound **4** was isolated as predominant α-anomer, which was confirmed by ^{1}H and ^{13}C NMR analysis. Therefore, we finished the synthesis of **4** from L-fucose in 8 steps with a 28% overall yield.

A number of elegant approaches toward the synthesis of fluorinated sugars haven been reported for mechanistic and biological studies,[28, 29, 42] but fluorinated L-fucose analogs bearing fluorine atoms at both the 2 and 6-positions of L-fucose have not been described. The synthesis of peracetylated 2-deoxy-2,6-difluoro-L-fucose (**3**) was shown in Scheme 1, panel C. Deoxyfluorination and electrophilic fluorination strategy were used to introduce the fluorine at C6 and C2 of fucose, respectively. The preparation of the fluorinated precursor (**13**) was readily achieved through deoxyfluorination of **7** with DAST in the presence of 2,4,6-collidine in refluxing dichloromethane.[33] Subsequent acidolysis followed by acetylation gave the peracetylated 6-fluoro-L-fucose (**2**)[13, 33] in 92% yield over 2 steps. Treatment of **2** with hydrogen bromide, subsequent reductive elimination afforded 6-fluoro-L-fucal (**14**) in 85% yield

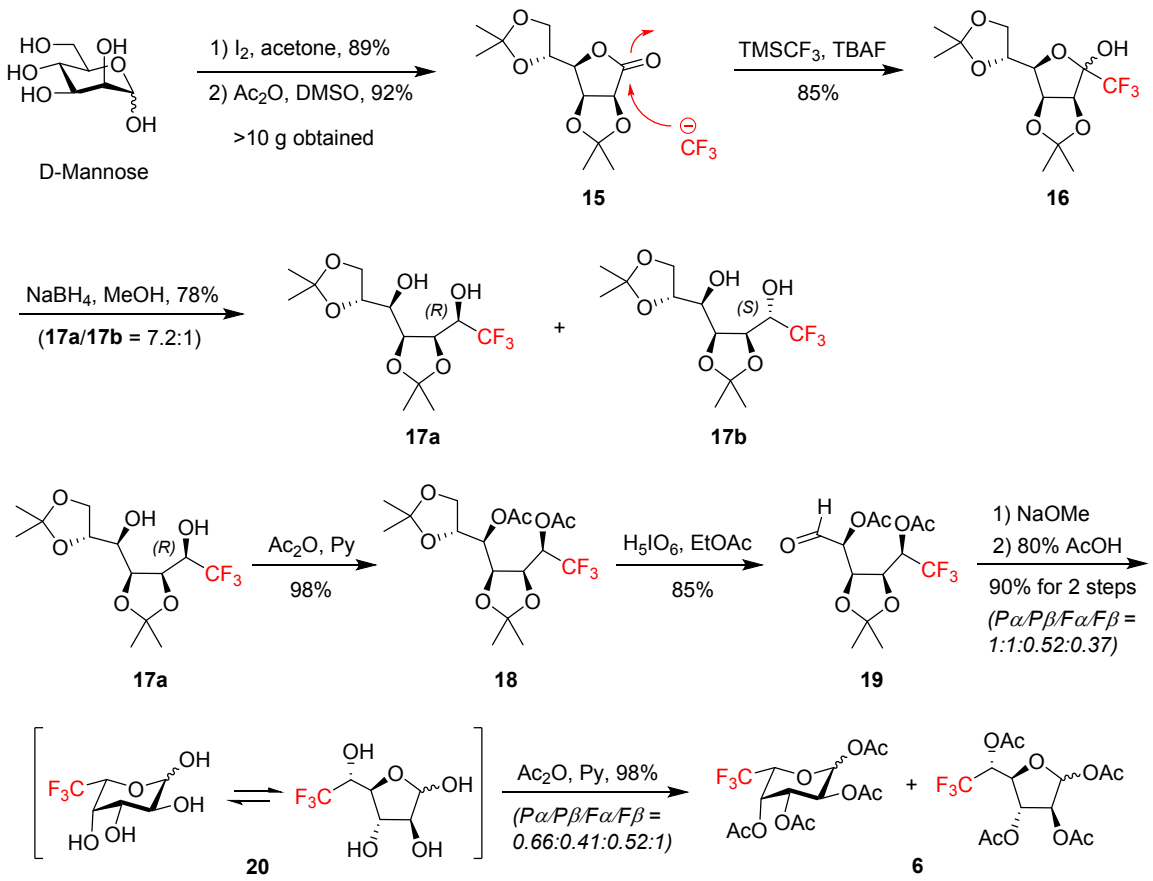
in two steps. Subsequent fluorination and acetylation afforded the peracetylated 2,6-difluoro-L-fucose (**5**) in 71% yield in 2 steps. Thus, the synthesis of **5** was completed in 7 steps with 42% overall yield from **7**.

Scheme 1. Synthesis of Peracetylated Fluorinated L-Fucose Analogs **2-5.**



Synthesis of Peracetylated 6,6,6-Trifluoro-L-Fucose 6. The first synthesis of 6,6,6-trifluoro-L-fucose was reported by Toyokuni and co-workers [43] starting from a rare sugar D-lyxose. This approach required dethioacetalization with stoichiometric amount of $HgCl_2$ and generated a 1:1 mixture of 6,6,6-trifluoro-L-fucose and its epimer 6,6,6-trifluoro-D-altrose, which was difficult to separate. After that, several groups reported different approaches to the synthesis of the target molecule.[44-46] However, most of the previous syntheses suffered from low stereoselectivity and/or low yield. Inspired by the elegant synthesis from Goddard-Borger group[46], we initiated a modified synthesis from D-mannose (Scheme 2). Isopropylidene protection of D-mannose and subsequent Swern oxidation provided mannolactone (**15**)[47] on a 10-gram scale. Treatment of **15** with trifluoromethyl(trimethyl)silane and TBAF afforded a mixture of diastereomers (**16**) in a ratio of 3.5:1, which was confirmed by NMR analysis. Next, we examined several reagents for the stereoselective reduction of hemiketal **16**, including $NaBH_4$, $LiBH_4$ and $LiAlH_4$. We found that $NaBH_4$ in refluxing methanol was the best, which yielded the desired diastereomer **17a** as the major product (**17a/17b,** 7.2:1). Acetylation of the major diastereomer **17a** provided **18** in 98% yield. Remarkably, periodic acid-promoted regioselective hydrolysis of the isopropylidene group and simultaneous oxidative cleavage of the resulting diol enabled the transformation of **18** to aldehyde **19** in a one-pot manner. Removal of the O-acetyl and isopropylidene groups in**19** afforded the 6,6,6-trifluoro-L-fucose (**20**) in 90% yield in two steps. Global acetylation of free sugar **20** completed the synthesis of **6**. Notably, replacement of the methyl group in fucose with the trifluoromethyl group increased the ratio of the furanose isomer. NMR analysis indicated that compound **20** and **6** were a mixture of α,β-isomers of pyranose and furanose, which was in consistence with the previously reported observation.[45] Thus, the synthesis of **6** was completed in 9 steps from D-mannose in a 36% overall yield. The structures of key reaction intermediates and all fluorinated fucose analogs (**1–6**) were confirmed by ^{1}H, ^{13}C, and ^{19}F NMR spectroscopy and MS analysis (see the Supporting Information).

Scheme 2. Synthesis of Peracetylated 6,6,6-Trifluoro-L-fucose **6**



Inhibition of Proliferation of Cancer Cells by the Fluorinated Fucose Analogs (1-6). We initially screened for inhibition of cell viability by the fluorinated L-fucose analogs (**1-6**) in four different human cancer cell lines of solid tumors: MDA-MB-231, PANC-1, HeLa, and HCT116, which were derived from triple-negative breast adenocarcinoma, pancreatic epithelioid carcinoma, cervical adenocarcinoma, and colorectal carcinoma, respectively. Previous studies investigating cellular inhibition by 2-deoxy-2-fluoro-L-fucose analogs have used 100-500 μM[12, 17] concentrations. To identify more potent hits, we screened our compounds in each cell line at 100 μM and 10 μM for 72 hrs before cell viability was determined by the Alamar blue assay. We found that inhibition ($p < 0.05$) in all four cancer cell lines was demonstrated by peracetylated 6,6-difluoro-L-fucose (**3**) and peracylated 6,6,6-trifluoro-L-fucose

(**6**) with 100 μM treatment (Figure 3). Interestingly, **3** strongly inhibited cell viability in HCT116 colorectal cancer cells by a mean of 75% (Figure 3D), whereas in each of the other cell lines, **6** exhibited a slightly stronger effect than **3**. Of note, peracetylated 6-fluoro-L-fucose (**2**) showed inhibition of HCT116 cells, and to a lesser degree, HeLa cells (Figure 3C, D). These results demonstrate that the analogs fluorinated solely at the C6 position are capable of inhibiting cell viability. In contrast, we did not observe apparent inhibition by compounds fluorinated only at the C2 position.

Given that HCT116 colorectal cancer cells exhibited the most sensitivity to C6 fluorinated analogs, we investigated a dose-dependent effect of the 6-fluorinated L-fucose analogs in this cell line. We tested compounds **2**, **3**, and **6** across a range of concentrations and determined the relative IC_{50} values of analogs **2, 3,** and **6**. The 6,6-difluoro-L-fucose (**3**) was found to be the most potent with an IC_{50} of 43 μM, while the IC_{50} values for the 6,6,6-tifluoro-L-fucose (**6**) and the 6-fluoro-L-fucose (**2**) were 58 μM and 159 μM, respectively (Figure 3E).

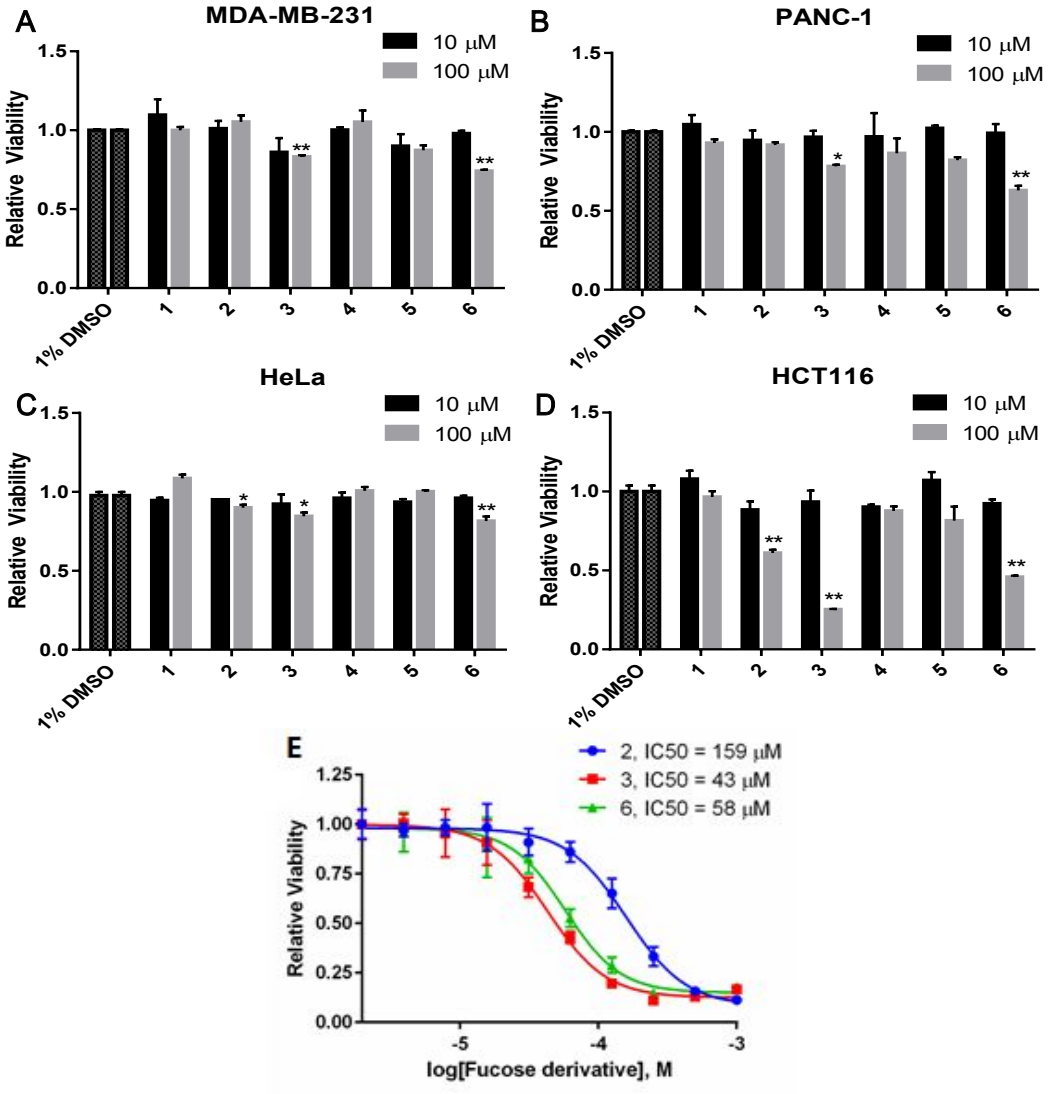


Figure 3. Screen of fluorofucose Analogs 1-6 in four cancer cell lines. Cells were treated with fluorofucose analogs for 72 hrs. at concentrations indicated. Viability was examined with Alamar blue assay. **A** and **B.** Cells were inhibited by 100 μM compound **3** and **6**. **C** and **D.** Cells were significantly inhibited by both **2**, **3** and **6**. HCT116 cells experienced dramatic inhibition by both compounds **3** and **6** at 100 μM. * indicates $p<0.05$, ** indicates $p<0.01$. **Panel E.** Comparison of mono, di, and tri-fluorinated fucose compounds at the C6 position. Dose response curves were generated using a titration of compounds from 1 mM to 4 μM in HCT116 cells. All C6-fluorinated analogs had a dose-dependent effect. The difluorinated analog resulted in the most potent IC_{50} of 43 μM.

It has been well demonstrated that angiogenesis plays an important role in tumor growth and metastasis.[48, 49] We were therefore interested to know if the synthetic fluorinated L-fucose analogs could

inhibit the viability of Human Umbilical Vein Endothelial Cells (HUVECs), an *in vitro* proxy of angiogenesis. The assays of the effects on the viability of HUVECs were performed as described above with analogs **1-6.** As with HCT116 cells, the analogs fluorinated at the C6 position, including the 6-fluoro-L-fucose (**2**), the 6,6-difluoro-L-fucose (**3**), and the 6,6,6-trifluoro-L-fucose (**6**), all showed strong inhibitory activity on HUVEC viability. Once again, analogs **1, 4** and **5,** fluorinated at the C2 position, did not show apparent inhibition of HUVEC at 100 μM. Interestingly, HUVEC showed increased sensitivity to both **3** and **6**, which demonstrated significant inhibitory activity at 10 μM (Figure 4).

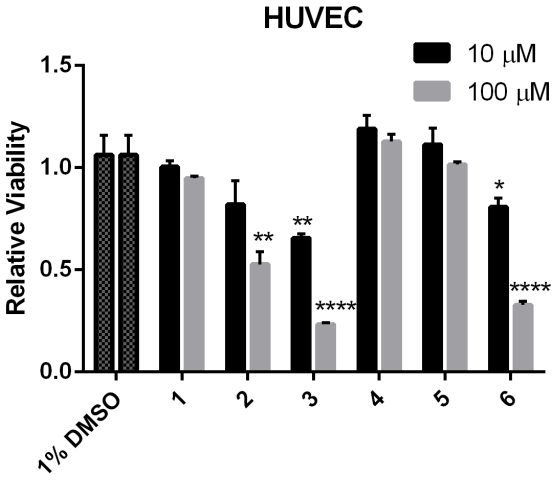


Figure 4. Screen of fluorinated L-fucose analogs 1-6 in HUVECs. HUVEC cells were treated with indicated concentrations for 72 hrs before being analyzed by Alamar blue. Cells were strongly inhibited by all compounds fluorinated at the C6 position at 100 μM. Cells were also significantly inhibited by 10 μM analogs di- and tri-fluorinated on C6. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, **** indicates $p<0.0001$.

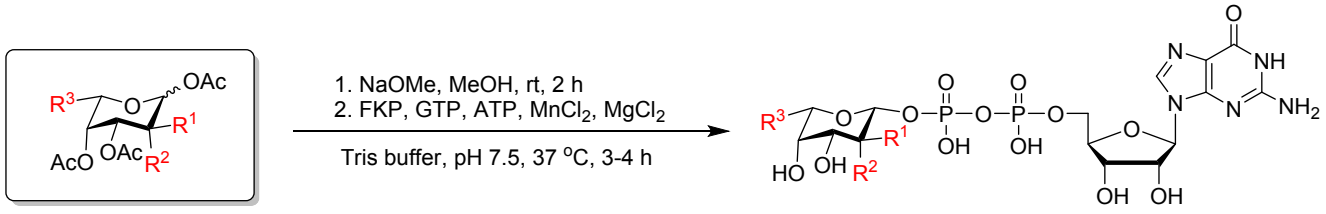
Our results demonstrated that the peracetylated 6,6-difluoro-L-fucose (**3**) and 6,6,6-trifluoro-L-fucose (**6**) showed significant inhibitory activity against the proliferation of colon cancer cell line, while the peracetylated 2-deoxy-2-fluoro-L-fucose (**1**), at 100 μM, had no apparent effect on all the cancer cell lines tested. Our results are consistent with the report described by Paulson and co-workers.[13] Their

experiments have demonstrated that **1** did not inhibit the proliferation of HL-60, Ramos, and CHO-K1 cells at up to 500 μM.[13] Korytnyk and co-workers have previously studied the metabolic effects of halogenated L-fucose and D-galactose analogs on growth, viability, and incorporation of D-glucosamine, D-galactose, L-fucose, and L-leucine into macromolecular components (proteins and glycoconjugates) in murine leukemia L1210, murine lymphoma P288, and human mammary tumor SW613 cells.[20] Although the fluorinated L-fucose analogs have been found to effectively inhibit the incorporation of L-[3H]fucose into the macromolecular components, the 6-fluoro-L-fucose had only moderate inhibition effect on cell growth of murine leukemia L1210 cells but had no inhibitory effect on human mammary tumor SW613 cells. This study has also demonstrated that O-acetylation increased the inhibitory activities of 2-chloro-L-fucose and 2-bromo-L-fucose analogs against murine leukemia L1210 cells, as acetylated sugars has greater cellular permeability than the free sugars.[20] Our observations that the peracetylated 6-fluoro-L-fucose analog (**2**) has only marginal inhibitory effect on the tumor cell lines so far tested are consistent with the reported results. In addition, our results further indicated that the peracetylated 2-deoxy-2-fluoro-L-fucose (**1**) also had only marginal inhibitory activity on HUVECs. In contrast, the 6-fluorinated fucose analogs (**2**, **3**, **6**) exhibited significant inhibitory effect on HUVECs under the same testing conditions.

Chemoenzymatic Synthesis of Fluorinated GDP-L-Fucose Analogs. To investigate the putative mechanism of cell proliferation inhibition by the synthetic fluorinated L-fucose analogs (**1**-**6**), we performed chemoenzymatic synthesis of the corresponding fluorinated GDP-fucose analogs and tested their inhibitory activity on FUT8, the key enzyme responsible for core-fucosylation (α1,6-fucosylation) of N-glycans. Thus, the peracetylated fluorinated L-fucose analogs (**1**-**6**) were de-O-acetylated and the resulting free fluorinated L-fucose analogs were converted into the corresponding fluorinated GDP-fucose analogs (**22**-**27**) using a bifunctional enzyme, L-fucokinase/GDP-fucose pyrophosphorylase (FKP)

as the catalyst [50] (Scheme 3). We found that most of the fluorinated L-fucose analogs (**1**, **2**, **3**, **5**, and **6**) were successfully converted into the corresponding fluorinated GDP-fucose analogs (**22**, **23**, **24**, **26**, and **27**) in excellent yields (66-87% in 3 steps). However, the 2-deoxy-2,2-difluoro-L-fucose derivative (**4**), after de-O-acetylation, was found to be a very poor substrate for the FKP enzyme, which gave only a marginal yield for the formation of the corresponding GDP-fucose derivative (**25**) under the reaction conditions. This result suggests that the 2-deoxy-2,2-difluoro-L-fucose analog might not be efficiently incorporated in the salvage biosynthetic pathway in cells to produce the corresponding GDP-fucose derivative, partly explaining its lack of inhibition of cell proliferation. All the fluorinated GDP-fucose analogs (**22**, **23**, **24**, **26**, and **27**), except the 2-deoxy-2,2-difluoro-L-fucose analog (**25**) that was not produced in sufficient amount, were purified by gel filtration chromatography and characterized by NMR and MS analysis. The GDP-2-deoxy-2-fluoro-L-fucose (**22**),[33] the GDP-6-fluoro-L-fucose (**23**) [33, 50], and the GDP-6,6,6-trifluoro-L-fucose (**27**)[17] have been synthesized previously by different methods.[17, 33, 50] The identity of the two new compounds, GDP-6,6-difluoro-L-fucose (**24**) and GDP-2-deoxy-2,6-difluoro-L-fucose (**26**) were characterized by ^{1}H, ^{13}C, ^{19}F, and ^{31}P-NMR analysis and MS analysis (See Supporting Information).

Scheme 3. Chemoenzymatic Synthesis of Fluorinated GDP-Fucose Analogs (**22-27**) using the Fluorinated L-fucose Analogs as the Starting Materials



1 R^1 = F, R^2 = H, R^3 = CH_3
2 R^1 = OH, R^2 = H, R^3 = CH_2F
3 R^1 = OH, R^2 = H, R^3 = CHF_2
4 R^1 = R^2 = F, R^3 = CH_3
5 R^1 = F, R^2 = H, R^3 = CH_2F
6 R^1 = OH, R^2 = H, R^3 = CF_3

22 R^1 = F, R^2 = H, R^3 = CH_3 (80%)
23 R^1 = OH, R^2 = H, R^3 = CH_2F (87%)
24 R^1 = OH, R^2 = H, R^3 = CHF_2 (72%)
25 R^1 = R^2 = F, R^3 = CH_3 (<5%)
26 R^1 = F, R^2 = H, R^3 = CH_2F (66%)
27 R^1 = OH, R^2 = H, R^3 = CF_3 (75%)

Inhibition of FUT8-Catalyzed Core-Fucosylation by the Fluorinated GDP-L-Fucose Analogs. The inhibitory effects of the fluorinated GDP-L-fucose analogs on the FUT8 catalyzed fucosylation were assessed using GDP-fucose as the donor substrate and a synthetic N-glycan, $GlcNAcMan_5GlcNAc_2$-AsnFmoc (Gn-Man_5-AsnFmoc), as the acceptor substrate (Figure 5A). The fucosylation was measured using the Malachite green phosphate assay through quantitative detection of the phosphate ions released from the calf intestinal alkaline phosphatase (CIP) catalyzed hydrolysis of the GDP, which was formed as a by-product of the FUT8 catalyzed fucosylation (Figure 5A). The Malachite green phosphate assay is a universal method for kinetics, high-throughput screening and inhibition studies of glycosyltransferases. [51] We optimized a previously reported method for assessing the FUT8 catalyzed core-fucosylation.[52] The results of inhibition by the fluorinated GDP-fucose analogs were shown in Figure 5B.

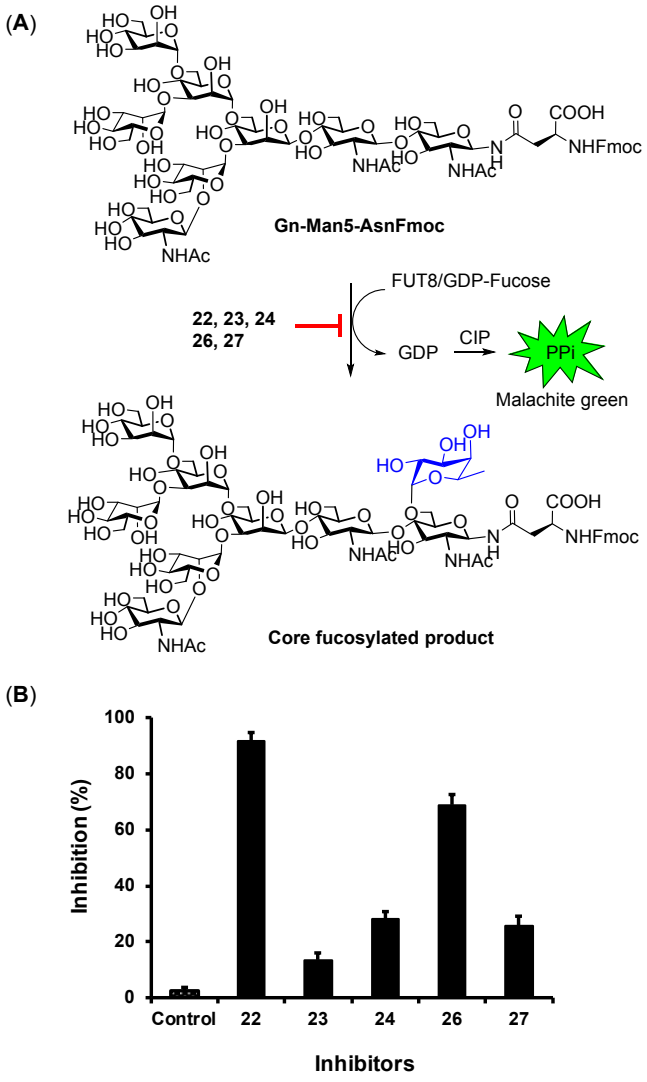


Figure 5. Inhibition of FUT8 catalyzed core-fucosylation by the synthetic fluorinated GDP-fucose analogs. (A) the reaction scheme for the FUT8-catalyzed core fucosylation and the Malachite green phosphate assay for assessment of the inhibition. (B) the relative inhibition potency of the synthetic fluorinated GDP-fucose analogs. The inhibition experiments were performed under the following conditions: A mixture of GDP-Fuc (50 μM), GlcNAcMan5-AsnFmoc (50 μM), CIP (3U), $MgCl_2$ (1 mM), and FUT8 (10 ng/μL) in a buffer (HEPES, 20 mM, pH 7.4, 80 μL) containing the respective fluorinated GDP-L-fucose analog (5 μM) was incubated at 37 ^{o}C in a 96-well flat-bottom plate (Santa Cruz Biotechnology) for 2 h. The GDP generated from the fucosylation reaction was measured by the Malachite green phosphate assay (see Supporting Information for details). The inhibition experiments were performed independently in triplicates. Standard curve was created for calibration and calculation of inhibition potency.

The inhibition of FUT8 activity was assessed by using a fixed concentration (5 μM) of the respective fluorinated GDP-L-fucose analog. The GDP-2-deoxy-2-fluoro-L-fucose (**22**) and the GDP-2-deoxy-2,6-difluoro-L-fucose (**26**) showed the most potent inhibitory activity against FUT8, reaching 92% and 70% inhibition at 5 μM, respectively. It was observed that an additional fluorination at the 6-position, as in compound **26**, did not lead to an enhancement of inhibitory activity, but resulted in a moderate decrease in the inhibitory efficiency against FUT8, in comparison with **22**. Interestingly, the three 6-fluorinated L-fucose analogs, including GDP-6-fluoro-L-fucose (**23**), GDP-6,6-difluoro-L-fucose (**24**), and GDP-6,6,6-trifluoro-L-fucose (**27**), showed only moderate inhibitory activities (13-28% at 5 μM) against FUT8 (Figure 5B). To the best of our knowledge, this is the first comparative study of a series of fluorinated GDP-fucose analogs on inhibition of FUT8 for core fucosylation. Previously, Wong and co-workers have reported that GDP-2-deoxy-2-fluoro-L-fucose (**22**) and GDP-6-fluoro-L-fucose (**23**) are competitive inhibitors (with a Ki of 4-38 μM) against several fucosyltransferases, including FUT3, FUT5, FUT6, and FUT7 [33]. Senter and co-workers have reported that the GDP-2-deoxy-2-fluoro-L-fucose (**22**) is an inhibitor of FUT8 and could potently inhibit core-fucosylation of recombinant monoclonal antibodies when it is present in the mammalian expression cell culture. [14]

While the observed potent inhibitory activity of the GDP-2-deoxy-2-fluoro-L-fucose analog (**22**) against FUT8 is expected and consistent with previous report, [14] the finding that the three 6-fluorinated GDP-L-fucose analogs (**23**, **24** and **27**) showed only moderate inhibitory activity of FUT8 is somewhat unexpected. The lack of correlation between the apparent inhibitory activity of the peracetylated 6-fluoro-L-fucose (**2**), 6,6-difluoro-L-fucose (**3**), and 6,6,6-trifluoro-L-fucose analog (**6**) against cell proliferation (Figure 3 and Figure 4) and the inhibition of FUT8 by the corresponding GDP derivatives (**23**, **24** and **27**) (Figure 5) suggests that other cellular factors, rather than FUT8, might be the major targets of the 6-fluorinated L-fucose analogs (**2**, **3**, and **6**) to achieve their cell proliferation inhibitory

activities. Given the fact that the GDP-6,6,6-trifluoro-L-fucose (**27**) has previously been shown to be a potent allosteric inhibitor of GMD, a key enzyme in the *de novo* biosynthesis of GDP-L-fucose, which significantly suppresses the biosynthesis of GDP-L-fucose,[17] our results suggest that the significant inhibition of proliferation of human colon cancer and angiogenetic cells by the peracetylated 6,6-difluoro-L-fucose (**3**) and 6,6,6-trifluoro-L-fucose analog (**6**) most likely result from the targeting of GMD by their corresponding GDP derivatives (**24** and **27**, respectively). Of course it should not be ruled out that the mechanism of action of the fluorinated L-fucose analogs could also involve targeting other key components, including the α,12- and α1,3/1,4-fucosyltransferases involved in the biosynthesis of tumor-associated glycoepitopes such as Lewis^b and sialyl Lewis^a (Figure 1) and the FX enzyme (GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase), another key enzyme in the *de novo* biosynthesis of GDP-L-fucose (Figure 2). Taniguchi and co-workers have previously demonstrated that 6-alkynyl L-fucose, a potent cellular fucosylation inhibitor, achieves its inhibitory activity by selectively binding and inhibiting FX, which leads to depletion of the GDP-L-fucose pool.[16] Thus, the mechanism by which L-fucose analogs to achieve inhibition of cellular fucosylation and the inhibition of cell proliferation appears to be more complicated than what was previously thought. In particular, the mechanism and correlation of fucosylation and cell growth in colon cancer remains to be characterized.[53, 54] Miyoshi and co-workers have shown that HCT116 cells have low cellular fucosylation due to an inactive mutation in GMD, and that the deficiency of GMD actually facilitates cancer cell progression by modulating the TRAIL signaling pathway, leading to an escape from NK cell-mediated tumor surveillance.[53] However, Fang and co-workers have demonstrated in another report that FUT8 plays a key role in colorectal carcinoma, and that targeting FUT8 by MiR-198 dramatically represses tumor growth and metastasis in colorectal cancer.[54]

Future studies should be directed to understanding the mechanism by which the fluorinated L-fucose analogs differentially inhibit cellular fucosylation and suppress cancer cell proliferation. These include: 1) investigation of the potential inhibition effects of those synthetic fluorinated GDP-fucose analogs on different types of fucosyltransferases, such as FUT1 (for α1,2-fucosylation) and FUT3 (for α1,3/4-fucosylation); 2) analysis of the inhibitory activity of the GDP-fluorinated-L-fucose analogs on GMD and FX, two key enzymes involved in the *de novo* GDP-L-fucose biosynthesis; 3) analysis of the changes in the cellular metabolites in the presence of the fluorinated L-fucose analogs; and 4) glycoproteomic analysis of the changes in various tumor-associated fucosylated glyco-epitopes of cancer cells in the presence of the fluorinated L-fucose analogs.

CONCLUSION

A facile synthesis of a panel of C2 and/or C6 fluorinated fucose analogs was achieved using L-fucose, L-galactose, or D-mannose as the starting material. Cell-based assays revealed that the 6,6-difluoro-L-fucose and 6,6,6-trifluoro-L-fucose analogs showed potent inhibitory activity against proliferation of human colon cancer cells and the angiogenic HUVEC cells, while the previously reported 2-fluoro-L-fucose did not show apparent effect on the two types of cell lines. This is the first comparative study of an array of fluorinated L-fucose analogs for the inhibition of the proliferation of human cancer cells and the angiogenic HUVEC cells. Notably, all the 6-fluorinated fucose analogs exhibited significant inhibitory activity against HUVEC cells, suggesting that they may inhibit tumor angiogenesis *in vivo*. The apparent no correlation between the inhibition of cell proliferation and the inhibition of FUT8 by the fluorinated fucose analogs and their GDP derivatives, respectively, suggests that other important factors, instead of FUT8, are most likely the targets of the fluorinated L-fucose analogs. These may include the key enzymes (GMD and FX) essential for GDP-fucose biosynthesis and/or other fucosyltransferases such as FUT1 and FUT3 involved in the synthesis of Lewis type tumor-

associated glycol-epitopes. Future work should be directed to the understanding of the mechanism of action of selected fluorinated fucose analogs. The present comparative study serves as a starting point for designing new fluorinated L-fucose analogs as inhibitors against cancer cell proliferation. This work also provides insights in designing inhibitors of other glycosylation processes that are potential drug targets for cancer and other diseases.

METHODS

Details of materials, methods, and experimental procedures are provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Detailed experimental procedures, synthesis of the fluorinated L-fucose analogs used in the study; chemoenzymatic synthesis of the GDP-fluorinated-L-fucose derivatives, copies of ^{1}H, ^{13}C, ^{19}F and ^{31}P NMR spectra.

AUTHOR INFORMATION

Corresponding Author

*E-mail: wang518@umd.edu

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

ACKNOWLEDGMENT

We thank R. Zhang for technical assistance and P. Wu for providing the FKP plasmid. This work was supported in part by the National Institutes of Health (NIH grants R01 GM080374 to LXW and P30 CA006973 to JOL).

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Graphic Abstract for TOC